Inhibition of non-O157 Shiga toxin *Escherichia coli* in African fermented foods by probiotic bacteria

By

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DECLARATION

I, Olanrewaju Emmanuel Fayemi declare that the thesis, which I hereby submit for the degree PhD Food Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

November, 2015

ABSTRACT

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By

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Non-O157 Shiga toxin producing *Escherichia coli* (STEC) serotypes are increasingly being associated with outbreaks of foodborne infections and illness. This study evaluated the *in vitro* probiotic characteristics of *Lactobacillus plantarum* strain B411 and dominant lactic acid bacteria (LAB) isolated from traditional African fermented maize gruel (ogi). Subsequently, the effects of acid adaption and those potential probiotic bacteria on the growth and survival of non-O157 STEC strains in fermented goat's milk and traditional African fermented cereal-based foods were investigated.

Two of the 14 lactic acid bacteria (LAB) strains that were isolated from ogi together with *L. plantarum* strain B411 possess hydrophobic cell surface with ability to coaggregate with pathogens as well as antimicrobial activities against non-O157 STEC strains and *E. coli* ATCC 25922. The three strains with *in vitro* probiotic attributes also exhibited high level of adhesion to Caco-2 cells and the two LAB strains from ogi spontaneous fermentation were genetically similar to other reported potential probiotic bacteria.

Though prior adaptation to acid enhanced acid tolerance of non-O157 STEC strains in Brain Heart Infusion (BHI) broth at low pH, it was detrimental to the survival in fermented goat's milk. The growth of both acid adapted (AA) and non-acid adapted (NAA) non-O157 STEC strains was not inhibited by the single strain potential probiotic *L. plantarum* B411 in fermented goat's milk. However, combination of yoghurt starter culture and potential probiotic *L. plantarum* B411 for the fermentation of the goat's milk had a bacteriostatic effect on the NAA non-O157 STEC strains while the growth of AA non-O157 STEC strains were substantially inhibited.

Acid adaptation did not contribute to the survival of non-O157 STEC strains in ogi as the survival of AA and NAA non-O157 STEC strains was similar during ogi fermentation and processing. This suggests that prior adaptation to acid may not be necessary for the survival of non-O157 STEC strains in ogi fermentation. However, the use of potential probiotic *L. plantarum* B411 in combination with spontaneous fermentation significantly inhibited the growth of both AA and NAA non-O157 STEC strains in ogi.

The NAA non-O157 STEC strains survived higher than AA non-O157 STEC strains in the sorghum motoho fermented with the potential probiotic *L. plantarum* strain FS2 or *P. pentosaceus* strain D39 isolated from ogi spontaneous fermentation. While the combination of the *L. plantarum* FS2 and *P. pentosaceus* D39 for the fermentation of the sorghum motoho resulted in substantial inhibition of AA non-O157 STEC strains.

This study shows that certain LAB strains from traditional fermented foods possess desirable *in vitro* probiotic properties and could be considered as potential probiotic strains. It also shows that NAA non-O157 STEC strains could develop acid adaptation in traditional African fermented foods and may not be inhibited by potential probiotic or bacteriocin producing bacteria. It is therefore proposed that in order to prevent the growth of non-O157 STEC strains in traditional African fermented foods, the use of bacteriocin producing starter culture must be combined with other additional hurdles such as prior acid adaptation as practised during backslopping.

DEDICATION

To the covenant keeping God,

My mother (Late Princess Adediwura Florence Fayemi)

My ever loving and supporting wife (Mrs Olanrewaju Bosede Fayemi) and

my beloved boys (Dave and Dan Olanrewaju).

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1. INTRODUCTION

Traditional lactic acid fermented foods are an important part of the diet in Africa and fermented foods of various types serve as complementary foods for infants and children, and the major meal of adults (Galati et al., 2014). Traditional fermented foods and the microorganisms that contribute to the fermentation process are increasingly being recognised for their health and nutritional benefits (Chilton et al., 2015). They are believed to aid in the control of some diseases, in particular intestinal disorders and diarrhoea (Aderiye and Laleye, 2003; Mathara et al., 2004; Franz et al., 2014). Fermented foods, particularly those produced under controlled fermentation, have a good record of safety and are rarely implicated in outbreaks of diseases (Omemu and Adeosun, 2010). However, natural fermentation processes practised in Africa are based largely on experience and knowledge gained through trial and error, which routinely allowed participation of diverse microorganisms (Galati et al., 2014). Therefore, involvement of pathogenic microorganisms during production cannot be totally ruled out, especially during fermentation under poor hygiene conditions and lack of potable water, a common occurrence in Africa (Oguntoyinbo et al., 2014). Various foodborne pathogens such as *Bacillus cereus*, *Clostridium perfringens*, *Aeromonas* spp., *Staphylococcus aureus* and *E. coli* serotypes have been isolated from cereal-based traditional fermented foods (Nyatoti et al., 1997; Kunene et al., 1999). Studies have shown that foodborne pathogens can survive in cereal-based fermented foods, which are mostly consumed as complementary infant foods in many African communities (Bakare et al., 1998; Kalui et al., 2010; Omemu and Adeosun, 2010; Oguntoyinbo, 2014; Kwaw, 2014).

Shiga toxin producing *Escherichia coli* (STEC), also known as enterohaemorrhagic *E. coli* (EHEC) is a group of pathogenic *E. coli* strains producing one or more Shiga toxins (*Stx*) (Monaghan et al., 2011). Studies have shown that spontaneous fermentation could not adequately eliminate STEC O157:H7 from cereal-based traditional fermented foods (Tadesse et al., 2005; Kwaw, 2014) and in fermented goat milk (Dlamini and Buys, 2009). Non-O157 STEC serotypes are increasingly being associated with outbreaks of foodborne infections and illness (Gould et al., 2013; Rund et al., 2013; Preubel et al., 2013; Bettelheim and Goldwater, 2014). Prevalence of infections by non-O157 STEC serotypes had been reported to be highest among children and could be five times higher than that of *E. coli* O157:H7 (Gould et al., 2013; Bettelheim and Goldwater, 2014).

Acid adaptation enhances the survival of STEC in acidic or fermented foods (Mathusa et al., 2010). Several studies have been done to determine acid adaptation of *E. coli* O157:H7 (Ryu and Beuchat, 1998; Guraya et al., 1998; Hsin-Yi and Chou, 2001; Beutin et al., 2007; Dlamini and Buys, 2009). However, little is known about the behaviour and stress response of non-O157 STEC when exposed to acid stress. Non-O157 STEC serotypes may not behave similarly to *E. coli* O157:H7 when exposed to acid stress or to food environments (Smith and Frantamico, 2012).

Currently, there is no effective treatment available for STEC infections in people and the use of antibiotics is not generally recommended due to the concern that they will induce *Stx* production, thus worsening the symptoms (Rund et al., 2013; Mohsin et al., 2015). Probiotic bacteria have been recommended as the only possible treatment for the STEC infections in people (Rund et al., 2013; Mohsin et al., 2015). Traditional fermented foods are predominantly fermented by the lactic acid bacteria (LAB) which could be potential probiotic starter culture to prevent the growth of non-O157 STEC and ensure the safety of traditional fermented foods commonly used as weaning food products (Waters et al., 2015). However, there is paucity of information on the role of probiotics and acid stress on non-O157 STEC serotypes in traditional African fermented foods.

Therefore, this study will determine the effect of acid adaption and potential probiotic bacteria on the survival of non-O157 STEC strains in fermented goat's milk and traditional African fermented cereal-based products.

2. LITERATURE REVIEW

The purpose of this review is to assess the significance of traditional African fermented foods and the associated safety challenges resulting from the fermentation process. It also focuses on the role of STEC as foodborne pathogens with emphasis on non-O157 STEC serotypes. This involves understanding the pathogenicity, virulence factors and the diversity non-O157 STEC serotypes. Research into the acid stress response in STEC is also discussed. Lastly, the possible application of probiotic bacteria as a potential intervention that can help prevent the growth of non-O157 STEC in traditional African fermented food products is reviewed.

2.1 Food fermentation in Africa

In developing countries, particularly in Africa which is characterized by high temperatures and humidity, and a scarcity of potable water, fermentation of various substrates is still widely utilized as a means of food preservation (Galati et al., 2014). Fermentation is widely used in African because its provides low cost for food preservation and enhance flavours to existing staples as well as a means of improving the nutritional quality and digestibility of the substrates (Olasupo et al., 2010; Smid and Hugenholtz, 2010; Egwim et al., 2013). In Africa, food fermentation represents an important way to improve food safety and combat food foodborne diseases that are prevalent in many of its resource-disadvantaged regions (Franz et al., 2014). In addition, the ability of fermentation process to soften food texture and alter its composition in such a way that it will require minimal energy and less fuel for cooking makes fermentation a highly desirable technique in the many rural communities of Africa where resources for cooking are scarce (Chelule et al., 2010).

Fermentation of foods in Africa is still based on traditional techniques handed from generation to generation (Sanni et al., 2013). Lactic acid fermentation is probably the oldest and most commonly practised technology among the African people (Lei and Jakobsen, 2004). Traditional African fermented foods are usually produced at household level by uncontrolled natural or spontaneous fermentation with frequent usage of backslopping techniques to initiate fermentation (Tou et al., 2006; Vieira-Dalode et al., 2007). Natural fermentation relies on the ability of dominant microorganisms to out-compete or produce metabolites that can readily inhibit pathogenic microorganisms (Kalui et al., 2009; Omemu et al., 2010; Oguntoyinbo, 2014). Further, fermentation of substrates especially cereals makes the final product suitable to serve as weaning food because the bacteria associated with the

fermentation process also produce vitamins and amino acids during their growth (Chelule et al., 2010).

2.1.1 Cereal-based fermented foods

Fermentation of cereals, such as pearl millet (*Peninsetum glaucum*), maize (*Zea mays*) and sorghum (*Sorghum bicolor*) to produce gruel, dough and porridge is predominant throughout Africa, where they are consumed as complementary food for babies and also serve as main meals and beverages for adults (Blandino et al., 2003; Tou et al., 2007; Kalui et al., 2010; Egwim et al., 2013). Information on the various non-alcoholic cereal-based African fermented products are summarised in Table 2.1. Cereal-based fermented foods are sources of non-digestible carbohydrates that may selectively stimulate the growth of *Lactobacilli* and *Bifidobacteria* present in the colon, thereby acting as prebiotics (Martensson et al., 2001).

Cereal-based fermented products are the most common foods for infants and adults in many rural communities across Africa. This is because cereals such as maize or sorghum are readily available (Chelule et al., 2010). Cereal-based lactic fermented foods in Africa are classified on the basis of either the raw cereal type used for preparation, or the texture of the fermented product (Nout, 2009). The two types of cereal-based fermented foods majorly produced in Africa are alcoholic food beverages and non-alcoholic foods (Olasupo et al., 2010). The non-alcoholic fermented foods are the focus of this review. Fermented cereal foods that are largely consumed across Africa include *ogi* (Nigeria/West Africa), *uji* (Kenya), *mahewu* and *motoho* (Southern Africa), *kenkey* (Ghana), and *ben-saalga* (Burkina Faso), *togwa* (Tanzania), *bogobe* (Botswana) among others (Table 2.1). However, fermented maize gruel (ogi) and sorghum beverage which were the focused of this research will be discussed in detail.

Table 2.1: Traditional African fermented foods, the substrate used for their production and microorganisms associated with the fermentation process

Fermented food	Substrate used	Country	Associated microorganisms	Reference
Non-alcoholic cereal based fermented foods				
Bushera or *Obesera*	Finger millet, sorghum	Uganda	*Lactobacillus plantarum*, *Lactobacillus paracasei* subsub *paracasei*, *Lactobacillus fermentum*, *Lactobacillus brevis*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactococcus lactis* subsp *lactis*, *Leuconostoc mesenteriodes*, *Weissella confusa*, *Enterococcus* spp., *Pediococcus pentosaceus*, *Leuconostoc lactis*, *Streptococcus infantarius* subsp. *infantarius*, *Saccharomyces cerevisiae*	Mukisa et al., 2012; Muyanja et al., 2003
Degue, Ben-saalga	Pearl millet	Burkina Faso	*Pediococcus* spp., *Lactobacillus* spp. *Lactobacillus fermentum, Leuconostoc*, and *Weissella* genera	Tou et al., 2006; Humblot and Guyot, 2009;
Hussuwa	Sorghum	Sudan	*Lactobacillus fermentum, Pediococcus acidilactici, Enterococcus faecium,*	Yousif et al., 2010
Injera	Sorghum	Ethiopia	*Candida gullermondii*, *Pullaria* sp., *Aspergillus* sp., *Penicillium* sp., *Rhodotorulla* sp., *Homodendrum* sp.	Ashenafi, 2006; Steinkraus, 1983
Kisra,	Sorghum	Sudan	*Pediococcus pentosaceus, Lactobacillus confusus, Lactobacillus brevis, Lactobacillus* sp., *Erwinia ananas, Klebsialla pneumonia, Enterobacter cloacae, Candida intermedia, Debaryomyces hansenii, Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., *Rhizopus* sp.	Mohammed et al., 1991
Koko, kenkey	Maize, sorghum, pearl millet	Ghana	*Weissella confusa, Lactobacillus fermentum, Lactobacillus* salivarus, *Pediococcus pentosaceus, Pediococcus acidilactici* and *Lb. paraplantarum* and yeast	Lei and Jakobsen, 2004
Kunu-zaki	Pearl millet, sorghum	Nigeria	*Weissella confusa, Lactobacillus fermentum, Lactobacillus amylolyticus, Lactobacillus delbrueckii* subsp. *bulgaricus, Bacillus* spp., and *Lactococcus lactis* spp *lactis*, *Streptococcus lutetiensis* and *Streptococcus gallolyticus* subsp. *macedonicus*	Oguntoyinbo et al., 2012
Mahewu	Maize, sorghum, pearl millet	South Africa, Zimbabwe and Lesotho	*Lactobacillus delbrueckii* subsp. *bulgaricus, Lactococcuslactis* subsp. *lactis Lactobacillus delbrueckii* subsp *delbrueckii, Leuconostoc* spp., and Heterofermetative *Lactobacilli*	Blandino et al., 2003; Steinkraus, 1997
Mawe	Maize	Benin	*Lactobacillus fermentum, Lactobacillus cellobiosus, Lactobacillus brevis, Lactobacillus confuses, Lactobacillus buchneri, Lactococcus lactis, Leuconostoc mensenteroides, Candida krusei, Candida kefyr,* and *Saccharomyces cerevisiae*	Hounhouigan et al., 1993, 1994.

Table 2.1 (continued)

Fermented food	Substrate used	Country	Associated microorganisms	Reference
Non-alcoholic cereal based fermented foods				
Motoho	Sorghum	Southern Africa	Unknown	Gadaga et al., 2014
Ogi	Maize, sorghum, pearl millet	Nigeria	*Pediococcus pentosaceus, Lactobacillus fermentum, Lactobacillus plantarum, Saccharomyces cerevisiae, Candida krusei, Lactococcus raffinolactis, Lactococcus garviae, Lactococcus* sp., *Lactobacillus paracasei* subsp. *tolerans, Pediococcus pentocaseous* subsp. *intermedius, Lactobacillus suebicus, Streptococcus* sp., *Lactobacillus brevis*	Odunfa and Adeyele, 1985;Teniola et al., 2005; Sanni et al., 2013
Togwa	Sorghum	Tanzania	*Lactobacillus plantarum, Lactobacillus brevis Lactobacillus fermentum, Lactobacillus cellobiosus, Pediococcus pentosaceus, Weissella confusa, Issatchenkia orientalis, Saccharomyces cerevisiae, Candida pellicullosa, Candida tropicalis*	Mugula et al., 2003
Ting	Sorghum	Botswana/South Africa	*Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus rhamnosus, Lactobacillus parabuchneri, Lactobacillus casei, Lactobacillus coryniformis, Lactobacillus curvatus Weissella cibaria, Lactobacillus harbinensis, Lactobacillus reuteri*	Madoroba et al., 2009; 2011; Sekwati-Monang and Ganzle, 2011
Uji	Maize, sorghum, finger millet	Kenya	*Lactobacillus plantarum, Lactobacillus fermentum, Lactobacillus cellobiosus, Lactobacillus buchneri, Pediococcus acidilactici, Pediococcus Pentosaceus* and *Lactobacillus mesenteroids*	Mbugua and Njega, 1991; Onyango et al., 2003, 2004
Fermented dairy food				
Amasi	Milk	Southern Africa	*Lactobacillus helveticus, Lactobacillus plantarum, Lactobacillus delbrueckii subsp. lactis, Lactobacillus paracasei* subsp. *paracasei, Lactobacillus paracasei* subsp. *pseudoplantarum, Lactobacillus lactis, subsp lactis, Lactobacillus acidophilus, Leuconostoc mesenteroides* subsp. *mesenteriodes, Enterococcus faecum* and *Enterococcus faecalis*	Mutukumira et al., 1996; Feresu and Muzondo, 1990; Beukes et al., 2001; Kebede et al., 2007
Ergo	Milk	Ethiopia	*Streptococcus thermophilus, Streptococcus acidominus, Enterococcus faecalis* var. *liquefaciens, Streptococcus bovis, Strep mitis, Streptococcus agalactiae, Lactoccus Cremoris, Leuconostoc dextranicum, Leuconostoc lactis, Lactobacillus xylosus, Lactobacillus. Latis.*	Assefa et al., 2008
Kule naoto	Milk	Kenya	*Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus paracasei*, *Lactobacillus acidophilus*, *Lactococcus* spp., *Enterococcus* spp., *Leuconostocs* spp.	Mathara et al., 2004
Wara	Milk	West Africa	*Lactobacillus delbrueckii, Serratia marcesens, Lactobacillus casei, Corybacterium* spp., *Staphylococcus aureus*	Adeleke et al., 2014
Omashikwa	Milk	Namibia	*Lactobacillus rhamnosus, Lactococcus lactis ssp. Lactis, Lactobacillus plantarum*	Bille et al., 2007; Heita and Cheikhyoussef, 2014

Ogi

Ogi is a traditional fermented gruel processed from maize, sorghum or pearl millet and has been produced on a semi-industrial scale. In many West African communities, *ogi* is the most important major traditional fermented food for weaning infants especially among the low income earners that cannot afford imported baby food (Abdus-Salaam et al., 2014). Along the West African coastal region the product is given other names such as *eko*, *agidi*, *kamu*, *akamu*, *koko* and *furah* depending on the substrate used and the form in which it is eaten (Blandino et al., 2003). The production of *ogi* is usually through uncontrolled spontaneous fermentation with LAB, yeasts and moulds being responsible for the fermentation, although *L. plantarum* is the predominant microorganism (Teniola et al., 2005; Omemu and Adeosun, 2010). The stages of traditional *ogi* production include steeping (soaking the grains in water for 24-72 h, wet milling and sieving) and souring (sedimentation of the filtrate for 12-48 h) to obtain fermented *ogi* (Teniola and Odunfa, 2002). The wet *ogi* usually has a smooth texture, a sour flavour akin to that of yoghurt and a characteristic aroma that differentiates it from other cereal-based fermented foods (Omemu, 2011).

Pathogenic *E. coli* has been isolated at different stages during processing of *ogi* and also in the final product which is an indication that spontaneous fermentation may not guaranteed the safety of *ogi* used as local weaning food (Omemu and Adeosun, 2010). However, the occurrence and survival of non-O157:H7 STEC has not been reported in ogi., Further, the unhygienic conditions in many rural communities under which local production of *ogi* occurs and poor storage are limitations to the microbiological quality of *ogi* and pose a risk to human health (Omemu and Adeosun, 2010).

Motoho

Motoho is a fermented non-alcoholic, thin sorghum gruel, commonly used as refreshing beverage as well as a weaning food among the Basotho people of South Africa and Lesotho. It is usually produced with red non-tannin type sorghum which gives the product its characteristics brownish colour (Gadaga et al., 2014). *Motoho* is usually produced via spontaneous fermentation which can yield products with inconsistent quality (Gadaga et al., 2014). The traditional preparation of motoho involves mixing the sorghum meal with warm water to make thin slurry. Occasionally, a traditional starter culture called *tomoso* (previously fermented sorghum slurry) is added as inoculum (usually 1 part of *tomoso* to 20 parts of the

slurry) to accelerate fermentation and the mixture is allowed to ferment for 24-48 h and the fermented product is then boiled for 20-30 minutes (Gadaga et al., 2014).

Although *motoho* has been successfully produced commercially, the scientific literature on the microbiology of *motoho* and the standardization of its preparation is very limited. Sakoane and Wash (1987) observed that *Salmonella typhi* inoculated at low dilution were able to grow in autoclaved *motoho*, raising concerns about the safety of the product.

2.1.2 Microorganisms associated with cereal-based fermented foods

Fermentation is the process by which a substrate is subjected to biochemical modification resulting from the activity of microorganisms and their enzymes (Gotcheva et al., 2000). The microorganisms that are commonly associated with cereal-based traditional African fermented foods are shown in Table 2.1. Yeasts, LAB, fungi, or mixtures of these are mainly responsible for natural cereal-based fermentation. Generally, it can be stated that yeasts perform carbohydrate metabolism, whereas bacteria contribute to the sensory and safety aspects of the final products (Todorov and Holzapfel, 2015). At the early stages of fermentation, contaminating microorganisms may increase slowly in number and compete for nutrients in order to produce metabolites (Holzapfel, 2002). Cereal-based fermented foods are dominated by LAB or a mixed population of LAB and yeasts (Galati et al., 2014). The LAB commonly associated with cereal-based traditional African fermented foods generally include the genus *Lactobacillus*, *Leuconostoc*, *Weissella*, *Pediococcus* (Table 2.1). Among the LAB, lactobacilli are predominantly associated with cereal fermentation where their acidification process serve as a preservative and other fermentation by-product improve the quality of the fermented product (Sanni et al., 2013). The dominant lactobacilli in various cereal-based traditional fermented foods are *L. plantarum* and *L. fermentum* strains, followed by species of *Pediococcus* (Olasupo et al., 2010; Njeru et al., 2010; Yousif et al., 2010; Oguntoyinbo and Narbad, 2012; Owusu-Kwarteng et al., 2012; Franz et al., 2014).

The involvement of yeasts in co-existence with other microorganisms especially LAB in the spontaneous fermentation of different types of cereal-based fermented foods has been reported (Table 2.1). Yeasts are responsible for sugar fermentation, production of secondary metabolites, and inhibition of the growth of mycotoxin-producing moulds as well as possession of several enzymatic activities such as lipolytic, proteolytic, pectinolytic and

urease activities (Tamang and Fleet, 2009). The commonly isolated species of yeasts from a wide range of cereal-based African fermented foods include *S. cerevisiae*, *Rhodotorula graminis*, *C. krusei, C. tropicalis, Geotrichum candidum* and *Geotrichum fermentum* (Steinkraus, 2002; Mugula et al., 2003; Teniola et al., 2005; Omemu, 2011).

2.1.1 Traditional fermented dairy products

Milk from cattle, sheep and goats is typically fermented in Eastern and Southern Africa, and some regions in North Africa and occasionally West Africa. Camel's milk is mainly fermented in Northern Africa and the Sudan region (Olasupo et al., 2010; Karenzi et al., 2013; Franz et al., 2014). Milk fermentation as practised in many rural areas in Africa is mainly by traditional, small scale 'sour milk technologies' to convert milk into various products for extending the shelf life (Table 2.1).

Amasi is a traditional fermented milk fermented in Southern Africa (Gadaga et al., 1999; Mufandaedza et al., 2006). Traditional *amasi* is produced by fermenting milk spontaneously at ambient temperature for one to four days. Backslopping is occasionally used to select for strains that are best for fermentation. The quality of *amasi* varies with regions and season due to variations in fermenting temperatures and fermenting microorganisms (Gadaga et al., 2007). It has been shown that commercial *amasi* may be an ideal vehicle for the delivery of probiotics (Master et al., 2008). Numerous types of LAB and other microorganisms such as yeasts have been cited to be responsible for fermentation in *amasi* (Table 2.1). The survival of *E. coli* O157:H7 in traditional and commercial amasi for a period of 3 days has been reported (Dlamini and Buys, 2009)

Ergo, a traditional fermented sour milk in Ethiopia, is usually produced by natural fermentation of milk at ambient temperature, without the addition of starter cultures (Assefa et al., 2008). The use of backslopping as a starter in highland areas where ambient temperature is relatively low has been reported (Kassa, 2008). Lactic acid bacteria genera that are associated with the fermentation of *ergo* include *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Entrococcus* and *Streptococcus* (Assefa et al., 2008). High counts of Enterobacteriaceae and coliforms (< log 4 cfu/mL) have been reported in *ergo* (Yilma, 2012). Tsegaye and Ashenafi (2005) reported that *E. coli* O157:H7 can survive the fermentation and processing of e*rgo*. The effect of lower pH of e*rgo* in controlling the proliferation of pathogenic microorganisms has been shown to be more effective after 24 hours of incubation (Ashenafi, 2006). However, at this stage, the *ergo* is considered to be too sour for direct

consumption since *ergo* coagulates within 24 hours and is preferably consumed at this stage for its good flavour (Ashenafi, 2006).

Kule naoto, traditional fermented milk produced in Kenya and Tanzania, is the major daily diet of the Maasai community. It constitutes an important part of the daily diet of the Maasai community in Kenya which rarely consume fruits or grains (Mathara et al., 2004). Kenyans appreciate the product for its excellent natural taste and aroma and believe that consumption helps in protecting against diarrhoea and constipation (Franz et al., 2014). *L. plantarum* is the dominant microorganism in the fermentation of *kule naoto*, followed by *Enterococcus*, *Lactococcus* and *Leuconostoc* species (Mathara et al., 2004).

Wara is a soft unripened cheese produced by the addition of plant extracts of Sodom apple (*Calotropis procera*) to unpasteurized whole cow's milk (Adeyemi and Umar, 1994; Uzeh et al., 2006). *Wara* is eaten fresh, used as a meat substitute in stews, or fried and eaten as a snack (Osuntoki and Korie, 2010). It is mostly produced in homes, especially in villages where the shelf-life and safety of the product cannot be guaranteed. Pathogenic bacteria such as *Staphylococcus aureus* and *E. coli* have been reported in *wara* and were attributed to poor hygiene practised by handlers during product preparation (Uzeh et al., 2006).

2.1.2 Safety challenges associated with traditional African fermented foods

Spontaneous or natural fermentation of foods as commonly practised in Africa has been reported to provide an antagonistic environment against pathogenic bacteria due to the production of organic acids that lower pH to a level inhibitory to some pathogenic organisms (Omemu et al., 2007; Olasupo et al., 2010; Abdus-Salaam et al., 2014; Lei and Jacobsen, 2004; Amoa-Awua et al., 2007; Kalui et al., 2009). Some of the microorganisms that are associated with the traditional African fermented foods could enhance the safety quality of the products by inhibiting foodborne pathogens. However, there is lack of control of the fermentation process that unavoidably results in significant variation in the quality and microbiological safety of the fermented foods (Oguntoyinbo, 2014).

Traditional fermented foods are mostly prepared using rudimentary equipment and processes are done without good hygiene practices (GHP). Hazard analysis critical control points (HACCP) are also unknown to many small-scale food processors, both in rural and urban centres (Ehiri et al., 1997). Occurrence of foodborne pathogens such as *Bacillus cereus*,

Clostridium perfringens, *Aeromonas* spp., *Staphylococcus aureus* and various *E. coli* serotypes have been reported in cereal-based traditional fermented foods (Kunene et al., 1999; Nyatoti et al., 1997).

The presence of potentially pathogenic microorganisms in cereal-based traditional fermented products, especially those that do not undergo a thermal process before consumption, represents a great risk to human health (Galatia et al., 2014). For instance, in some communities in south-western Nigeria, uncooked ogi is diluted with water and administered to people with diarrhoea (Steinkraus, 2002; Aderiye and Laleye, 2003; Omemu et al., 2010). There is also a common practice of diluting cooked traditional fermented foods with unwholesome water that may not be potable before feeding it to infants. These practises increase the possibility of traditional fermented complementary foods of being contaminated with pathogens (Bakare et al., 1998). Contaminated traditional complementary foods have been reported as the major causes of diarrhoea, acute gastroenteritis and outbreaks of infectious communicable diseases in infants and young adults in developing countries (Jay et al., 2005).

2.2 *Escherichia coli*

The German bacteriologist-paediatrician Theodore von Escherich was the first to discover *Escherichia coli* (*E. coli*) in the gut in 1885 and called it *Bacterium coli commune* (Bettelheim, 2007). Since then it has become one of the most studied microorganisms (Bettelheim, 2007). *E. coli* belongs to the Enterobacteriaceae family and the *Escherichia* genus. It is a facultative anaerobic, rod-shaped, Gram-negative bacterium, non-spore-former, and some strains may be motile with peritrichous flagella (Holt et al., 1994). *E. coli* was initially regarded as a commensal bacterium that co-exists with its human host in the intestine in a mutually beneficial relationship (Tchaptchet and Hansen, 2011). It was first associated with human illness in the early 1940s, when it was linked to infant diarrhoea (Bray and Beavan, 1948).

E. coli can be classified based on the unique polysaccharide antigens expressed on the outer membrane (O antigens) and by the protein antigens on their flagella (H antigens). *E. coli* is capable of growing over a wide range of temperature (between 7 ^{o}C and 46 ^{o}C), although the optimum temperature is 37 ^{o}C. It is also capable of growing over a wide range of pH values, from pH 4.5 to pH 9 (ICMSF, 1996). However, the nature of the acidifying agent, the

serotype and particular environmental conditions are very crucial for the growth of *E. coli* in acidic environment (Grant et al., 2011).

2.2.1 Pathotypes of *E. coli*

Pathogenic *E. coli* that are capable of causing diarrhoea have been grouped into six major pathotypes by Okeke (2009) based on their virulence properties, mechanisms of pathogenicity, clinical symptoms or disease manifestation, and the presence of distinct O and H antigens (Table 2.2). The *E. coli* pathotypes that are capable of causing gastrointestinal infections and diarrheal disease are enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), diffuse adhering *E. coli* (DAEC), enteroaggregative (EAggEC) and Shiga toxin producing *E. coli* (STEC), often referred to as enterohaemorrhagic *E. coli* (EHEC). Among all the aetiological agents implicated in the causation of diarrhoea, STEC has the potential to cause the most severe clinical symptoms (Eblen, 2007). It is believed to be the second most frequent implicated pathogens after rotavirus (Gómez-Duarte et al., 2013). STEC have the capacity to cause bloody diarrhoea, haemolytic-uraemic syndrome (HUS) and Attaching and effacing (A/E) lesions in the intestine (Melton-Celsa et al., 2012).

Table 2.2: Classification and characteristics of diarrheagenic *E. coli* pathotypes (adapted from Okeke, 2009)

Pathotypes	Evidence for pathogenicity	Adherence pattern	Principal virulence factors
Enteropathogenic *E. coli* (EPEC)	Epidemiologic associations, outbreaks, human volunteer challenge	Localized	Locus for enterocyte effacement (LEE) encoding intimate adhesin, intimin, type III secretion system and secreted effectors, secreted effectors encoded outside the LEE, bundle forming pili and other virulence-plasmid genes, EspC enterotoxin
Enterotoxigenic *E. coli* (ETEC)	Epidemiologic associations, human volunteer challenge	Weak diffuse	Heat stable toxin (ST), Heat labile toxin (ST), colonization factor antigens (CFAs), longus pilus, Tia invasin
Enteroinvasive *E. coli* (EIEC) and *Shigella*	Epidemiologic associations, outbreaks	Variable	Invasion plasmid encoding type III secretion system and secreted effectors, IcsA, *Shigella* enterotoxins, Shiga toxins (*Stx*; *Sh. dysenteriae*), pathoadaptive deletions in house-keeping genes
Shiga toxin producing *E. coli* (STEC) or Enterohaemorrhagic *E. coli* (EHEC)	Epidemiologic associations, outbreaks, individual illnesses	Variable	LEE, Shiga toxins (*Stx*), virulence plasmid encoded enterohaemolysin, accessory adhesins
Enteroaggregative *E. coli* (EAEC)	Epidemiologic associations, outbreaks, human volunteer challenge	Aggregative	Dispersin, Aggregative Adherence Fimbriae (AAF; multiple types), Plasmid-encoded toxin, EAEC heat-stable toxin (EAST-1), accessory adhesins
Diffusely-adherent *E. coli* (DAEC)	Epidemiologic associations	Diffuse	Diffuse adhesin (Daa), AIDA adhesion, other adhesins

2.2.2 Shiga toxin producing *E. coli* (STEC)

Shiga toxin-producing *E. coli* (STEC) are also known as EHEC. They were first described in 1977 during identification of the virulence factors that were responsible for the pathogenesis of diarrheal disease caused by *E. coli* (Konowalchuk et al., 1977). The terms Shiga toxin (*Stx*) and verotoxin are interchangeable and they refer to the production of cellular cytotoxins (Mathusa et al., 2010). STEC are so named because they produce Shiga toxin 1 (*Stx1*) and/or Shiga toxin 2 (*Stx2*) (Farrokh et al., 2013). They are zoonotic foodborne and waterborne pathogens that are of serious public health concern due to their ability to cause a number of life-threatening diseases (Kanki et al., 2011; Coombes et al., 2011).

2.2.2.1 Pathogenicity and virulence factors in STEC

Pathogenicity in STEC is linked to several virulence factors which allow the organism to attach and colonize the bowel, invade tissues, and produce toxins that contribute to disease symptoms and disease progression (Grant et al., 2011). The ability of STEC to cause serious disease in humans depends on their ability to produce *Stx* (Eblen et al., 2007; Farrokh et al., 2013). Grant et al. (2011) identified *Stx* production, attaching and effacing (A/E) and plasmid mediated factors as virulence factors associated with STEC infections in humans.

Shiga toxins (*Stx*) production

Shiga toxin 1 is very similar to the type 1 toxin of *Shigella dysenteriae,* while *Stx*2 is genetically and immunologically distinct with 55–60% similarity in genetic and amino acid sequence (Ethelberg et al., 2007). STEC strains that produce *Stx2* are likely to cause haemolytic ureamic syndrome (HUS) than those that produce *Stx1* alone (Friedrich et al., 2002). Shiga toxin belongs to the A_1B_5 family of toxins that consists of the enzymatically active A-subunit within a pentameric complex of B-subunits (O'Brien et al., 1992). The B-subunit binds the toxin to a glycosphingolipid on the surface of host vascular endothelial cells, whereas the A subunit is a N-glycosidase, which cleaves adenine residues in the rRNA of host cell ribosomes causing protein synthesis inhibition and cell death (Betz et al., 2012). Shiga toxins are capable of binding the cellular receptors and inhibiting the protein synthesis in several organs such as kidney, brain and liver, causing severe diseases (Smith and Fratamico, 2014). Among various virulence factors involved in non-O157 STEC pathogenicity, the combined effect of *eae* and *Stx* genes has been associated with enhanced virulence (Mathusa et al., 2010).

Exposure to these toxins is generally through the consumption of contaminated foods (Rangel et al., 2005). The presence of STEC in food could result in high levels of *Stx* production and consumption of such contaminated food has the potential to pose a serious health risk (Weeratna and Doyle, 1991; Rasooly and Do, 2010). Rasooly and Do (2010) revealed that thermal treatment such as pasteurization did not inactivate *Stx* in food. In addition to *Stx*, STEC strains additionally contain the locus of enterocyte effacement (LEE), a plasmid-borne enterohaemolysin and other virulence genes (Table 2.2).

Attaching and effacing (A/E)

Shiga toxin producing *E. coli* have the capacity to intimately attach to and efface intestinal epithelial cells, a histopathological feature known as the attaching and effacing lesion (A/E) (Melton-Celsa et al., 2012). STEC have the ability to colonize the large intestine and produce a characteristic induced A/E lesson by bacterial secretion systems (Grant et al., 2011). These systems include structural components of a type III secretion system (TTSS), intimin, and translocated intimin receptor (Tir) and other effector proteins (Garmendia, et al., 2005). In addition, some STEC strains can tightly attach and form A/E lesions to intestinal epithelial cells through an adhesin called intimin, which is encoded by the *eae* gene (Elhadidy and Mohammed, 2013). Most STEC infections that are associated with severe HUS are caused by A/E pathogens that carry the locus of enterocyte effacement (LEE) (Newton et al., 2009). The proteins needed for the A/E lesion are encoded within this large pathogenicity island (i.e LEE) (Melton-Celsa et al., 2012). Therefore, STEC containing LEE are characterized by their ability to attach to the host intestinal mucosa and destroy the surrounding microvillus brush border, which causes substantial cytoskeletal rearrangements within the enterocyte (Frankel et al., 1998). After attachment, *Stx* is absorbed into the host cell through a transcellular pathway (Nataro and Kaper, 1998). It has been shown that some non-O157 STEC serotypes without LEE, such as STEC O113:H21, are associated with sporadic and outbreak cases of severe disease, which is indistinguishable from that caused by STEC O157:H7 (Elliot et al., 2001).

Plasmid mediated factors

Many STEC serotypes possess a highly conserved plasmid, such as pO157, pSFO157 and pO113 (Brunder et al., 2006; Newton et al., 2009). Large plasmids encoding EHEC haemolysin (*Ehx*) are found in almost all disease-associated STEC strains (Newton et al., 2009). The possible function of pO157 in pathogenesis is unclear (Melton-Celsa et al., 2011).

However, *in vitro* and *in vivo* studies demonstrated a possible role of pO157 in adherence to host epithelial cells (Dziva et al., 2007). Abu-Ali et al. (2010) and Karmali et al. (2003) suggested that strains that contain an intact large plasmid or express high levels of plasmid-encoded products are more often associated with epidemics and HUS than those without pO157. Non-O157 STEC O26:H11 and O113:H21 serotypes possess a large plasmid which is similar to plasmids in *E. coli* O157:H7 (Newton et al., 2009).

2.2.2.2 Acid stress in STEC

Acid stress can be described as the combined biological effect of low pH and weak (organic) acids, such as acetate, propionate and lactate present in food as a result of fermentation, or alternatively, when added as preservatives on pathogenic bacteria (Zhao et al., 1993; Smith and Fratamico, 2012). The survival of foodborne pathogens in a particular food and in the gastrointestinal tract depends on their resistance to acid stress (Sainz et al., 2005). STEC strains are considered to be one of the greatest microbiological challenges to the food industry because they are highly resistant to stress conditions, including low pH which enhances their survival in acidic foods (Beutin et al., 2007; Elhadidy and Mohammed, 2013). Pre-exposure of bacteria to mild acidic conditions may induce adaptation to subsequent lethal levels of acid stress (Rodriguez-Romo and Yousef, 2005). This phenomenon is known as acid resistance.

The acid tolerance response (ATR) is characterized by the resistance of bacteria to a lethal acid stress if the organisms are pre exposed (adapted) to a non-lethal level of acid for a period of time before exposure to a lethal pH (Foster and Hall, 1990). Acid resistance (AR) in *E. coli* is the ability to withstand an acid challenge of pH 2.5 or less and is a trait generally restricted to stationary-phase cells (Castanie-Cornet et al., 1999). It has been shown that exposure of *E. coli* log phase cells to pH 5.5 and pH 4.5 induces the synthesis of acid shock proteins (ASPs) which most likely are responsible for the induction of ATR (Paul and Hirshfield, 2003).

Studies have evaluated the effect of acid stress on the growth of *E. coli* O157:H7 (Dong and Schellhorn, 2009; Vanaja et al., 2010) but there is a paucity of information concerning the effect of acid stress on the growth and survival of the non-O157 STEC serotypes in food. Mathusa et al. (2010) suggested that the stress interventions that are detrimental to *E. coli* O157:H7 may also inactivate non-O157 STEC serotypes. However, Smith and Frantamico

(2012) argued that non-O157 STEC serotypes may not behave similarly to *E. coli* O157:H7 when exposed to acid stress or to food environments. For instance, Bergholz and Whittam (2007) demonstrated that the transcription levels of the glutamate decarboxylase system genes (*gadA* and *gadB*) that codes for acid tolerance were higher in *E. coli* O157 than in non-O157 STEC. The study of Enache et al. (2011) reported that *E. coli* O157:H7 had higher D-values than the individual six non-O157 STEC serotypes (O26, O45, O103, O111, O121, O145) when exposed to the acid stress in apple juice. Bergholz and Whittam (2007) also found that *E. coli* O157:H7 can survive in acidic environments of fermented foods and simulated gastric acidity than non-O157 STEC serotypes.

In contrast, Molina et al. (2003) showed that some non-O157 STEC serotypes such as O91:H21, O171:H2, O26:H11 and O111:H¯ can survive acidic condition better than *E. coli* O157:H7. Large et al. (2005) used the oxidative system and the glutamate and arginine decarboxylase systems to evaluate the survival of *E. coli* O157:H7 and non-O157 STEC serotypes. These authors found that *E. coli* O157:H7 survival rates were lower than those of the non-O157 STEC strains in minimal medium at low pH and concluded that *E. coli* O157:H7 strains are not exceptionally acid resistant in comparison to certain strains of non-O157 STEC serotypes. Further, Berry et al. (2004) demonstrated that a greater number (2 to 2.5 fold) of the *E. coli* O157:H7 cells were injured by the 6-h exposure to pH 2.5 than the non-O157 STEC regardless of the absence or presence of glucose in the growth medium.

Mechanism of acid resistance in STEC

The three major acid-resistance (AR) mechanisms that can protect STEC cells against low pH as identified in *E. coli* O157:H7 are: oxidative or glucose-repressed system (AR-1), glutamate decarboxylase-dependent system (AR-2) and arginine decarboxylase-dependent system (AR-3). The oxidative system is induced when STEC strains are grown to the stationary phase in glucose-free Luria-Bertani (LB) broth buffered to pH 5.5. The glutamate and arginine decarboxylase-dependent systems are required for protection at extreme low pH of 2.5 (Lin et al., 1995, 1996; Hersh et al., 1996; Smith et al., 2012). All three systems were identified in stationary-phase cells. The induction of the AR-1, AR-2, and AR-3 systems enables STEC to resist the extreme acidic conditions such as those encountered in the stomach and in acidic food products (Smith et al., 2012).

The oxidative or glucose-repressed system also known as the AR-1 system depends on the alternative sigma factor (σ^S), encoded by the *rpoS* gene (Battesti et al., 2011). The stationary phase σ^S (*rpoS*), global regulatory protein and cAMP receptor protein (CRP) are all required for induction of AR-1 (Battesti et al., 2011). The *rpoS* gene controls the expression of more than 50 proteins that are mostly involved in the general stress response in STEC, including acid resistance (DebRoy et al., 2011). In many situations, the AMP cyclic receptor protein is needed for transcriptions (Castanie-Cornet et al., 1999). Variation in *rpoS* induction levels has been shown to be responsible for the variability in the acid resistance ability of different *E. coli* O157:H7 strains (Bergholz and Whittam, 2007).

The glutamate decarboxylase system (AR-2) requires glutamate, one of the two glutamate decarboxylase genes (*gad*A or *gad*B) and the *gad*C gene encoding for glutamate/g-aminobutyric acid antiporter for protection at the extreme of pH 2.5 (Castanie-Cornet et al., 1999; Smith et al., 2012). Glutamate is transported into the cell via the antiporter *gad*C and is decarboxylated by *gad*A or *gad*B to γ-aminobutyric acid with the uptake of a proton. The incorporation of the proton by γ-aminobutyric acid production decreases the internal pH of the bacterial cell. The γ-aminobutyric acid is then transported out of the cell via *gad*C in exchange for a glutamate entering the cell (Foster, 2004). The glutamate decarboxylase system is believed to be the most effective mechanism for the protection of EHEC from the acid conditions of the stomach (Smith et al., 2012).

The arginine decarboxylase system (AR-3) requires arginine, the arginine decarboxylase gene (*adiA*), the arginine/agmatine antiporter gene (*adiC*), and the regulator *cysB* (Castanie-Cornet et al., 1999; Smith et al., 2012). Arginine is transported into the cell via the *adi*C antiporter and is decarboxylated by *adi*A to agmatine with uptake of a proton. Agmatine is then transported out of the cell via *adi*C in exchange for arginine entering the cell (Foster, 2004).

Other defence mechanisms which have been reported to protect *E. coli* cells at low pH include the internal pH homeostasis system (Foster, 2004) and the system for repairing and protecting essential cellular components (Choi et al., 2000). Further, growth in mild acid may also be enhanced by components of metabolism and transport such as the lysine and ornithine decarboxylase systems and by cyclopropane fatty acid synthase (Yuk and Marshall, 2004; Dlamini and Buys, 2009; Zhao and Houry, 2010). It has been shown that some non-O157

STEC strains utilize a chaperone-based acid stress response (*HdeA* and *HdeB*) to combat acidic conditions, which is lacking in *E. coli* O157:H7 (Smith and Fratamico, 2012).

2.2.2.3 Occurrence and survival of STEC in fermented and acidic foods

Fermentation prevents the growth of pathogenic microorganisms in fermented foods by disrupting the internal environment of the cells such that growth is no longer possible (Beales, 2004). Undissociated organic acids have the ability to penetrate the bacterial cell and release protons after dissociation within the bacteria cells, leading to an increase in intracellular acidity and eventual cell inactivation (Chung et al., 2006; Jones, 2012). However, the ability of some pathogenic bacteria such as *E. coli* to adapt to acidic environment in fermented foods and subsequently grow has been of great concern over the years (Deng et al., 1998). The survival of pathogens in fermented foods has also raised issues concerning the safety of fermented foods with emphasis on the possibility of failure of natural fermentation to enhance food preservation (Oguntoyinbo, 2014).

Acidic ready-to-eat foods, such as apple cider (pH 3.6-4.0) and beef sausage (pH 4.5-5.0), have been implicated in STEC foodborne illnesses and outbreaks (Besser et al., 1993; Tilden et al., 1996; Ethelberg et al., 2009). It has been shown that *E. coli* O157:H7 can survive the acid stress of fermented foods such as unpasteurized orange juice (pH 3.9-4.2) (Parish, 1997), goat's milk amasi (Dlamini and Buys, 2009), traditional Africa fermented maize porridge (Kwaw, 2014) and *Borde*, an Ethiopian fermented millet beverage (Tadasse et al., 2005). Tsegaye and Ashenafi (2005) confirmed that *E. coli* O157:H7 may also survive during the processing and storage of fermented dairy foods such as lactic cheese and *ergo*. However, most of these survival studies in fermented foods have been undertaken using *E. coli* O157:H7 strains and little is known about the survival of non-O157 STEC serotypes in fermented foods.

2.2.3 *E. coli* O157:H7 serotype

In 1982, infection with *E. coli* O157:H7 strains was first linked to haemorrhagic colitis (HC) and HUS, a new foodborne zoonosis (Riley et al., 1983). Since then, *E. coli* O157:H7 has been the most common STEC serotype implicated in sporadic and epidemic human diarrheal disease (Coombes et al., 2011). *E. coli* O157:H7 is a Gram-negative rod-shaped enteric bacterium which produces *Stx1* and *Stx2* virulence factors (Chauret, 2011). It is the most

widely recognized serotype of pathogenic *E. coli* associated with foods (Grants et al., 2011). This pathogen has received the most attention by the scientific community because of its apparent ability to survive food processing procedures that had previously assured food safety, and also its association with several large outbreaks of human illness with severe manifestations (Eblen, 2007; Grant et al., 2011). However, identification of a heterogeneous group of *E. coli* that express an O surface antigen other than 157 as foodborne pathogens is on the increase worldwide (Gould et al., 2013; Wang et al., 2013; Bettelheim and Goldwater, 2014).

2.2.4 Non-O157 STEC as an emerging foodborne pathogen.

Non-O157 STEC serogroups have been recognised as emerging pathogens and increasingly linked to foodborne infections and illnesses worldwide (Farrokh et al., 2013). Non-O157 STEC accounts for a substantial portion of all STEC infections worldwide (CDC, 2007; Hedican et al., 2009). It has been estimated that 20-50% of all STEC infections can be attributed to non-O157 strains, but the percentages vary from country to country and among regions within a country (Mathusa et al., 2010). Non-O157 STEC serotypes are a challenging problem because, unlike O157, they have no unique or distinguishing physiological features or phenotypic characteristics to readily distinguish them from other non-pathogenic *E. coli* strains (Grant et al., 2011).

Some groups of non-O157 STEC strains are capable of causing outbreaks and severe disease such as HUS and HC, whereas others are associated with mild diarrhoea (Coombes et al., 2011). The diversity of the serotypes that cause disease, the infective dose as well as the ability of the pathogen to survive in foods and in the host gut also influence the pathogenicity of non-O157 STEC (Grant et al., 2011; Mellies et al., 2007). Studies have shown that the number of non-O157 STEC infections can nearly equal or in some regions exceed that of STEC O157:H7 infections (Couturier et al., 2011; Cooley et al., 2013). Therefore, non-O157 STEC serotypes pose just as great risk to public health as *E. coli* O157:H7 (Eblen, 2007) but their infections have been under-recognised and under-reported around the world (Hadler et al., 2011).

2.2.4.1 Epidemiology and outbreaks of non-O157 STEC

Outbreaks attributed to non-O157:H7 STEC have been reported in the USA, Europe, Australia, South America and Japan (Table 2.3). The reported cases of non-O157 STEC serotypes in foods from different countries revealed a wide variation in prevalence estimates (Hussein and Sakuma, 2005; Erickson and Doyle, 2007). More than 50% of all confirmed STEC infections in Europe are caused by non-O157 STEC serotypes (Atkinson et al., 2006). In Canada, 63% of the total STEC infections were caused by non-O157 STEC (Thompson et al., 2005), and 60% in the USA (Hale et al., 2012). Non-O157 STEC was responsible for 27% of the total STEC-induced infections in Ireland in 2008 (Garvey et al., 2015). Outside the UK, USA and Canada, the most frequently reported non-O157 STEC serotypes have been O26:H11, O103:H2, O111:H- and O145:H- (Paton and Paton, 1998; Caprioli et al., 1994).

The majority of the outbreaks of non-O157 STEC reported so far occurred in developed countries and there are no available data on the incidence or outbreaks of non-O157 STEC serotypes in Africa. Studies on the epidemiology of non-O157 STEC infections are limited by the fact that generally in outbreaks in which STEC are suspected, most investigations stop when an *E. coli* O157:H7 is found and identified (Bettelheim and Goldwater, 2014). However, there may be a much larger group of non-STEC present and such outbreak may be erroneously labelled as due to STEC O157:H7.

2.2.4.2 Diseases associated with non-O157 STEC

STEC are associated with various diseases such as diarrhoea, HC, potentially fatal HUS and thrombotic thrombocytopenic purpura (TTP) (Rahal et al., 2015). The symptoms range from mild non-bloody diarrhoea in healthy adults to more significant health outcomes which sometimes can be fatal in young, old or immunocompromised individuals (Eblen, 2007). HUS is characterized by acute renal failure, thrombocytopenia and microangiopathic haemolytic anaemia and is more common in children less than 5 years of age and the elderly (Gould et al., 2013). Many severe cases of haemorrhagic diseases and deaths caused by STEC are frequently associated with *E. coli* O157:H7 (Bettelheim and Goldwater, 2014).

However, investigations into the incidence of non-O157 STEC as disease agents indicate that these pathogens are a significant cause of STEC-induced illnesses (Smith et al., 2012; Bettelheim and Goldwater, 2014). The outbreak of STEC O104:H4 in Germany and France

in 2011 clearly showed the public health significance of these serotypes (Bettelheim et al., 2014). Non-O157 STEC is capable of causing illnesses indistinguishable from *E. coli* O157:H7 induced disease and many other foodborne enteric infections (Johnson et al., 2006). More than 380 non-O157 STEC serotypes have been associated with human disease (Rahal et al., 2015), while over 100 serotypes have been associated with outbreaks and sporadic cases of gastrointestinal diseases and HUS (Smith and Fratamico, 2012).

To date, there is no consensus regarding the treatment of STEC infections (Mohsin et al., 2015). The use of antimicrobial agents is usually avoided in the treatment of STEC infections since they are believed to induce bacterial cell lysis and release of stored toxins. Some antimicrobials have also been reported to enhance toxin synthesis and production from these organisms (Rahal et al., 2015). Therefore, alternate preventive or therapeutic strategies for STEC mediated infections and diseases need to be investigated (Mohsin et al., 2015).

Table 2.3: Reported outbreaks of non-O157 STEC (adapted and modified from Kasper et al., 2010)

Country	Serotype	Associated food	Reference
Argentina	O8:H19, O39:H49, O60:H19, O113:H21	Frozen hamburgers, soft cheese, ground beef.	Guth et al., 2003
Australia	O111:NM, O128:H2	Sausage	CDC, 1995b, Bettelheim et al., 1999
Austria	O26:H-	Raw cow's milk,	Allerberger et al., 2003
Belgium	O145, O26	Ice cream	De Schrijver et al., 2011
Brazil	O82:H8, O113:H21, O46:H38, O65:H48, O116:H21	Ground beef and hamburgers.	Guth et al., 2003
Denmark	O26	Beef sausage	Ethelberg et al., 2007
France	O119:B14	Cheese, bovine and caprine milk	Deschenes et. al., 1996
	O103	Cheese, raw caprine milk	Ammon et al., 1997
	O104:H4	Sprouts	Frank et al., 2011
Germany	O157:H-	Sausage	Ammon et al., 1999
	O26:H11	Beef	Werber at al., 2002
	O104:H4	Sprouts	Frank et al., 2011
Japan	O118:H2, O26:H11	Salads, watermelon, sprouts, spinach.	Hiruta et al., 2001
	O111:NM	Meat	Kato et al., 2005
	O115:H NM	Chicken and egg	Etoh et al., 2009
Norway	O103:25	Lamb sausage	Schimmer et al., 2008
USA	O104:H21	Milk	CDC, 1995a
	O111:H8	Salad bar	CDC, 1996a
	O111	Apple cider	Bopp, 2008;
	O111:NM		CDC, 2006

2.3 Probiotics

The original observation of the role of some selected bacteria in prevention and alleviation of gut diseases is attributed to Eli Metchnikoff, the Russian born Nobel Prize recipient, who worked at the Pasteur Institute at the beginning of the last century (FAO/WHO, 2002). He hypothesized that the long and healthy lives of Bulgarian peasants were the outcome of their consumption of fermented milk and milk products (Metchnikoff, 1908). However, it was not until the mid-1960s that the term probiotic came into wide usage (Ozdemir, 2009). Probiotics are non-pathogenic microorganisms that when ingested in adequate amounts, confer health benefits to the host (FAO/WHO, 2002). They play a key role in enhancing resistance to colonization by exogenous, potentially pathogenic bacteria (Holzapfel and Schillinger, 2002; Helland et al., 2004). It has been shown that probiotic foods not only have several potential health benefits but also have nutritional benefits (Sharma and Ghosh, 2006). However, it has been recommendation that high number of probiotics (more than 6 log CFU/ml) must reach the GIT in order to exert the beneficial effects to human health (Boylston et al., 2004).

LAB are the major probiotic microorganisms because they are autochthonous in the gastrointestinal tract (GIT) of healthy people (Tannock, 1999). LAB probiotics include various species of *Lactobacillus*, *Bifidobacterium*, and *Streptococcus*, as well as *Lactococcus* (Morrow et al., 2012; Argyri et al., 2013). Members of the genera *Lactobacillus, Bifidobacterium* and *Streptococcus* are the most common probiotics used in commercial fermented and non-fermented dairy products (Heller, 2001).

2.3.1 Mechanisms of action of probiotics

The health-improving properties of probiotics are still not completely understood (Chaucheyras-Durand et al., 2010), but are commonly suggested to involve competition for nutrients and adhesion sites in GIT, a decrease in the pH and production of antimicrobial molecules (Medellin-Pena et al., 2009; Maltby et al., 2013; Franz et al., 2014). Oelschlaeger (2010) proposed three general modes of action of probiotics: Direct effect on other microorganisms, Inactivation of microbial products such as toxins, and Modulation of the host defence mechanism. An illustration of the main mechanisms of inhibition of enteric bacteria and enhancement of barrier function by probiotics as proposed by Ng et al. (2009) is shown in Figure 2.1.

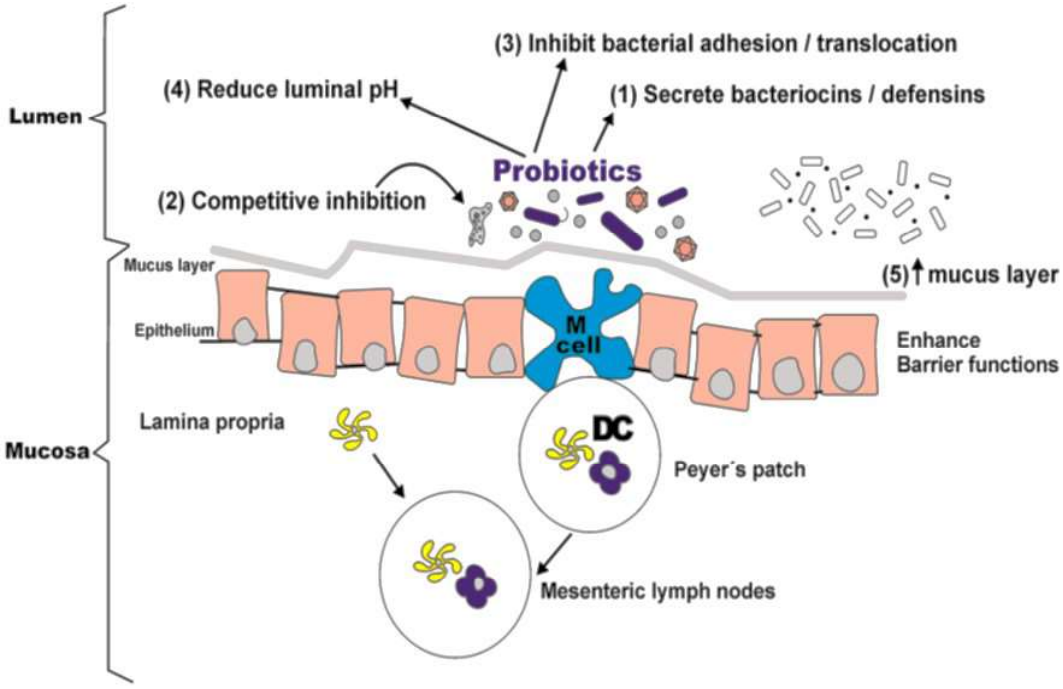

Figure 2.1: Schematic representation of the antimicrobial activities of probiotics in the intestinal mucosa (Ng et al., 2009). (1) the production of bacteriocins/defensins, (2) competitive inhibition of pathogenic bacteria, (3) inhibition of bacterial adherence or translocation, (4) reduction of luminal pH and (5) increasing the mucus production

2.3.1.1 Direct effect on other microorganisms

This mechanism is responsible for the prevention and therapy of infections as well as restoration of the microbial equilibrium in the gut (Oelschlaeger, 2010). It involves the production of substances such fatty acids, organic acids, hydrogen peroxide, and diacetyl (butanedione), acetoin and also small, heat-stable inhibitory peptides known as bacteriocins (Simova et al., 2009). The inhibitory activity of bacteriocins varies. Some inhibit other lactobacilli or taxonomically related Gram-positive bacteria, and some are active against a much wider range of Gram-positive and Gram-negative bacteria as well as yeasts and moulds (Nemcova, 1997).

Probiotics are known to resist colonization of the gut by pathogenic bacteria through creation of a physiologically restrictive environment with respect to pH, redox potential, and hydrogen sulphide production (Ng et al., 2009). The lowering of pH due to organic acids (especially lactic and acetic acids) produced by these bacteria in the gut also has a bacteriocidal or bacteriostatic effect (Vasiljevic and Shah, 2008). Besides exerting its activity through lowering the pH and through its undissociated form, organic acid produced by probiotics also functions as a permeabilizer of the Gram-negative bacterial outer membrane, allowing other compounds to act synergistically with lactic acid (Alakomi et al., 2000; Niku-Paavola et al., 1999). The capacity to produce different antimicrobial compounds is critical for the effective competitive exclusion of pathogen survival in the human GIT (Ouwehand and Salminen, 1998).

Probiotic bacteria also produce so-called deconjugated bile acids which are derivatives of bile salts (Oelschlaeger, 2010). Deconjugated bile acids show a stronger antimicrobial activity compared to the bile salts synthesized by the host organism. How the probiotics protect themselves from these ‘‘selfmade’’ metabolites or if they are resistant to deconjugated bile acids at all remains to be elucidated (Oelschlaeger, 2010)

2.3.1.2 Inactivation of microbial toxins

Probiotic effects may be based on actions affecting microbial products such as toxins, host products such as bile salts and food ingredients. Such actions may result in inactivation of toxins and detoxification of host and food components in the gut (Oelschlaeger, 2010). The effectiveness of probiotics in the treatment of diarrhoea is mostly linked to their ability to

protect the host against toxins. This protection can result from inhibition of toxin expression in pathogens (Carey et al., 2008; Mohsin et al., 2015). Probiotics are also capable of disrupting host-infectious agent/toxin interactions by occupying cellular receptors themselves, by producing decoy receptors that take up the toxins or by modifying the local milieu, hence making these interactions unfavourable (Corr et al., 2009).

Various mechanisms suggested for the toxins inhibition by probiotics include; binding to bacterial cell components (peptidoglycan complex, polysaccharides), reaction with bacterial metabolites, genotoxin conjugation with bacterial metabolites and bioconversion by bacterial enzymes to non-reactive moieties (Cenci et al., 2005). Toxin inhibition is commonly considered a strain-dependent feature among probiotic bacteria which share numerous functional properties (Burns and Rowland 2004; Commane et al., 2005).

2.3.1.3 Modulation of host immune system

The GIT is a complex ecosystem which contains numerous bacterial cells of different phenotypes lining the epithelial wall which express complex metabolic activities (Zboril, 2002). Modulation of the immune system is significant in the prevention of infectious diseases and for the treatment of (chronic) inflammation in the digestive tract (Oelschlaeger, 2010). Probiotic bacteria are capable of tempering the host inflammatory response to infection and are considered to be important mediators of immune-regulation in the gastrointestinal environment (Corr et al., 2009; O'Hara et al., 2006). This immunomodulatory role is an important factor governing the immune clearance of gastrointestinal pathogens and in preventing the establishment of post infectious inflammatory conditions (such as irritable bowel syndrome, IBS) in the gastrointestinal tract (Corr et al., 2009).

The presence of probiotic bacteria in the gut has been proposed to beneficially affect the immune system through the action of signal receptors expressed on the surface of epithelial cells (Isolauri et al., 2001; Vasiljevic and Shah, 2008). The innate immune system via toll-like receptors (TLRs) recognizes a large group of chemical structures in pathogens such as lipopolysaccharides (LPS) and lipoteichoic acids which enables them to recognize foreign objects that trigger a cascade of immunological defence mechanisms, such as the production of pro- and anti-inflammatory cytokines (Oelschlaeger, 2010). TLRs are expressed mainly by macrophages and dendritic cells (DCs), but also include a variety of other cell types such as B cells and epithelial cells. Probiotic bacteria regulate mucosal immune responses through

induction of anti-inflammatory cytokines such as IL-10 and TGF-b, while decreasing expression of pro-inflammatory cytokines, such as TNF and IFN-g (Corr et al., 2007). Oelschlaeger (2010), however, emphasised that there seems not to be one probiotic bacteria that exhibit all three principles, at least not to the extent that it could be a remedy or therapy for prevention of the many GIT diseases.

2.3.2 Inhibitory activities of probiotics against STEC pathogenicity and infections

Inhibition in *Stx* release has been proposed as an effective mechanism for prevention of STEC infection and HUS (Mohsin et al., 2015). The down-regulation of *Stx* gene expression is a potential factor for reducing *Stx* and vero cytotoxicity in STEC (Brunder and Schmidt, 1999). The work of Mohsin et al. (2015) indicated that probiotics display strong inhibitory effects on growth, *Stx* gene expression, amount and cytotoxicity of STEC strains. Carey et al. (2008) who worked on the effect of probiotics on the *Stx* genes expression in *E. coli* O157:H7 revealed that probiotic strains of *Lactobacillus*, *Pediococcus* and *Bifidobacterium* have the ability to down-regulate *Stx*2 gene expression. Reduction in *Stx*-induced cytotoxicity had also been reported when a probiotic was administered (Tahamtan et al., 2011).

Certain probiotic *Lactobacillus* strains had been shown to exert significant inhibitory effect on the growth of the STEC O157:H7 when co-incubated (Gough et al., 2006; Hugo et al., 2008). The production of acetate, butyric and lactic acids by probiotic strains has been shown to be an important factor in their antimicrobial activities against STEC (Ogawa et al., 2001; Fukuda et al., 2011). Gopal et al. (2001) reported that the combination of organic acids and proteinaceous substances such as bacteriocins produced by certain probiotic *Lactobacilli* strains effectively inhibited the growth of *E. coli* O157:H7, through synergistic action. The extent to which a probiotic will confer protection on patients with STEC infections depends on the probiotic strain and its ability to modify the GIT environment (Rahal et al., 2015).

2.3.3 Probiotic potential of LAB associated with traditional fermented foods

The predominant microorganisms involved in the spontaneous or natural fermentation of foods are LAB (Lei and Jakobsen, 2004). Lactic acid bacteria are Gram-positive microorganisms, devoid of cytochromes and preferring anaerobic conditions but are aerotolerant, fastidious, acid-tolerant, and strictly fermentative, producing lactic acid as a main end product (Stiles and Holzapfel, 1997). The most important genera are: *Lactobacillus*, *Lactococcus*, *Enterococcoccus*, *Streptococcus*, *Pediococcus*, *Leuconostoc*, and *Bifidobacterium*. Traditional African fermented foods have potential as probiotic products, because of high levels of associated LAB with possible health beneficial effects (Lei and Jakobsen, 2004). Some LAB that are involved in fermentation of African foods have been documented as having the health benefit of shortening the duration of diarrhoea (Rosenfeldt et al., 2002). Reduction in the incidence of antibiotic-associated diarrhoea was reported when k*oko* sour water (KSW) from fermented pearl millet porridge in Ghana was administered to children (Lei et al., 2006). Traditional fermented foods have a high level of bacteria with a large bio-diversity and there is a possibility that some of these bacteria could be potential probiotic candidates (Muyanja et al., 2003; Sanni et al., 2013)

Various LAB strains that are found in traditional African fermented foods had been examined as potential probiotic candidates that could be used to supplement traditional fermentation (Table 2.4). A study by Jacobsen et al. (1999) revealed that *L. plantarum* and *L. fermentum* strains from maize dough exhibited antimicrobial activity against pathogenic bacteria, survived exposure to low pH and bile salt and adhered to Caco-2 cells. Sanni et al. (2013) examined the probiotic potential of dominant LAB in *ogi*, and found that species of *Pediococcus* and *Lactobacillus*; especially *L. plantarum* produced antimicrobial metabolites, and were tolerant towards bile and low pH and showed good adherence capacity to mucus-secreting HT29-MTX cell lines. *In vitro* evaluation of *L. paracasei*, *L. plantarum*, *L. rhamnosus* and *L. acidophilus* strains from *kule naoto*, a traditional fermented milk produced in Kenya and Tanzania, showed high survival rate under simulated gastric acidic conditions and physiological bile salt concentrations and up to 70% adhesion to HT29 MTX cells (Mathara et al., 2004). LAB strains isolated in *kimere*, a Kenyan spontaneously fermented pearl millet dough, showed that some of the strains possessed probiotic characteristics (Njeru et al., 2010). Opere et al. (2003) demonstrated that *Lactobacillus* starter cultures from a fermented cereal gruel prevented shigellosis in a murine animal model. In their study, 33 to

100% of mice fed with a diet of an African fermented complementary food for young children containing *L. acidophilus* and *L. pentosus* survived, depending on the starter strain or combination of strains used to ferment the cereal gruel. In contrast all control mice died upon *Shigella dysenteriae* infection. Several other studies on evaluation of the probiotic characteristics of LAB from traditional African fermented foods such as *ogi*, *boza*, *togwa* showed that many of the dominant strains, especially lactobacilli and pediococci could be potential probiotic starter cultures (Nout et al., 1989; Adebolu et al., 2012; Todorov and Dicks, 2006; Kivanc et al., 2011). Therefore, this research will evaluate the probiotic potential of *L. plantarum* and *P. pentosaceus* isolated from ogi.

2.3.4 Probiotic potential of *Lactobacillus plantarum*

L. plantarum is a rod-shaped, Gram-positive lactic acid bacterium. It is commonly found in the human and other mammalian gastrointestinal tracts, saliva, and various food products. It can grow at temperatures between 15-45˚C and at pH levels as low as 3.2. *L. plantarum* is a facultative hetero-fermentative organism that ferments sugars to produce lactic acid, ethanol or acetic acid, and carbon dioxide under certain conditions and selective substrates (Quatravaux et al., 2006). Depending on the carbon source, this bacterium can switch from using hetero-fermentative and homo-fermentative ways of metabolism (Zago et al., 2011). Various special therapeutic or prophylactic properties have been associated with *L. plantarum*, such as reduced incidence of diarrhoea, reduced pain and constipation associated with irritable bowel syndrome, reduced bloating, flatulence, ability to displace entero-pathogens from Caco-2 cells among others (Parvez et al., 2006; Candela et al., 2008).

L. plantarum is an important species in the fermentation of various cereal-based products (Ashenafi and Busse, 1991). *L. plantarum* is considered a probiotic because it secretes antimicrobial compounds such as bacteriocins that can inhibit pathogenic Gram-positive and Gram-negative bacteria (Cebeci and Gurakan, 2003). It also possesses a mannose-specific adhesion, which allows it to adhere to the epithelial lining in the human intestine and compete with pathogenic bacteria for nutrients. *L. plantarum* is acid tolerant and can utilise fermentable substrates in plant-based materials (Steinkraus, 2002). These traits, in addition to its proven ability to survive gastric transit and colonize the GIT of humans and other mammals, make *L. plantarum* a good probiotic candidate for the treatment of various gastrointestinal diseases such as diarrhoea (Sunanliganon et al., 2012).

Table 2.4: Overview of LAB strains studied as potential candidate probiotic strains for use in different African traditional fermented cereal foods (adapted and modified from Franz et al., 2014)

Strains	Source of strains	Probiotic evaluation	Substrate used for the product	Region / country in which the fermented African food product is commonly consumed	Reference
Lactobacillus plantarum, Lactobacillus fermentum	Fermented maize dough	Only *in vitro* studies	Maize	Ghana	Jacobsen et al., 1999
Lactobacillus plantarum, Pediococcus spp.	Ogi	Only *in vitro* studies	Maize	Nigeria	Sanni et al., 2013
Pediococccus spp., *Lactobacillus* spp., *Lactobacillus fermentum*	Pearl millet slurry	Only *in vitro* studies	Pearl millet	Burkina Faso, Ghana	Turpin et al., 2011
Lactobacillus fermentum	Kimere: pearl millet dough	Only *in vitro* studies	Pearl millet	Kenya	Njeru et al., 2010
Lactic Acid Bacteria, mainly *Weissella confusa* and *Lactobacillus fermentum*	Koko and koko sour water: from pearl millet porridge	Fermented cereal, maize gruel, Koko sour water	Maize	Northern Ghana	Lei and Jakobsen, 2004; Lei et al., 2006

2.3.1 Probiotic potential of *Pediococcus pentosaceus*

P. pentosaceus is a Gram-positive, facultative anaerobic, non-motile and non-spore-forming member of LAB. Like other LAB, *P. pentosaceus* is acid tolerant, cannot synthesize porphyrins, and possesses a strictly fermentative metabolism with lactic acid as the major end product (Semjonovs and Zikmanis, 2008). It is one of the most frequently isolated species from traditional fermented foods (Teniola et al., 2005; Banwo et al., 2012). It has a long history of safe consumption in human food (Semjonovs and Zikmanis, 2008). *In vivo* evaluation of the probiotic potential of *P. pentosaceus* strain MP12 which was isolated from pickled cabbage, showed that it possessed probiotic characteristics with regard to its antagonistic activity against *Salmonella* spp. in mice and was able to adhere to, and survive on the mouse intestinal epithelium (Chiu et al., 2008). Jonganurakkun et al. (2008) demonstrated that *P. pentosaceus* strain NB-17 from pickled eggplant effectively modulated immune response *in vitro* and it survived well through the digestive tract of rats. There is however, a paucity of literature on the probiotic potential of *P. pentosaceus* from fermented cereals.

2.4 Conclusions

This review establishes that traditional fermented foods play important roles in the diet of people in Africa and support the livelihood. Processing and consumption of traditional fermented foods in Africa are associated with safety challenges because they are mostly produced through uncontrolled natural or spontaneous fermentation processes. Traditional African fermented foods are predominantly fermented by LAB some of which could be potential probiotic starter cultures for improving their safety.

This review clearly shows the public health significance of STEC as foodborne pathogens that can tolerate the acid stress of fermented foods. To date, there is no treatment for STEC infections in humans but LAB strains from traditional fermentation of cereal products hold promise as potential probiotic bacteria that could be used to prevent the growth and survival of non-O157 STEC in such products. However, there is little scientific information available on the stress response and survival of non-O157 STEC in traditional African fermented foods. Thus, a good understanding of the effect of probiotic bacteria on survival and fate of this pathogen in the traditional African fermented foods is critical to minimizing its risk to public health.

3. HYPOTHESES AND OBJECTIVES

3.1 Hypotheses

Adaption to acid will enhance the survival of non-O157 shiga toxin producing *E. coli* in traditional fermented food products. Acid adaptation will induce a decrease in the percentage of unsaturated fatty acids and increase in the percentage of saturated fatty acids of the cell membrane in non-O157 STEC thereby increasing membrane rigidity and proton permeability into the acid adapted cell, as reported for *E. coli* O157:H7 (Brown et al., 1997; Dlamini and Buys, 2009). Acid adaptation may also increase the ratio of cis-vaccenic acid to palmitic acid at acidic pH, causing a decrease in membrane fluidity which will enhance acid tolerance in non-O157 STEC (Yuk and Marshall, 2004).

Probiotic bacteria will inhibit the growth of acid adapted non-O157 STEC in fermented foods. The low external pH of fermented foods will lead to reduction in internal pH of the acid adapted cells, which subsequently will reduce the activity of acid sensitive enzymes, damages proteins and DNA in cells (Adam and Moss, 2000). Probiotic bacteria will also produce secondary metabolites such as bacteriocins and other antimicrobials that will inhibit the growth and also prevent the acid-induced DNA repair in acid adapted cells (Borregaard and Arneborg, 1998; Yuk and Marshall, 2004). Cellular damage which may occur during acid adaptation will enhance the susceptibility of acid adapted non-O157 STEC to inhibition by antimicrobial metabolites produce by probiotic bacteria (Hsin-Yi and Chou, 2001).

3.2 Objectives

To determine the probiotic potential of *L. plantarum* and *P. pentosaceus* strains that are associated with the traditional African fermented maize gruel in order to predict their usefulness as probiotic starter culture for the fermentation of traditional fermented foods.

To determine the effect of potential probiotic *L. plantarum* strain B411 on the survival of acid adapted and non-acid adapted non-O157 STEC in traditional fermented goat's milk.

To determine the effect of potential probiotic *L. plantarum* and *P. pentosaceus* strains on the survival of acid adapted and non-acid adapted non-O157 STEC in ogi, a fermented maize gruel and sorghum motoho, a non-alcoholic fermented sorghum beverage.

4. RESEARCH CHAPTER

4.1 Research Chapter 1: Effect of *Lactobacillus plantarum* bacteria on the survival of acid tolerant non-O157 Shiga toxin producing *E. coli* (STEC) strains in fermented goat's milk

4.1.1 Abstract

The ability of goat's milk fermented with a *Lactobacillus plantarum* strain B411, and in combination with commercial starter culture, to inhibit acid adapted (AA) and non-acid adapted (NAA) environmental non-O157 STEC strains was investigated. Acid adapted and NAA non-O157 STEC strains were not inhibited in the *L. plantarum* fermented goat's milk while the goat's milk fermented with the combination of *L. plantarum* and starter culture inhibited AA more than NAA non-O157 STEC strains. Environmental acid tolerant non-O157 STEC strains were not inhibited by *L. plantarum*, starter culture as well as combination of starter culture with *L. plantarum* unless they were subjected to prior acid adaptation such as backslopping.

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4.1.2 Introduction

Goat's milk plays an important role in nutrition and wellbeing of people in developing countries, where it provides basic nutrition and subsistence to rural people (Park and Haenlein, 2007). It has higher digestibility, lower allergenic properties compared to cow's milk and also contains antibacterial characteristics (Haenlein and Wendorff, 2006). Probiotics are defined as viable microorganisms that following consumption with food, have potential for improving the health and nutrition of the consumer (Gourbeyre et al., 2011). Bacterial probiotics include various species of *Lactobacillus*, *Bifidobacterium*, and *Streptococcus*, as well as *Lactococcus lactis* and some *Enterococcus* species (Argyri et al., 2013).

Lactobacillus has a long history of safe use in food and it plays a major role in fermented milk and other food products (Karska-Wysocki et al., 2010). Probiotics have been examined for their effectiveness in the prevention and treatment of diverse spectrum of gastrointestinal disorders such as antibiotic-associated diarrhoea (Rolfe, 2000). They have also been shown to aid in control of diarrhoea in children (McNaught and MacFie, 2001) and the use of fermented milks containing *Lactobacillus rhamnosus* GG has been shown to shorten the duration of diarrhoea in infants (Marteau et al., 2001). Reduced incidence of diarrhoea was also reported in day-care centres when *L. plantarum* was administered to the children (Vanderhoof, 2000). Dairy fermented products, such as fermented milk and yoghurt including goat's milk (Argyri et al., 2013) have been regarded as the best matrices to deliver probiotics.

Goats have been regarded as a natural reservoir for both *E. coli* O157 and non-O157 shiga toxin producing *E. coli* (STEC) and raw goat's milk may serve as a vehicle of such pathogens transmission (Rey et al., 2006). Non-O157 STEC strains have emerged as important foodborne pathogens worldwide (Wang et al., 2013) and the consumption of dairy products may represent an important route of non-O157 STEC infections in humans (Rangel et al., 2005). It has been shown that non-O157 STEC strains were not eliminated from lactic cheese made with raw goat's milk (Caro et al., 2007) probably because they can tolerate the low pH and the presence of undissociated weak organic acids in fermented food (Ryu and Beuchat, 1998; Elhadidy and Mohammed, 2013). This is because adaptation to acid by *E. coli* can significantly enhance their survival in acidic foods and alter other physiological characteristics of the cell (Rowan, 1999).

Non-O157 STEC infections may induce a range of illnesses from mild gastroenteritis to critical illnesses, including haemorrhagic colitis, haemolytic-uraemic syndrome (HUS) and death, either as sporadic cases or in outbreaks (Smith and Fratamico, 2012). Although the survival of *E. coli* O157:H7 in fermented goat milk (Dlamini and Buys, 2009) and in yoghurt (Ogwaro et al., 2002) has been documented, however, there is paucity of information on the effect of *L. plantarum* on the non-O157 STEC strains in fermented goat milk. Therefore, the aim of this study was to determine the effect of goat's milk fermented with a *L. plantarum* strain B411 on acid tolerant non-O157 STEC strains from environmental sources.

4.1.3 Methodology

4.1.3.1 Source of the milk

Fresh Saanen goat's milk was sourced from the experimental farm of the University of Pretoria, Pretoria, South Africa. The goats were milked using standard milking machines under appropriate hygienic condition. The milk was collected in 1 L sterile Schott bottles immediately after milking and transferred to the laboratory within 30 min. Six portions (100 mL each) were then supplemented with skim milk (3%) (Oxoid, Basingstoke, UK) and gelatin (0.5%) (Davis, Gauteng, South Africa) for stability and pasteurised at 63 ^{o}C for 30 min

4.1.3.2 Bacterial preparation and culture conditions

Acid adaption of the non-O157 STEC isolates

The presence of Shiga toxin 1 (*Stx*1), Shiga toxin 2 (*Stx*2) and intimin (*eae*) genes in the environmental non-O157 STEC strains used in this study had previously been determined (Aijuka et al., 2014). The stock cultures were stored in cryovial beads (Pro-lab Diagnostic, Austin, TX) at -75 ^{o}C. Seventeen (17) environmental non-O157 STEC strains were subjected to acid adaptation as follows; the non-O157 STEC strains were resuscitated in Tryptone Soy Broth (TSB) (Merck, Darmstadt, Germany) for 18 h before inducing acid adaptation and subsequently acid tolerance. The working cultures were prepared by inoculating 1 mL of the resuscitated cultures into 100 mL of TSB buffered with 100 mM Morpholino propanesulphonic acid (MOPS) (Merck) to pH 7.4 and incubated at 37 ^{o}C for 18 h. The procedure of Buchanan and Edelson (1996) was then used to prepare acid adapted (AA) and non-acid adapted (NAA) non-O157 STEC strains. Acid adaptation was induced in the non-

O157 STEC strains by inoculating 1 mL of the working cultures into 100 mL of TSB supplemented with 1% glucose (Merck) (TSB+G) and with the pH adjusted to 4.5 (using 2 M lactic acid). The TSB+G was held at 37 ^{o}C in a water bath shortly before inoculation with non-O157 STEC strains. While for NAA non-O157 STEC strains used as control, TSB without glucose (TSB-G) buffered with 100 mM MOPS with a pH 7.4 was inoculated with 1 mL of the working cultures. Both were immediately incubated for 18 h at 37 ^{o}C. The viability was determined by plating on Sorbitol MacConkey (SMAC) agar (Oxoid) and the plates were incubated at 37 ^{o}C for 24 h.

Acid tolerance of the non-O157 STEC test strains

After acid adaptation for 18 h, 8 non-O157 STEC strains with high acid adaptation potential were selected and exposed to lethal acid shock. Cells were harvested by centrifugation at 5000 g for 15 min at 4 ^{o}C and re-suspended in fresh Brain Heart Infusion (BHI) broth (Oxoid) previously acidified to pH 2.5 using 2 M lactic acid and then incubated at 37 ^{o}C for 2 h. The viability was determined after 0, 60, 90 and 120 min of exposure to lethal acid shock by plating appropriate dilutions on SMAC agar (Merck), incubated at 37 ^{o}C for 24 h and the percentages survival were calculated. After acid tolerance, strains MPU(W)8(3), MPU(W)9(1) and MPU(W)5(2) were then selected for this study. The selection was based on the strains that had more than 50% survival after exposure to lethal acid shock for 2 h. The MPU(W)8(3) and MPU(W)9(1) non-O157 STEC strains were serotyped as O138:K81 while the MPU(W)5(2) strain was serotypes as O83:K-

Starter culture and L. plantarum *strains*

A commercial starter culture (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*) (Cape Food Ingredients, Noordhoek, South Africa) and *L. plantarum* strain B411 (obtained from the Council for Scientific and Industrial Research [CSIR], Pretoria, South Africa) were used for this study. The probiotic characteristics of the *L. plantarum* strain B411 was determined in Research Chapter 2 (Table 4.3). The *L. plantarum* B411 and starter stock culture were activated in MRS broth (de Man, Rogosa and Sharp, 1960) incubated at 37 ^{o}C for 18 h to obtain stationary phase cells. The 18 h cultures of *L. plantarum* B411 and starter culture were centrifuged at 5000 × g for 15 min at 4 ^{o}C and standardised using McFarland Standard ampules (BioMerieux, Marcy l'Etoile, France) to obtain cells at 10^{8} cfu/mL before suspending in the pasteurised and chilled goat's milk.

4.1.3.3 Inoculation of the goat's milk with non-O157 STEC strains

The 3 acid tolerant non-O157 STEC strains (MPU[W]8[3], MPU[W]9[1], MPU[W]5[2]) selected for the study were subjected to acid adaptation as previously described to obtain AA and NAA cells. After 18 h, the resulting cell suspensions were centrifuged at 5000 × g for 15 min at 4 ^{o}C and suspended in 0.1% buffered peptone water (BPW) (Merck). A cocktail of the AA or NAA non-O157 STEC strains was and standardised with McFarland Standard ampules (BioMerieux) to obtain cells at final inoculum level 10^6 cfu/mL after suspending in the chilled goat milk.

4.1.3.4 Fermentation of goat's milk and enumeration of lactic acid bacteria (LAB) and non-O157 STEC strains during survival studies

Goat's milk (100 mL) was inoculated with 10^6 cfu/mL of the commercial starter culture. The second portion (100 mL) of the pasteurised milk was inoculated with 10^6 cfu/mL of commercial starter culture in combination with the *L. plantarum* B411 while a third portion was only inoculated with *L. plantarum* B411 (10^6 cfu/mL). Each treatment was prepared in duplicate and inoculated with a cocktail of either AA or NAA non-O157 STEC strains to obtain final inoculum level 10^6 cfu/mL and incubated at 30 ^{o}C for 6 h. The inoculation of non-O157 STEC strains was performed when the pH of the milk reached 4.5. The non-O157 STEC strains and lactic acid bacteria (LAB) were enumerated at 0, 2, 4 and 6 h of incubation on SMAC and MRS agar, respectively. The SMAC agar plates were incubated at 37 ^{o}C for 24 h while MRS agar plates were incubated anaerobically using anaerobic jar together with anaerocult system (Merck) at 37 ^{o}C for 48 h.

4.1.3.5 Changes in the pH during the survival of AA and NAA non-O157 STEC strains in the fermented goat's milk

The changes in the pH of the fermented goat's milk were determined using a Digital pH meter, Hanna pH meter 211 (Hanna instruments, USA)

4.1.3.6 Statistical analysis

All experiments were performed three times and results were analysed using multifactor analysis of variance (ANOVA) to determine whether factors such as fermentation treatment, acid adaptation and time affected the survival of non-O157 STEC strains. Fisher's Least Significant Difference Test (LSD) was used to determine significant differences between the treatments.

4.1.4 Results

4.1.4.1 Acid tolerance of the non-O157 STEC strains

There were significant ($p < 0.05$) differences in the level of survival of non-O157 STEC strains that were challenged at pH 2.5. All the strains exhibited acid adaptation at higher pH 4.5 but after 120 min of exposure at pH 2.5, three of the strains did not survive while the percentage survival of the remaining strains ranged between 29 and 57% (Table 4.1).

4.1.4.2 Effect of *L. plantarum* B411 on AA and NAA non-O157 STEC strains in fermented goat's milk

The goat's milk fermented with the *L. plantarum* B411 did not inhibit the growth of either AA or NAA non-O157 STEC strains. The initial counts of AA non-O157 STEC strains in the goat's milk fermented with the *L. plantarum* B411 increased from 5.3 ± 0.3 $\log_{10}$ cfu/mL to 6.8 ± 0.1 $\log_{10}$ cfu/mL after 6 h of incubation. Similarly, the viable counts of the NAA non-O157 STEC strains in the goat's milk fermented with *L. plantarum* B411 also increased significantly from 5.6 ± 0.2 $\log_{10}$ cfu/mL to 6.6 ± 0.1 $\log_{10}$ cfu/mL after 4 h of incubation and then remained constant up to 6 h (Fig. 4.1). The presence of acid adapted cells had no substantial effect on the pH as there was no notable difference in the pH of *L. plantarum* B411 fermented goat milk inoculated with AA or NAA non-O157 STEC strains (Fig. 4.2). The initial pH of 5.7 for both AA and NAA non-O157 STEC strains decreased to pH 5.4 and 5.5, respectively, after 6 h of incubation at 30 ^{o}C.

Table 4.1: The acid tolerance of acid adapted non-O157 STEC strains in Brain Heart Infusion (BHI) broth at pH 2.5 (acidified with 2 M lactic acid) and the percentage of survival after 2 h of exposure at 37 ^{o}C

Strain	Microbial count (Log_{10} cfu/mL)				% survival* after 120 min
	0 min	60 min	90 min	120 min	
MPU(W)8(3)	$6.60^{a} \pm 0.08$[1]	$5.42^{ab} \pm 0.01$	$4.19^{b} \pm 0.01$	$3.28^{d} \pm 0.04$	50 ± 3
MPU(W)9(3)	$6.66^{a} \pm 0.01$	$5.64^{ab} \pm 0.06$	$3.11^{a} \pm 0.04$	$2.26^{b} \pm 0.03$	34 ± 2
MPU(W)8(4)	$6.73^{a} \pm 0.08$	$4.05^{c} \pm 0.07$	nd	nd	0
MPU(W)5(3)	$6.12^{a} \pm 0.01$	$5.60^{ab} \pm 0.08$	$3.31^{a} \pm 0.10$	$1.80^{a} \pm 0.03$	29 ± 3
NW(W)5(1)	$6.67^{a} \pm 0.02$	$4.31^{cd} \pm 0.03$	nd	nd	0
MPU(W)9(1)	$6.80^{a} \pm 0.06$	$5.30^{a} \pm 0.10$	$4.68^{b} \pm 0.10$	$3.89^{c} \pm 0.07$	57 ± 3
MPU(W)5(7)	$6.75^{a} \pm 0.02$	$6.04^{b} \pm 0.10$	$3.12^{a} \pm 0.10$	nd	0
MPU(W)5(2)	$6.84^{a} \pm 0.04$	$5.00^{a} \pm 0.03$	$4.49^{b} \pm 0.05$	$3.70^{c} \pm 0.10$	54 ± 3

[1]Means and standard deviations n =3. Values with different superscript in the same column are significantly different at $p \leq 0.05$.
* % Survival = (Log_{10} cfu/mL at 2 h / Log_{10} cfu/mL at 0 h) ×100
nd = not detected. Detection limit = 1 Log_{10} cfu/mL

4.1.4.3 Effect of starter culture on AA and NAA non-O157 STEC strains in fermented goat's milk

Acid adaptation had a significant ($p \leq 0.05$) effect on the on the survival of the non-O157 STEC strains in the goat's milk fermented with the starter culture. The AA non-O157 STEC strains in goat's milk fermented with the starter culture decreased significantly ($p \leq 0.05$) from 5.6 ± 0.1 $\log_{10}$ cfu/mL to 4.2 ± 0.2 $\log_{10}$ cfu/mL after 6 h. While the counts of NAA non-O157 STEC strains in the goat's milk fermented with the starter culture only decreased by 0.4 $\log_{10}$ cfu/mL after 4 h of inoculation and then remained constant up to 6 h (Fig. 4.1). Similar to what was observed in the goat's milk fermented with only *L. plantarum* B411, the presence of acid adapted cells had no substantial effect on the pH of the starter culture fermented goat's milk inoculated with AA or NAA non-O157 STEC strains. The pH declined from 4.6 to 4.3 after 6 h for both starter culture fermented goat's milk inoculated with AA and NAA non-O157 STEC strains (Fig. 4.2).

4.1.4.4 Effect of starter culture combined with *L. plantarum* strain B411 on AA and NAA non-O157 STEC strains in fermented goat's milk.

The addition of *L. plantarum* B411 and acid adaptation had a significant ($p \leq 0.05$) effect on the survival of non-O157 STEC strains in the goat's milk fermented with the combination of the starter culture and *L. plantarum* B411 after 6 h of exposure. A significant ($p \leq 0.05$) reduction from 5.5 ± 0.2 $\log_{10}$ cfu/mL to 3.3 ± 0.2 $\log_{10}$ cfu/mL was recorded for the counts of AA non-O157 STEC strains after 6 h of inoculation while the counts of NAA non-O157 STEC strains only decreased by 0.5 $\log_{10}$ cfu/mL after 6 h in the goat's milk fermented with the combination of starter culture and *L. plantarum* B411 (Fig. 4.1). The reduction in the pH was similar to what was recorded in the goat's milk fermented with the starter culture. The initial pH 4.6 decreased to 4.2 after 6 h, for goat's milk fermented with the combination of starter culture and *L. plantarum* B411, inoculated with AA or NAA non-O157 STEC strains (Fig. 4.2).

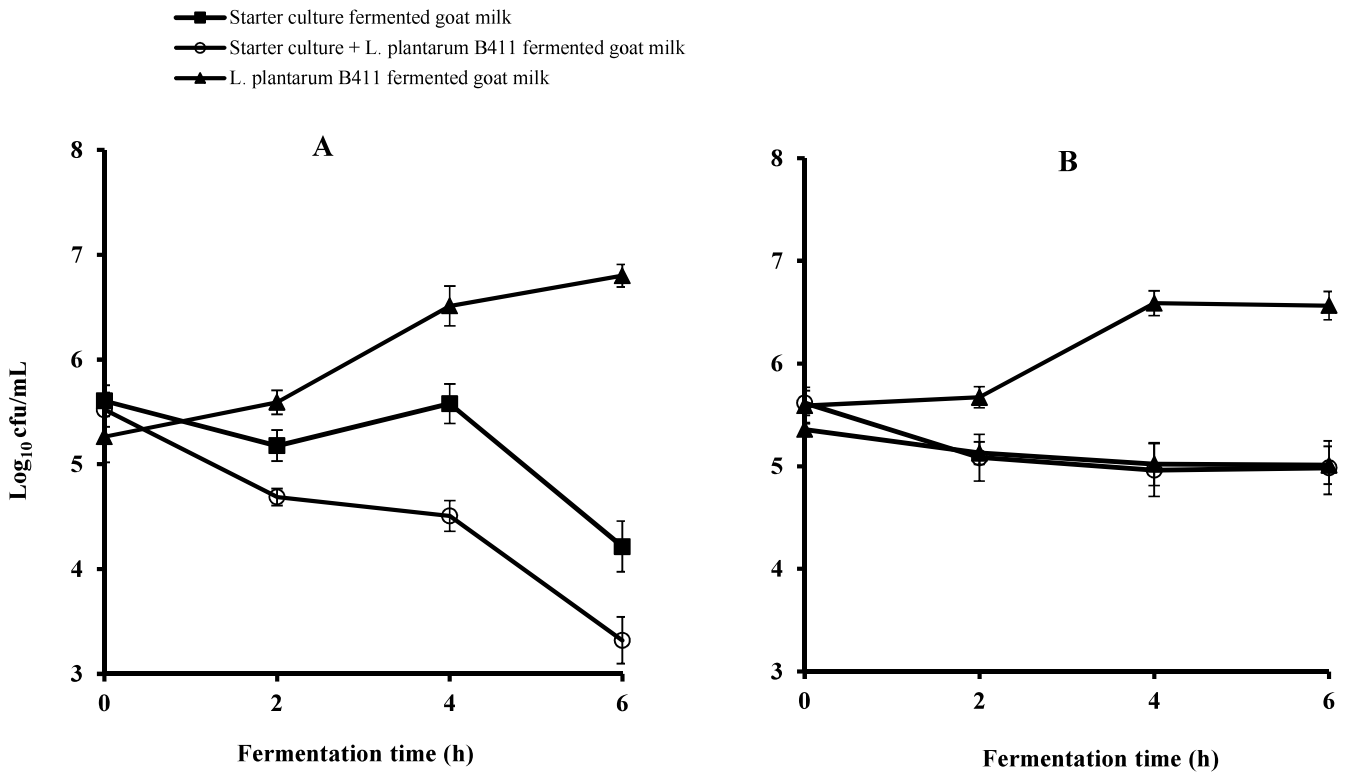

Figure 4.1: The effect of goat's milk fermented with *L. plantarum* B411, starter culture (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarus* subsp. *thermophilus*) and combination of starter culture with *L. plantarum* B411 for 6 h at 30 ^{o}C on (A) survival of acid adapted and (B) non-acid adapted environmental acid tolerant non-O157 STEC strains. Results are expressed as mean ± standard deviation (n = 3).

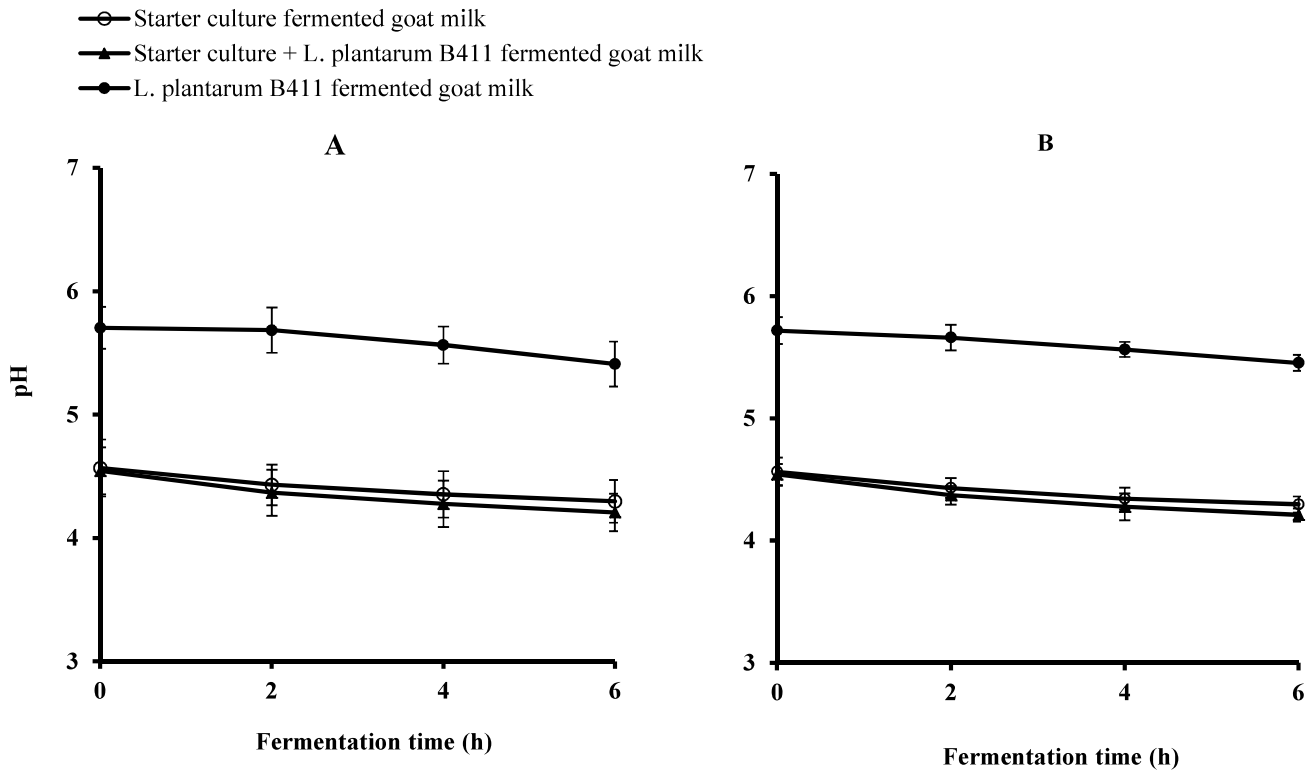

Figure 4.2: Changes in the pH during the fermentation of goat's milk with starter culture (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarus* subsp. *thermophilus*), *L. plantarum* B411 and combination of starter culture and *L. plantarum* B411 for 6 h at 30 ^{o}C, inoculated with acid adapted (A) or non-acid adapted (B) environmental acid tolerant non-O157 STEC strains. The pH of the goat's milk fermented with starter culture and combination of starter culture with *L. plantarum* B411 at the point of inoculating the non-O157 STEC strains was 4.5. Results are expressed as mean ± standard deviation (n = 3).

4.1.4.5 Enumeration of LAB in fermented goat's milk inoculated with either AA or NAA non-O157 STEC strains.

Fermentation of the goat's milk with starter culture and in combination with the *L. plantarum* B411 had a significant ($p \leq 0.05$) effect on the LAB counts. The LAB counts in the goat's milk fermented with only *L. plantarum* B411 inoculated with NAA non-O157 STEC strains increased from 7.7 ± 0.3 $\log_{10}$ cfu/mL to 8.4 ± 0.3 $\log_{10}$ cfu/mL after 6 h of inoculation and incubation at 30 ^{o}C. The LAB counts in the *L. plantarum* B411 fermented goat's milk inoculated with AA non-O157 STEC strains also increased from 7.7 ± 0.2 $\log_{10}$ cfu/mL to 8.3 ± 0.2 $\log_{10}$ cfu/mL after 4 h and remained constant after 6 h of incubation (Fig. 4.3). Similarly, there was no difference in the LAB counts of the goat's milk fermented with starter culture inoculated with AA or NAA non-O157 STEC strains. The LAB counts in the goat's milk fermented with the starter culture inoculated with AA and NAA non-O157 STEC strains increased slightly from 8.5 ± 0.2 $\log_{10}$ cfu/mL to 8.6 ± 0.2 $\log_{10}$ cfu/mL and from 8.4 ± 0.3 $\log_{10}$ cfu/mL to 8.6 ± 0.3 $\log_{10}$ cfu/mL, respectively, after 6 h of inoculation and incubation at 30 ^{o}C (Fig. 4.3). Similar to what was observed in the goat's milk fermented with the *L. plantarum* B411 and starter culture, there was no notable difference between the LAB counts in the goat's milk fermented with the combination of the starter culture and *L. plantarum* B411, inoculated with AA or NAA non-O157 STEC strains. The LAB counts in the goat's milk fermented with the combination of the starter culture and *L. plantarum* B411 inoculated with AA and NAA non-O157 STEC strains decreased from 8.8 ± 0.2 $\log_{10}$ cfu/mL to 8.6 ± 0.3 $\log_{10}$ cfu/mL and from 8.8 ± 0.2 $\log_{10}$ cfu/mL to 8.6 ± 0.2 $\log_{10}$ cfu/mL, respectively, after 6 h of incubation at 30 ^{o}C (Fig. 4.3).

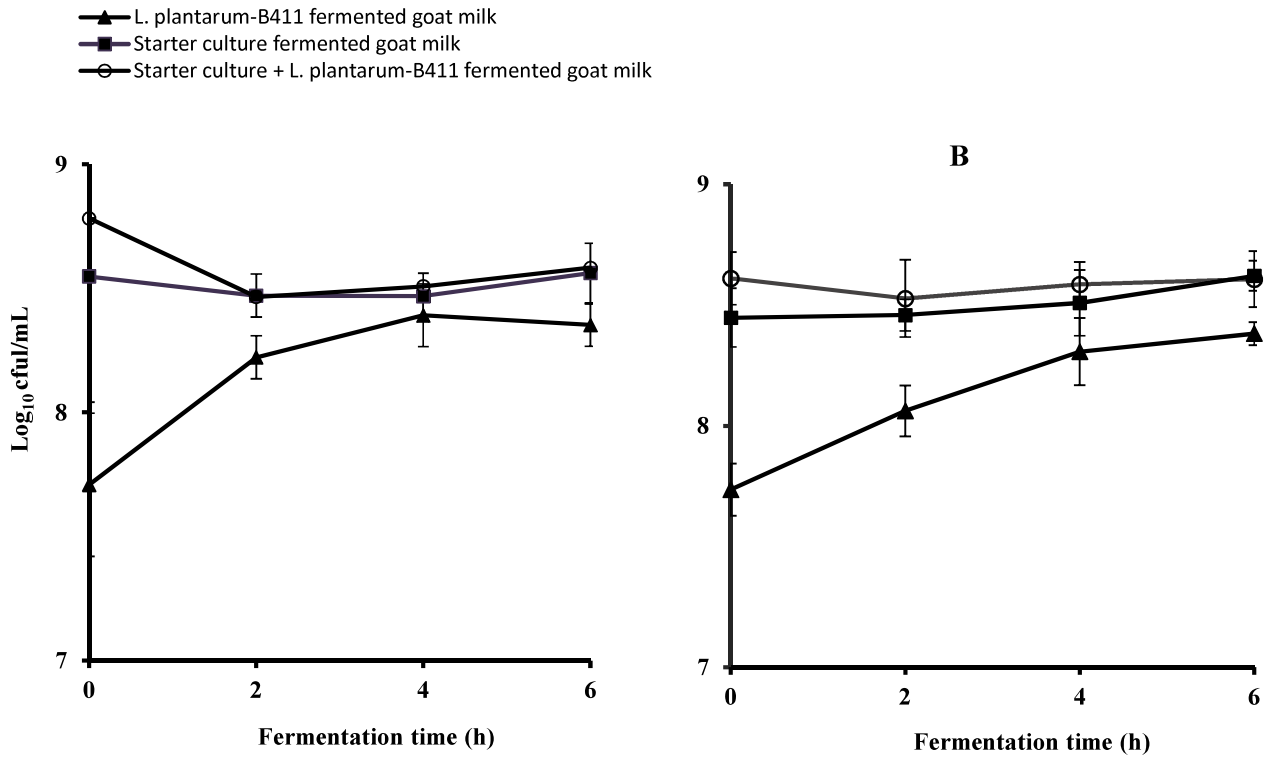

Figure 4.3: Lactic acid bacteria counts during the fermentation of goat's milk with *L. plantarum* B411, starter culture (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarus* subsp. *thermophilus*) and combination of starter culture with *L. plantarum* B411 inoculated with acid adapted (A) or non-acid adapted (B) environmental acid tolerant non-O157 STEC strains for 6 h at 30 ^{o}C. Results are expressed as mean ± standard deviation (n = 3).

4.1.5 Discussion

4.1.5.1 Acid tolerance potential of the non-O157 STEC strains

The survival of the 3 strains selected for this study at pH 2.5 for 2 h suggests the ability of these non-O157 STEC strains to pass through the stomach acidity barrier and possibly initiate infection (Gorden and Small, 1993). These strains can be regarded as highly acid tolerant based on their survival at pH 2.5. This is in accordance with the study of Benjamin and Datta (1995) who grouped EHEC into highly acid-tolerant (50 to 100% survival), moderately acid-tolerant (10 to 50% survival), and slightly acid-tolerant strains (10% survival) based on their survival at pH 2.5. The observed variations in acid tolerance of the non-O157 STEC strains at pH 2.5 is in agreement with the findings of Duffy et al. (2000) who reported that the behaviour of *E. coli* cells under acidic conditions varied among the strains of pathogenic *E. coli*.

4.1.5.2 Survival of AA and NAA non-O157 STEC strains in the goat's milk fermented with potential probiotic *L. plantarum* strain B411.

The goat's milk fermented with only *L. plantarum* B411 did not inhibit the growth of both AA and NAA non-O157 STEC strains. This could be attributed to the high pH and low acidification during exposure which enhanced adaption of non-O157 STEC strains. According to Dlamini and Buys (2009), high pH enhanced the survival of *E. coli* O157:H7 for 3 days in fermented goat's milk amasi. Kingamkono et al. (1995) reported that ETEC inoculated in lactic-fermenting food when the pH was > 5 developed acid-tolerance response (ATR) system that protects them against severe acid stress for a long period. The study of Gran et al. (2003) on the survival of *E. coli* in fermented milk products revealed that high pH and slow acid production induced acid adaptation and enhanced acid tolerance of acid adapted cells present in the inoculum from backslopping. Backslopping is a process in which small portion of a previous batch of fermented food is used to inoculate another batch of food to be fermented. The non-O157 STEC strains used in this study possess high level of acid tolerance at low pH, hence explaining the inability of the *L. plantarum* B411 fermented goat's milk with higher pH to inhibit the growth of both AA and NAA non-O157 STEC strains. This may be because the AA non-O157 STEC strains have developed acid tolerance during prior adaption to acid at lower pH thereby enhancing their survival at higher pH of the

L. plantarum B411 fermented goat's milk while the NAA non-O157 STEC strains adapted to the changing pH due to their ability to tolerate and grow at lower pH as reported for *E. coli* O157:H7 in fermented milk products (Dlamini and Buys, 2009).

4.1.5.3 Survival of AA and NAA non-O157 STEC strains in the goat's milk fermented with the starter culture.

The goat's milk fermented with the starter culture inhibited AA and NAA non-O157 STEC strains than *L. plantarum* B411 fermented goat's milk. The study of Dineen et al. (1998) on the survival of *E. coli* O157:H7 in the yogurt production process reported that starter culture appeared to synergistically reduce *E. coli* O157:H7 beyond the capability of either culture alone. Ogueke (2008) also reported that the inhibitory level exhibited by the commercial starter culture fermented milk on clinical *E. coli* isolates was higher than by the milk fermented with a single strain of *Lactobacillus* spp. The variation in the level of inhibition in their study was attributed to the higher amounts of antibacterial metabolites produced by the starter culture than by the individual strain of *Lactobacillus* spp when used for the fermentation of milk products. However, the inhibition of AA more than NAA non-O157 STEC strains in the goat's milk fermented with the starter culture could be attributed to the production of antimicrobial compounds by the starter culture coupled with the effect of prior adaptation to acid.

Hsin-Yi and Chou (2001) reported lower survival of a population of acid adapted *E. coli* O157:H7 ATCC 43889 than the non-acid adapted cells in a fermented milk drink after 48 h of exposure. Studies have shown that LAB starter cultures produce antimicrobials such as organic acids, bacteriocins, hydrogen peroxide, ethanol, and diacetyl which have potential to inhibit the growth of pathogenic bacteria during acidic fermentation (Stern et al., 2006). Furthermore, acid adaptation of non-O157 STEC strains in this study was performed with lactic acid before inoculation into the fermented goat milk. This could have also enhanced the susceptibility of AA non-O157 STEC strains to the inhibition by other organic acids apart from lactic acid produced during the fermentation of the goat milk with starter culture. This is in accordance with the findings of Ryu and Beuchat (1998) who suggested that the response of acid adapted cells depends on the type of acidulant used to induce acid adaptation. In their study, acid induction of *E. coli* O157:H7 was performed with lactic acid

before inoculation into apple cider and orange juice and this was reported to enhance the susceptibility of adapted cells to inhibition by other organic acids apart from lactic acid.

4.1.5.4 Survival of AA and NA non-O157 STEC strains in the goat's milk fermented with starter culture combined with potential probiotic *L. plantarum* strain B411.

The goat's milk fermented with the starter culture combined with *L. plantarum* B411 inhibited the growth of AA non-O157 STEC strains more than the goat's milk fermented with either starter culture or *L. plantarum* B411 alone. This could possibly be as a result of the starter culture enhancing the growth of the *L. plantarum* thereby resulting in the production and accumulation of various antimicrobial compounds and the weakening effects of prior adaptation to acid (Timmerman et al., 2004). Acid adaptation has been reported to increase susceptibility of *E. coli* O157:H7 to the antimicrobials produced by LAB starter cultures (Hsin-Yi and Chou, 2001). The study of Buchanan and Edelson (1996) revealed that acid adaptation did not enhance acid tolerance in an extremely acid tolerant *E. coli* O157:H7 strain due to the weakening effects of cellular damage during acid adaptation which exceeded the protective effect of acid shock proteins or other protective metabolic changes induced by low pH. According to Leyer et al. (1995), acid adaptation of *E. coli* O157:H7 resulted in injured or damaged cells while producing protective acid shock proteins leading to inability to survive when exposed to further harsh acidic environment in the presence of other antimicrobial metabolites.

Similar to the results observed in the starter culture fermented goat's milk, the NAA non-O157 strains survived more than the AA non-O157 STEC strains in the goat's milk fermented with the starter culture combined with *L. plantarum* B411 after 6 h of exposure. This can be attributed to the fact that the non-O157 STEC strains in this study possess a high level of acid tolerance at low pH and this could have possibly enhanced the survival of NAA non-O157 STEC strains during fermentation due to gradual adaptation to the changing pH. While the sudden shift of the AA non-O157 STEC strains to normal optimum growth conditions followed by the subsequent demand to re-adapt resulted in failure to acquire maximum adaptation as reported by Dlamini and Buys (2009). According to Ryu and Buchant (1998), regardless of prior adaptation to acidic environment, *E. coli* O157:H7 will again undergo physiological changes during subsequent exposure in response to other organic acids and antimicrobial compounds produced. A similar trend of survival was observed in

NAA non-O157 STEC strains inoculated into the goat's milk fermented with only starter culture or starter culture combined with probiotic. This could be attributed to the similar decrease in pH and increase in acidification levels of the two fermented goat's milk samples. Hence, the rate of adaptation of NAA non-O157 STEC strains to acid during the fermentation of the goat's milk with starter culture or with the combination of the starter culture and *L. plantarum* B411 seemed similar.

4.1.6 Conclusions

This study showed that non-O157 STEC strains from environmental sources vary in their acid tolerance ability and the acid tolerant strains may not be inhibited either by *L. plantarum*, commercial starter culture as well as a starter culture and *L. plantarum* combination. However, prior adaptation to acid enhanced the susceptibility of environmental acid tolerant non-O157 STEC strains to inhibition for instance during backslopping as practised during traditional fermentation of goat's milk. Therefore, acid adaptation may contribute to the safety of traditional fermented complementary food from environmental acid tolerant non-O157 STEC strains.

4.2 Research Chapter 2: Potential probiotic *Lactobacillus plantarum* can inhibit environmental non-O157 Shiga toxin producing *Escherichia coli* strains in traditional African fermented maize gruel (ogi)

4.2.1 Abstract

Non-O157 Shiga toxin producing *E. coli* (STEC) serotypes are emerging foodborne pathogens, considered to be the frequent cause of STEC related infections. The effect of a probiotic *Lactobacillus plantarum* strain on the survival of environmental acid adapted (AA) and non-acid adapted (NAA) non-O157 STEC strains during the production of traditional African fermented maize gruel (ogi) was investigated. Ogi was produced by spontaneous fermentation and in combination with probiotic *L. plantarum*. The growth of AA and NAA non-O157 STEC strains was significantly inhibited by 2.5 and 3.0 log reductions respectively in the ogi fermented with the probiotic *L. plantarum* after fermentation and processing, while they were not inhibited in the spontaneously fermented ogi. This use of probiotic starter culture could help ensure the safety of traditional African fermented cereal gruel, which is also used as a weaning food by preventing the growth of non-O157 STEC thereby reducing the occurrence of infant diarrhoea caused by non-O157 STEC.

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4.2.2 Introduction

Shiga toxin-producing *Escherichia coli* (STEC), also known as verocytotoxogenic *Escherichia coli* (VTEC), has emerged as a group of highly pathogenic *Escherichia coli* strains that are characterized by the production of one or more Shiga toxins (Monaghan et al., 2011). Several serotypes of STEC have been linked to foodborne illness (Mathusa et al., 2010). Non-O157 Shiga toxin-producing *E. coli* (non-O157 STEC) serotypes are increasingly recognized as emerging foodborne pathogens worldwide and their occurrence is five times higher than that of STEC O157:H7 (Gould et al., 2013). Non-O157 STEC is associated with both outbreaks and individual cases of severe illness and is considered as a major contributor to human disease (Bettelheim and Goldwater, 2014).

Contaminated food is the main principal vehicle for the transmission of STEC to humans (Erickson and Doyle, 2007) and it has been shown that spontaneous fermentation did not inactivate STEC O157:H7 in traditional Africa fermented foods such as maize porridge (Kwaw, 2014) and *Borde*, an Ethiopian fermented cereal beverage (Tadasse et al., 2005). Many of the foods associated with the past outbreaks of STEC O157:H7 were likely to also contain non-O157 STEC strains, but were not implicated because the focus was only on STEC O157:H7 (Mathusa et al., 2010). Non-O157 STEC serotypes are highly resistant to acidic stress and can tolerate the low pH of fermented food products (Elhadidy and Mohammed, 2013). This is because acid adaptation can significantly enhance the survival of pathogenic *E. coli* in acidic or fermented foods and alter the physiological characteristics of the bacteria (Rowan, 1999). Exposure to non-O157 STEC can lead to mild or watery diarrhoea, haemorrhagic colitis (HC), haemolytic-uremic syndrome (HUS) and death (Hughes et al., 2006). There is no effective prophylaxis and treatment available for STEC infections in humans and the use of antibiotics are not generally recommended as they may induce Shiga toxin (*Stx*) production, thus worsening the symptoms (Rund et al., 2013).

However, probiotic bacteria are gaining interest as an alternate therapeutic option for the prevention and treatment of STEC mediated foodborne infections in humans (Mohsin et al., 2015). It has been suggested that probiotic bacteria can limit non-O157 STEC infections by inhibiting the growth and down regulating the expression of the virulence factors (Mohsin et al., 2015; Rund et al., 2013). Certain probiotic *Lactobacillus* strains have been shown to exert

growth inhibitory and bactericidal activities on STEC O157:H7 serotype (Carey et al., 2008). Reduction in the incidence of diarrhoea caused by STEC has also been reported when fermented foods containing potential probiotic *L. plantarum* were administered to children (Parvez et al., 2006). Utilization of well-defined probiotic starter cultures with proven efficacy in traditional fermented foods could be an important avenue for the treatment of diarrhoea especially in the rural communities in Africa (Oguntoyinbo, 2014).

The survival of Enterotoxigenic *E. coli* (ETEC) strain in ogi, a West African traditional fermented cereal gruel, produced with a bacteriocin producing *Lactobacillus* strain has been reported (Olasupo et al., 1997). However, little is known about the stress response and behaviour of non-O157 STEC serotypes when exposed to the acidic stress of fermented cereal food products. Therefore this study investigated the effect of a potential probiotic *L. plantarum* and acid adaptation on the survival of environmental non-O157 STEC strains in the traditional African fermented maize gruel, ogi.

4.2.3 Materials and methods

4.2.3.1 Fermentation and processing of ogi

White maize grain was purchased at Oba market, Akure, Nigeria. After sorting and cleaning, the grains (400 g) were processed to ogi by steeping in potable tap water (1 L) and allowing to spontaneously fermenting for 72 h at 30 ^{o}C. After fermentation to pH 4.6, the steeped grain was wet-milled using a Waring blender and sieved through muslin cloth with about 300 µm pore size. The slurry was then transferred to a closed container to sediment and ferment further (sour) for 48 h at 30 ^{o}C to pH < 4.5.

4.2.3.2 Microbiological analysis during ogi spontaneous fermentation and processing without inoculation with non-O157 STEC strains

At 24 h intervals, changes in the microbial population (cfu/g) of the total aerobic bacteria (TAC), lactic acid bacteria (LAB), Enterobacteriaceae and yeasts and moulds were determined using nutrient agar (NA) (Merck, Darmstadt, Germany), MRS (De Man et al., 1960) and M17 agar (Oxoid, Basingstoke, UK), violet red bile glucose (VRBG) agar (Oxoid) and acidified (using 10% w/v tartaric acid to pH 3.5) potato dextrose agar (PDA) (Merck), respectively. Samples were enumerated by homogenizing the fermenting maize grains with

buffered peptone water (BPW) (Merck) and appropriate dilutions were made and spread plated. The NA and VRBG agar plates were incubated at 37 ºC for 24 h. The yeast and mould plates were incubated at 25 ºC for 3-5 days and the MRS agar plates were incubated anaerobically using anaerobic jar together with Anaerocult system (Merck) at 37 ^{o}C for 48 h.

4.2.3.3 Characterisation and identification of dominant *Lactobacillus plantarum* in ogi spontaneous fermentation and processing

Colonies were randomly picked from the highest dilution of MRS agar plates to determine the dominant LAB at different stages of ogi spontaneous fermentation and processing. Cellular morphology, Gram staining, catalase reaction and motility test were performed (Collins et al., 1989). The LAB isolates were then identified using Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) (Bruker Daltonics, Bremen, Germany). The raw spectra generated by the MALDI-TOF MS were analysed using the MALDI Biotyper 3.0 software package (Bruker Daltonics) and results of the pattern-matching process were expressed as proposed by the manufacturer. For strain classification, a score-oriented dendrogram was generated based on cross-wise minimum spanning tree (MSP) matching using the standard settings of the MALDI Biotyper 3.0 software.

4.2.3.4 Probiotic potential *L. plantarum* strain B411 and bacterial preparation

Determination of the probiotic potential of the L. plantarum *strain used for the production of ogi*

The probiotic ability of *L. plantarum* strain B411 (obtained from the Council for Scientific and Industrial Research (CSIR), Pretoria, South Africa) used for this study was determined. The acid and bile salt tolerance of the *L. plantarum* strain was determined as described by Succi et al. (2005). The hydrophobicity as measured by the microbial adhesion to hydrocarbons (MATH), autoaggregation and coaggregation were determined as described by Kos et al. (2003). The Antimicrobial activity of the *L. plantarum* strain was performed as described by Schillinger and Lucke (1989).

Bacteria adhesion to Caco-2 cells

Caco-2 cells were routinely grown in tissue culture dishes (9 cm^2) (Orange Scientific, Biovalley, South Africa) on microscopy cover glasses in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) heat-inactivated (30 min at 56°C) foetal calf serum, 100 U/mL penicillin, 100 mg/mL streptomycin, 0.1 mM nonessential amino acids, and 2 mM glutamine and incubated at 37°C in 5% carbon dioxide. For cell adhesion assay, Caco-2 cells were used for adherence assay after 14 days growth. The adhesion assay was performed as described by Jacobsen et al. (1999).

4.2.3.5 Preparation and inoculation of the ogi with potential probiotic *L. plantarum* strain B411 and non-O157 STEC test strains

The three non-O157 STEC strains selected were subjected to acid adaptation as described in Research Chapter 1 (4.1.3.2) to obtain AA and NAA non-O157 STEC cells. After acid adaptation for 18 h, the resulting cell suspensions were centrifuged at 5000 × g for 15 min at 4 ^oC and suspended in 0.1% BPW. A cocktail of the three non-O157 STEC strains was then prepared after centrifugation and standardised using McFarland Standard ampules (BioMerieux) before inoculating the steeped maize grains. The potential probiotic stock culture was activated in MRS broth incubated at 37 ^oC for 18 h to obtain stationary phase cells. The resulting cell suspension was then centrifuged at 5000 × g for 15 min at 4 ^oC and also standardised using McFarland Standard (BioMerieux) before inoculating the steeped maize grains. The potential probiotic *L. plantarum* B411 culture and a cocktail of the AA or NAA non-O157 STEC strains were standardised with McFarland Standard ampules (BioMerieux) to obtain cells at final inoculum level 10^6 cfu/mL after suspending in the fermenting maize grain.

4.2.3.6 Enumeration of LAB and detection of AA and NAA non-O157 STEC strains in ogi fermentation and processing

The survival of both AA and NAA non-O157 STEC strains were determined during ogi fermentation and processing by steeping maize grains in four separate closed containers. The first two containers were inoculated with *L. plantarum* strain B411 and with either AA or NAA non-O157 STEC strains, while the other two containers were only inoculated with either AA or NAA non-O157 STEC strains. They were then fermented at 30 °C for 72 h

followed by other unit operations (Table 4.2). All inoculations were done to obatain cells at final inoculum level 10^6 cfu/mL after suspending in the steeped maize grains. The non-O157 STEC strains and LAB were enumerated at 24 h intervals on SMAC agar and MRS agar respectively. SMAC agar plates were incubated at 37 ^oC for 24 h, while MRS agar plates were incubated as described (4.2.3.2). Titratable acidity and pH (% lactic acid equiv.) of the fermenting ogi were determined at 24 h intervals during the fermentation and processing.

4.2.3.7 Statistical analysis

All experiments were performed three times and results were analysed using multifactor analysis of variance (ANOVA) to determine whether factors such as fermentation treatment, acid adaptation and time affected the survival of non-O157 STEC strains. Fisher's Least Significant Difference Test (LSD) was used to determine significant differences between the treatments.

4.2.4 Results and Discussion

4.2.4.1 Microbial profile during ogi spontaneous fermentation and processing in the absence of non-O157 STEC strains

The predominant microorganisms during the steeping and souring of ogi fermentation and processing were LAB, while there was a high level of yeasts and moulds in the later stages of souring (Table 4.2). All the microbial populations increased throughout the steeping with the exception of Enterobacteriaceae which gradually decreased after initial increase within the first 24 h of fermentation. The Enterobacteriaceae increased by 1 $\log_{10}$ cfu/g during the souring period. A reduction in the pH and an increase in TA took place throughout the fermentation except at 0 h of souring, due to the addition of fresh potable tap water during wet milling.

The prevalence of LAB and yeasts in the spontaneous fermentation of ogi has been reported (Banwo et al., 2012; Omemu et al., 2007; Oyedeji et al., 2013). The increase in the population of Enterobacteriaceae in the early stages of the fermentation is in agreement with the findings of Mugula et al. (2003) who reported that Enterobacteriaceae are usually active and dominate the early stages of fermentation of cereal-based products due to the low acidity. The gradual decrease of the Enterobacteriaceae counts as the fermentation progressed can be attributed to the inhibitory effect of organic acids and other antimicrobial substances produced by LAB during fermentation (Abdus-Salaam et al., 2014). The level of Enterobacteriaceae in the final product suggests the presence of some pathogenic bacteria that can survive in spontaneously fermented ogi. Nwokoro and Chukwu (2012) reported a high level of Enterobacteriaceae in spontaneously fermented ogi and they concluded that ogi could pose a health risk to the consumers especially when used as a weaning food.

4.2.4.2 Characterisation of the *L. plantarum* strains in ogi spontaneous fermentation and processing

Comparative cluster analysis (dendrogram) for the *L. plantarum* in spontaneous fermentation of ogi revealed two distinct clusters (A1 and A2) with an 80% degree of relatedness (Fig. 4.4). The *L. plantarum* strains at the later stages of steeping and souring had higher degree of similarity than isolates at the early stages of steeping. There was also high degree of relatedness between the *L. plantarum* strains in cluster A2 and the probiotic *L. plantarum* strain B411.

Table 4.2: Effect of processing steps on the microbiological profile of spontaneously fermented ogi

Stage of ogi processing	pH	Titratable acidity (%)	Total aerobic bacteria (Log_{10} cfu/g)	Lactic acid bacteria (Log_{10} cfu/g)	*Streptococcus* and *Lactococcus* (Log_{10} cfu/g)	Enterobacteriaceae (Log_{10} cfu/g)	Yeasts and moulds (Log_{10} cfu/g)
Steeping period (h)							
0	$6.11^{d} \pm 0.04$[1]	$0.01^{a} \pm 0.00$	$2.67^{a} \pm 0.22$	$2.61^{a} \pm 0.27$	$2.18^{a} \pm 0.06$	$1.10^{a} \pm 0.46$	$2.66^{a} \pm 0.20$
24	$5.64^{c} \pm 0.04$	$0.16^{b} \pm 0.01$	$5.15^{b} \pm 0.18$	$4.58^{b} \pm 0.11$	$3.66^{b} \pm 0.11$	$4.45^{e} \pm 0.17$	$3.32^{b} \pm 0.26$
48	$4.86^{ab} \pm 0.04$	$0.34^{c} \pm 0.04$	$7.34^{c} \pm 0.29$	$7.69^{c} \pm 0.05$	$4.56^{cd} \pm 0.10$	$3.43^{cd} \pm 0.05$	$5.00^{c} \pm 0.56$
72	$4.62^{ab} \pm 0.12$	$0.38^{c} \pm 0.02$	$7.72^{c} \pm 0.60$	$8.80^{d} \pm 0.03$	$5.60^{e} \pm 0.10$	$3.25^{c} \pm 0.05$	$6.72^{d} \pm 0.51$
Souring Period (h) after wet milling and sieving with fresh tap water							
0	$6.18^{d} \pm 0.03$	$0.11^{ab} \pm 0.00$	$5.38^{b} \pm 0.33$	$7.47^{c} \pm 0.35$	$4.33^{c} \pm 0.26$	$2.27^{b} \pm 0.17$	$3.41^{b} \pm 0.10$
24	$4.66^{ab} \pm 0.11$	$0.14^{b} \pm 0.04$	$7.41^{c} \pm 0.38$	$8.47^{d} \pm 0.08$	$4.74^{d} \pm 0.10$	$4.04^{de} \pm 0.24$	$6.54^{d} \pm 0.30$
48	$4.36^{a} \pm 0.02$	$0.22^{bc} \pm 0.04$	$7.65^{c} \pm 0.28$	$8.48^{d} \pm 0.28$	$5.41^{e} \pm 0.20$	$3.28^{c} \pm 0.47$	$7.41^{e} \pm 0.01$

[1]Means and standard deviations n =3. Values with different superscript in the same column are significantly different at $p \leq 0.05$.

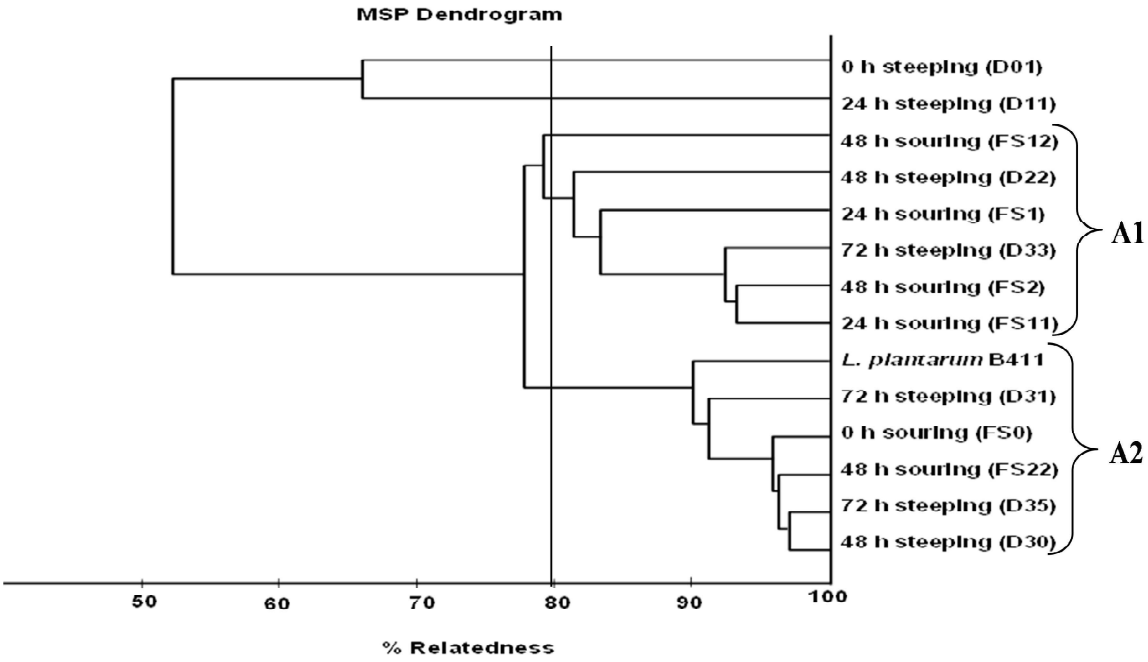

Figure.4.4: Score-oriented dendrogram showing genetic relationships between the dominant *L. plantarum* strains isolated during ogi spontaneous fermentation and the probiotic *L. plantarum* strain B411 used for the production of ogi. The vertical line represents clusters of isolates that showed 80% strain similarity which was taken as the threshold for closely related isolates. A1 and A2 indicate clusters of closely related *L. plantarum* isolates. *Isolate codes are next to the fermentation steps.*

The cluster analysis demonstrated that different strains of *L. plantarum* were involved during the fermentation and production of ogi. However, the high level of relatedness among the *L. plantarum* strains in the later stages of steeping and souring suggests that similar *L. plantarum* strains dominate the later stages of ogi spontaneous fermentation. Sanni et al. (2013) who worked on the characterization of LAB in spontaneous fermentation of ogi found a high degree of similarity among the *L. plantarum* strains. Banwo et al. (2012) also reported a high level of similarity among *L. plantarum* strains associated with Nigeria fermented foods including ogi. The high level of similarity between probiotic *L. plantarum* strain B411 and the dominant *L. plantarum* strains in the ogi fermentation suggests that the probiotic strain was a suitable starter culture for effective fermentation and production of ogi.

4.2.4.3 Probiotic potential of the *L. plantarum* strain used as starter culture for the production of ogi

The *L. plantarum* strain B411 had ability to coaggregate with non-O157 STEC strains and *E. coli* ATCC 25922 (Table 4.3). Further, the cell free supernatant (CFS) of the *L. plantarum* strain had broad spectrum of antimicrobial activity against non-O157 STEC strains and *E. coli* ATCC 25922 pathogenic indicator strains. The most susceptible to antimicrobial activity of the *L. plantarum* strain were non-O157 STEC strains, while the least inhibited was *E. coli* ATCC 25922. Additionally, the *L. plantarum* strain exhibited high level of tolerance at low pH and bile salt as well as high degree of hydrophobicity. It also demonstrated high level of autoaggregation and adhesion to enterocyte-like Caco-2 cells.

The levels of coaggregation of the *L. plantarum* strain are higher than those reported for commercial probiotic *L. rhamnosus* GG, *L. rhamnosus* Lc-705 and *L. paracasei* ATCC 25598 (Collado et al., 2008; Mirlohi et al., 2009; Xu et al., 2009). The ability of the *L. plantarum* strain to coaggregate with potential gut pathogens suggests it may have a host defence mechanism against infection in the gastrointestinal tract (GIT), a property ascribed to probiotic bacteria (Kos et al., 2003).

The antimicrobial activity of the filtered and neutralized supernatant of the potential probiotic *L. plantarum* strain B411 against non-O157 STEC strains and *E. coli* ATCC 25922 was not lost after treatment with catalase or adjustment of pH to 6.5 (Table 4.3). This suggests that the *L. plantarum* strain had the ability to inhibit non-O157 STEC strains at high pH in the absence of organic acids. The level of antimicrobial activity of the *L. plantarum* strain against

the pathogenic indicator strains was higher than that of commercial probiotic *L. acidophilus* LA-1 when tested against *E. coli* O157:H7 (Gopal et al., 2001). The antimicrobial activity of the *L. plantarum* strain B411 against the pathogenic indicator strains is an indication that the *L. plantarum* B411 has the potential to inhibit the growth or prevent the colonization of the gut by pathogenic bacteria (Kos et al., 2003; Suskovic et al., 2010).

The tolerance of the *L. plantarum* strain B411 to low pH and bile salt is similar to results reported by Mirlohi et al. (2009) but higher than the values obtained by Succi et al. (2005) for commercial probiotic *L. rhamnosus* GG. The *in vitro* acid and bile tolerance of the *L. plantarum* strain suggests its ability to survive through the acidic condition of the upper part of the gastrointestinal tract and exert its probiotic potential on the host (Orlowski and Bielecka, 2006). The *L. plantarum* B411 exhibited a similar level of hydrophobicity compared to that of the commercial probiotic *L. rhamnosus* GG ATCC 5310 and *L. rhamnosus* Lc-705 strains (Collado et al., 2007). The *L. plantarum* strain B411 can be regarded as a potential probiotic strain based on its level of attachment to Caco-2 cells which were higher than those reported for commercial probiotic strains *L. rhamnosus* GG and *L. paracasei* ATCC 25598 (Xu et al., 2009).

4.2.4.4 Enumeration of LAB and survival of AA and NAA non-O157 STEC strains during fermentation and processing of ogi

Addition of the *L. plantarum* B411 culture had a significant ($p < 0.05$) effect on the LAB counts during fermentation (Table 4.4). LAB counts in the ogi fermented spontaneously in combination with *L. plantarum* B411 inoculated with AA and NAA non-O157 STEC strains were higher than those in the spontaneously fermented ogi by 1.2 and 0.7 $\log_{10}$ cfu/g, respectively after 24 h of maize steeping (Fig. 4.5). However, there was no notable difference in the LAB counts of all the fermented ogi inoculated with AA or NAA non-O157 STEC strains throughout the remaining period of fermentation. Addition of potential probiotic *L. plantarum* strain B411 had highly significant ($p < 0.001$) effect on the survival of non-O157 STEC strains during the fermentation of ogi (Table 4.5).

Table 4.3: The probiotic potential of the *L. plantarum* strain B411 as determined by pH and bile salt tolerance, cell surface hydrophobicity (MATH), autoaggregation, coaggregation and adhesion to enterocyte-like Caco-2 cells in comparison to literature data.

E. coli indicator strain	Coaggregation (%)	Inhibition zone (mm)	Microbial growth at low pH and subsequent exposure to 3 % bile salt (Log10 cfu/ml)				Hydrophobicity (Microbial adhesion to hydrocarbon) %			Autoaggregation (%)		Adhesion to Caco-2 cells (%)	Reference
			at pH 2.5		at 3% bile salt		Xylene	Chloroform	Ethyl acetate	MRS	PBS		
			0 h	2 h	3 h	7 h							
MPU(W)8(3)	53.0^{a} ± 2.5	26.0^{b} ± 1.2											
MPU(W)9(1)	58.0^{ab} ± 1.2	29.0^{b} ± 0.8											
MPU(W)5(2)	64.0^{b} ± 2.8	28.0^{b} ± 1.2	8.65 ± 0.10	6.51 ± 0.58	7.23 ± 1.03	7.59 ± 0.20	70.0 ± 3.5	50.0 ± 2.5	61.0 ± 1.4	92.0 ± 2.5	62.0 ± 3.1	68.0 ± 3.5	This study[1]
ATCC 25922	54.0^{a} ± 1.7	17.0^{a} ± 0.7											
L. rhamnosus GG		-	8.8	6.5	-	5.0	33.9	67.1	30.0	30.0	< 30	-	Mirlohi et al., 2009 & Pinto et al., 2007
		-	-	< 4	< 4	-	47	47	20	-	-	23.2	Xu et al., 2009 & Succi et al., 2005
L. acidophilus LA-1 (against *E. coli* O157:H7)	8.07	-	-	-	-	-	-	-	-	-	-	-	Gopal et al., 2001
L. acidophilus LA-1		-	-	-	-	-	-	-	-	-	-	17	Jacobsen et al., 1999
L. rhamnosus GG (ATCC 5310)		-	-	-	-	-	64.4	-	-	-	27.0	-	Collado et al., 2008
L. rhamnosus Lc-705 (Valio)		-	-	-	-	-	67.1	-	-	-	18.2	-	Collado et al., 2008
L. paracasei ATCC 25598		-	-	-	-	-	30	48	18	-	< 40	11.9	Xu et al., 2009

[1]Means and standard deviations n =3. [1]Values with different superscript in the same column are significantly different at $p \leq 0.05$.
- No data

Table 4.4: Multifactor ANOVA of the effect of spontaneous fermentation and in combination with the potential probiotic *L. plantarum* B411 in the presence of acid adapted and non-acid adapted non-O157 STEC strains on the lactic acid bacteria populations during the production of ogi (n=3).

Treatment	Degrees of freedom	*P* value
Potential probiotic *L. plantarum* strain	1	0.046
Presence of acid adapted non-O157 STEC	1	0.168
Fermentation steps	6	0.000
Potential probiotic × Fermentation steps	6	0.000
Acid-adaptation × Fermentation steps	6	0.868
Potential probiotic × acid adaptation	1	0.783
Potential probiotic×acid-adaptation×Fermentation steps	6	0.900

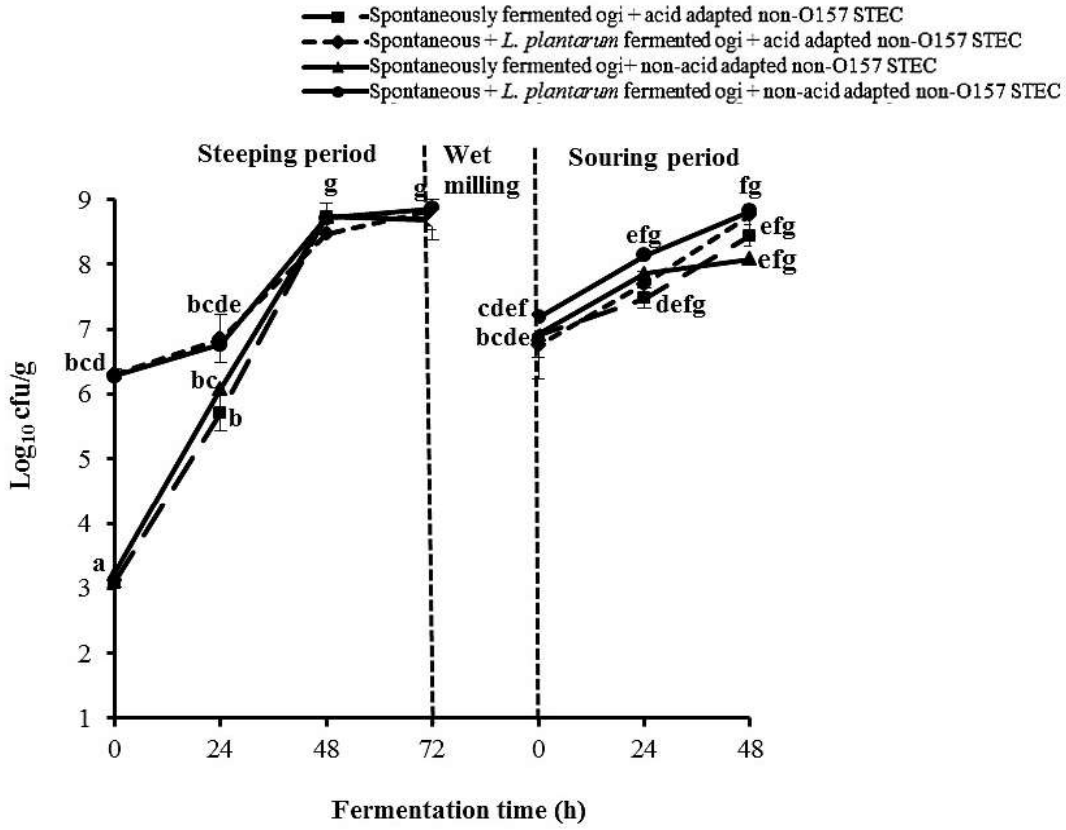

Figure 4.5: Effects of spontaneous fermentation and in combination with potential probiotic *L. plantarum* strain B411 in the presence of acid adapted or non-acid adapted non-O157 STEC strains on the lactic acid bacteria counts during the production of ogi. Results are means ± standard deviation (n = 3). *Data points with different superscript are significantly different at p ≤ 0.05.*

Table 4.5: Multifactor ANOVA of the effect of spontaneous fermentation and in combination with the probiotic *L. plantarum* on the survival of acid adapted (AA) and non-acid adapted (NAA) non-O157 STEC during the production of ogi (n=3).

Treatment	Degrees of freedom	*P* value
Potential probiotic (*L. plantarum* strain)	1	0.000
Acid adaptation	1	0.001
Fermentation steps (steeping & souring)	6	0.000
Potential probiotic × Fermentation steps	6	0.000
Acid-adaptation × Fermentation steps	6	0.000
Potential probiotic × acid adaptation	1	0.000
Potential probiotic×acid-adaptation×Fermentation steps	6	0.000

The AA and NAA non-O157 STEC strains were not inhibited in both ogi fermented spontaneously and in combination with *L. plantarum* B411 within the first 24 h of fermentation (Fig. 4.6). The AA and NAA non-O157 STEC strains were substantially inhibited by 2.5 and 3.0 log reductions, respectively in the final product of ogi fermented spontaneously in combination with the *L. plantarum* B411, while their growth was not inhibited in the spontaneously fermented ogi after steeping and souring. There was no notable difference in the survival of AA and NAA non-O157 STEC strains in either ogi fermented spontaneously or in combination with *L. plantarum* B411 throughout the fermentation.

The increase in the growth of both AA and NAA non-O157 STEC strains within the first 24 h of fermentation may be due to the high pH and probably low levels of antimicrobial substances. Bakare et al. (1998) attributed the increase in the viable counts of Enteroinvasive *E. coli* (EIEC) in ogi within the first 24 h of inoculation to the high pH and low accumulation of antimicrobial substances. Valenzuela et al. (2008) reported that the low acidity of *L. plantarum* fermented millet at the early stage of fermentation enhanced the survival of STEC O157:H7. Kingamkono et al. (1998) reported that ETEC and Enteropathogenic *E. coli* (EPEC) strains were not inhibited in lactic-fermenting cereal gruel at the beginning of the fermentation process due to high pH. The lack of inhibition of the growth of both AA and NAA non-O157 STEC strains in the spontaneously fermented ogi after wet milling can be attributed to the increase in pH and possible loss of antimicrobial substances after wet milling with fresh potable tap water, as reported by Mensah and Tomkins (2003). Oranusi et al. (2007) also reported that decanting of fermenting water during wet milling of ogi resulted in an increase in the pH and reduction in the LAB population which subsequently enhanced the growth of ETEC during souring and storage.

In addition, the AA and NAA non-O157 STEC strains could have developed acid tolerance during the long period of exposure to the low pH during steeping period of the spontaneously fermented ogi which subsequently enhanced their survival at higher pH after wet milling. The reduction in pH in all the fermented ogi inoculated with AA or NAA non-O157 STEC strains was not different from those obtained during the spontaneous fermentation in the absence of non-O157 STEC strains shown in Table 4.2. This suggests that the inhibition of AA and NAA non-O157 STEC strains in ogi fermented spontaneously in combination with *L. plantarum* B411 may not be as a result of the reduction in pH or increase in the acidity.

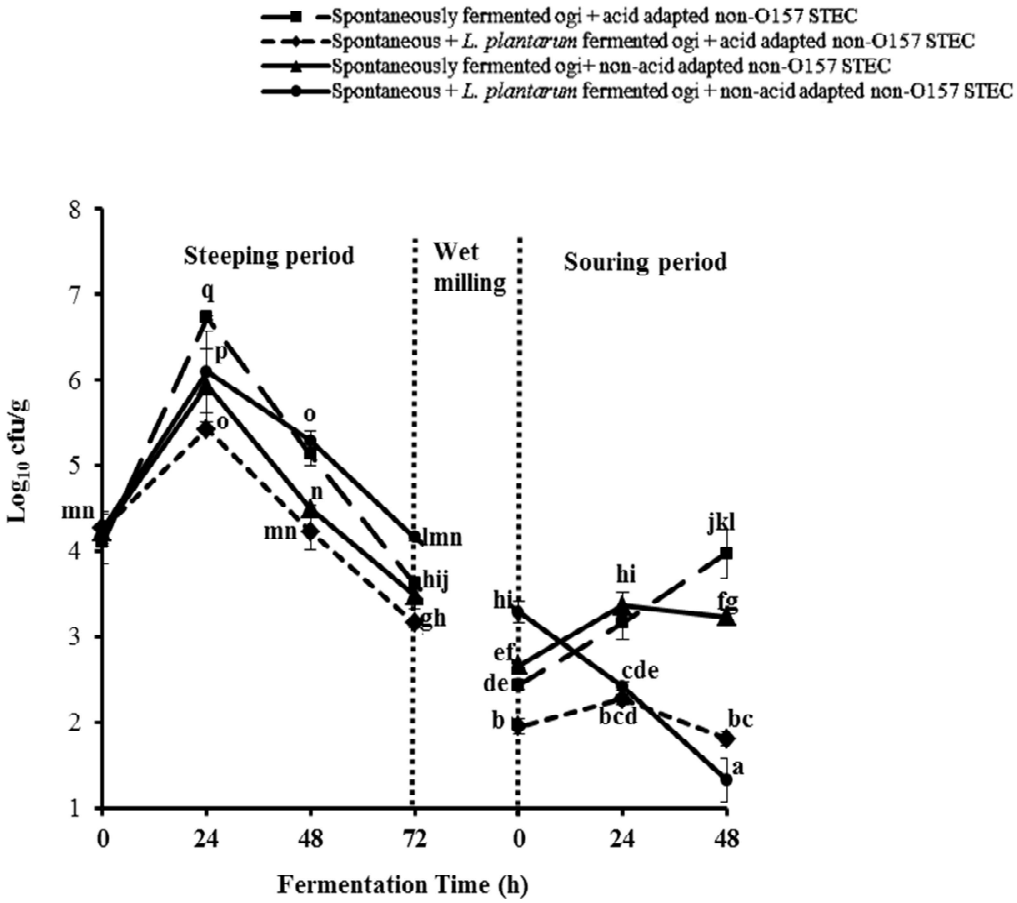

Figure 4.6: Effects of spontaneous fermentation and in combination with potential probiotic *L. plantarum* strain B411 on the survival of acid adapted and non-acid adapted non-O157 STEC strains during the production of ogi. Results are means ± standard deviation (n = 3). *Data points with different superscript are significantly different at $p \leq 0.05$.*

The *in vitro* evaluation of the antimicrobial activity of the *L. plantarum* B411 culture used for the production of ogi in this study showed a possible production of bacteriocins (crude supernatant) with antimicrobial activities against non-O157 STEC strains (Table 4.3). Thus, the inhibition of both AA and NAA non-O157 STEC strains in the ogi fermented spontaneously in combination with the probiotic *L. plantarum* can be attributed to the production of antimicrobial substances such as bacteriocins by the probiotic *L. plantarum* rather than organic acids. According to Gagnon et al. (2004), the antagonistic activity of probiotic bacteria against STEC O157 is more related to the ability of the probiotic strain to produce various antimicrobial substances than acid production. Rund et al. (2013) attributed the growth inhibition of non-O157 STEC strains when co-incubated with the probiotic bacteria to the production of microcins and not organic acids. However, contrary to this finding was the report of Ogawa et al. (2001) that the growth inhibitory and bactericidal activities exerted by probiotic *Lactobacillus* strains on STEC depend majorly on the production of organic acid and pH reductive effect.

Furthermore, the potential probiotic *L. plantarum* was at logarithmic growth phase throughout the souring period (Fig. 4.5) which may have enhanced the production of bacteriocins by the probiotic *L. plantarum* leading to the inhibition of both AA and NAA non-O157 STEC strains in the ogi fermented with probiotic *L. plantarum*. Todorov and Dicks (2006) reported that production of bacteriocins by *L. plantarum* starter culture used for the production of *boza*, a traditional fermented beverage made from maize and millet was highest at the logarithmic growth phase of the starter culture.

4.2.5 Conclusions

Inoculation of the steeped grains with potential probiotic *L. plantarum* starter culture at the onset of the spontaneous fermentation of ogi is more effective in preventing the growth of non-O157 STEC strains than the generally used spontaneous fermentation. Therefore utilization of such probiotic culture could help ensure the safety of this type of traditional African cereal gruel which is also used as weaning food and make an important impact on the health and well-being of children by reducing the occurrence of infant diarrhoea caused by non-O157 STEC.

4.3 Research Chapter 3: Probiotic potential of lactic acid bacteria isolated from ogi, a traditional non-alcoholic fermented maize gruel from West Africa

4.3.1 Abstract

The probiotic potential of lactic acid bacteria isolated from ogi, a fermented maize gruel was evaluated. Fourteen lactic acid bacteria (LAB) strains were examined for acid and bile tolerance, physicochemical properties of the bacterial cell surface as well as adhesion to Caco-2 cells and antimicrobial activity against selected enteric pathogens. The 16S rDNA genes of the strains with probiotic potential were sequenced and phylogenetic analysis was conducted for comparison with the reported potential probiotic strains. Six *Lactobacillus plantarum* and three *Pediococcus pentosaceus* strains showed bile and acid tolerance of > 6 $\log_{10}$ cfu/mL but only two strains possessed > 40% hydrophobicity. The strains with hydrophobic cell surface also demonstrated high aggregative ability and antimicrobial activities against non-O157 STEC strains and *E. coli* ATCC 25922 as well as high level of adhesion to Caco-2 cells. The strains with probiotic attributes exhibited genetic similarity with other potential probiotic bacteria. Certain LAB strains from ogi possess desirable *in vitro* probiotic properties and could be considered as potential probiotic strains. However, i*n vivo* studies are necessary to validate the colonization ability and immune stimulatory properties of the strains.

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4.3.2 Introduction

The health benefits of traditional fermented foods have been attributed to the associated fermentative microorganisms (Kalui et al., 2010; Sybesma et al., 2015). This is beacuse traditional fermented foods have a high level of bacteria with a large bio-diversity and there is a possibility that some of these bacteria could be potential probiotic candidates (Muyanja et al., 2003; Sanni et al., 2013). Certain *Lactobacillus* strains isolated from Ghana fermented maize were found to survive the simulated gastrointestinal conditions, adhere to Caco-2 cells and produced antimicrobial substances (Jacobsen et al., 1999). Kalui et al. (2009) demonstrated that the *L. plantarum* isolated from *ikii*, a Kenyan traditional fermented maize porridge can withstand the physiological challenges posed by the gastrointestinal tract (GIT) and may be able to colonise the GIT. The search for strains which show resistance to biological barriers of the human GIT, and possess physiological characteristics compatible with probiotic properties among LAB associated with the traditional African fermented foods, may lead to discovering potential probiotic strains from traditional African fermented foods for functional food products (Ugarte et al., 2006).

Bile and acid tolerance are considered as an important characteristic of probiotic *Lactobacillus* strains, which enables them to survive, grow, and exert their probiotic action in the gastrointestinal tract (Argyri et al., 2013; Guglielmetti et al., 2008). The ability to adhere to the intestinal epithelium is one of the main criteria for selecting potential probiotic strains, as this property allows probiotic bacteria to remain at least transiently in the intestinal tract and exert their probiotic effects on the host (Collado et al., 2008; Argyri et al., 2013). However, the adhesion of probiotic bacteria varies among strains and depends on the cell surface properties such as hydrophobicity also known as microbial adhesion to hydrocarbon (MATH) (Botes, 2008; Abdulla et al., 2014). This is because hydrophobicity of bacterial cell surface plays a key role in the first contact between a bacterial cell wall and intestinal epithelial cells (Schillinger et al., 2005). Further, the ability of probiotic strains to autoaggregate is also an essential pre-requisite for adhesion to intestinal epithelium, whereas their abilities to coaggregate with pathogens will enable them to form an effective barrier that prevents colonisation of epithelium by pathogenic bacteria (Del Re et al., 2000; Orłowski and Bielecka, 2006).

Despite ogi being a popular traditional fermented food in West Africa, the dominant LAB involved in its fermentation and production have not been fully evaluated to determine their

potential for probiotic attributes. Therefore, this study investigated the probiotic potential of LAB that are associated with the traditional African fermented maize gruel (ogi) in order to predict their usefulness as potential probiotic starter culture for the fermentation of traditional fermented foods.

4.3.3 Materials and methods

4.3.3.1 LAB and *E. coli* strains

Ogi was produced using traditional processed as outlined in Research chapter 2 (4.2.3.1). LAB strains isolated at various stages of the fermentation were identified with Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) (Bruker Daltonics, Bremen, Germany). Non-O157 STEC serotypes (O138 : K81 and O83 : K-) were isolated from environmental sources and the presence of Shiga toxin 1 (*Stx* 1), Shiga toxin 2 (*Stx* 2) and intimin (*eae*) genes had previously been determined (Aijuka et al., 2015), *E. coli* ATCC 25922 was also used as indicator strain.

4.3.3.2 Acid and bile tolerance of the LAB strains

Acid and bile salt tolerance of the LAB strains were determined as described by Succi et al. (2005) with modifications. The LAB strains were grown overnight in MRS broth (De Man et al., 1960) acidified with lactic acid to pH 4.5 and then 1 ml was inoculated in 100 ml of MRS broth acidified with 1.0 M HCl to pH 2.5. The respective broths were incubated at 37 ^{o}C and the survival of the LAB strains at 0, 1 and 2 h was determined on MRS agar incubated anaerobically using anaerobic jar together with Anaerocult system (Merck, Darmstadt, Germany) at 37 ^{o}C for 48 h. After 2 h of incubation in acidified broth (pH 2.5), the pH of the culture was adjusted to 6.5 (using 8% w/v sterile sodium bicarbonate solution) and 0.3% bile salt was then added to reproduce the conditions of the small intestine environment. The culture was further incubated at 37 ^{o}C for 5 h with constant agitation and the viability of the strains in the presence of bile salt was determined at 2 h intervals on MRS agar.

4.3.3.3 Hydrophobicity, autoaggregation and coaggregation assays

LAB strains for hydrophobicity also known as microbial adhesion to hydrocarbons (MATH) assay were selected based on their level of survival at low pH and in the presence of bile salts. Hydrophobicity was determined as described by Kos et al. (2003). Autoaggregation and

coaggregation were performed according to Del Re et al. (2000) as modified by Kos et al. (2003).

4.3.3.4 Antimicrobial activity of the LAB strains against pathogenic indicator strains

Antimicrobial activity of the LAB strains was performed as described by Schillinger and Lucke (1989) with modifications. The LAB strains were grown in MRS broth for 18 h at 37 °C. Cell-free supernatant (CFS) was obtained by centrifuging the culture at 5000 × g for 15 min at 4 ^{o}C, followed by filtration of the supernatant through a 0.2 µm pore size cellulose acetate filter. The pH of the filtered supernatants was adjusted to pH 6.5 with 1 M NaOH to neutralise the effect of organic acids. Inhibitory activity from the hydrogen peroxide was also eliminated with the addition of catalase (5 mg/ml). The antimicrobial activities of the CFS were determined against non-O157 STEC strains and *E. coli* ATCC 25922 by agar well diffusion method.

4.3.3.5 Adhesion to Caco-2 cells

Caco-2 cells were grown in tissue culture dishes (9 cm^2) (Orange Scientific, Biovalley, South Africa) on microscopy cover glasses in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) heat-inactivated (30 min at 56 °C) foetal calf serum, 100 U ml^{-1} penicillin, 100 mg ml^{-1} streptomycin, 0.1 mM nonessential amino acids, and 2 mM glutamine and incubated at 37 °C in 5% carbon dioxide. Caco-2 cells were used for adhesion assay after 14 days growth and adhesion assay was performed as described by Jacobsen et al. (1999). After adhesion assay, samples were prepared for SEM as described by Ranadheera et al. (2012) and viewed using a JEOL JSM-840 SEM (Tokyo, Japan).

4.3.3.6 16S rDNA Sequencing Analysis

The DNA was extracted and purified using the ZR Fungal/Bacterial DNA kit (Zymo Research). The 16S target region was amplified with Dream Tag DNA polymerase (Thermo Scientific) using the primers 16S-27F, 5′- AGAGTTGATCMTGGCTAG-3′ and 16S-1492R, 5′- CGGTTACCTTGTTACGACTT-3′ (Weisburg et al., 1991). Polymerase chain reaction (PCR) products were gel extracted (Zymo Research, Zymoclean Gel Recovery Kit), and sequenced in the forward and reverse directions on the ABI PRISM 3500XL Genetic Analyser. Sequencing products were purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit) and analysed using CLC Main Workbench 7 (CLC bio, Denmark) followed by

a Basic Local Alignment Search Tool (BLAST) searching at National Centre for Biotechnology Information (NCBI) (Altschul, et al., 1997).

The 16S rDNA gene sequences of *L. plantarum* and *P. pentosaceus* strains with probiotic attributes from the NCBI BLAST (Lv et al., 2014; Thamacharoensuk et al., 2013; Zheng et al., 2013; Senthong et al., 2012; Turpin et al., 2011) were obtained from the GenBank (NCBI). The sequences of FS2 and D39 strains were edited using Chromas (Goodstadt and Ponting, 2001) and consensus sequences were obtained using BioEdit Sequence Alignment Editor (version 7.2.5). The consensus sequences were aligned with the reference gene sequences from the GenBank using the Multiple Alignment of Fast Fourier Transform (MAFFT) online software (http://mafft.cbrc.jp/alignment/server/). After which the sequences were combined into a single file in BioEdit. Gaps and ambiguous bases were removed from each sequence pair and a neighbour-joining phylogenetic tree was constructed as described by Saitou and Nei (1987) in MEGA5 using Kimura 2 parameter method (Kimura, 1980; Tamura et al., 2011). The analysis involved 13 nucleotides sequences for each of the two strains and the confidence values of individual branches in the phylogenetic tree were determined using the bootstrap analysis based on 1000 replicates (Felsenstein, 1985).

4.3.3.7 Statistical analysis

All experiments were repeated three times and results were analysed using multifactor analysis of variance (ANOVA). Fisher's Least Significant Difference Test (LSD) was used to determine significant differences between the treatments at $p \leq 0.05$.

4.3.4 Results

4.3.4.1 Survival in the low pH and bile salt

The *L. plantarum* and *P. pentosaceus* strains showed varying degrees of tolerance to low pH and 0.3% bile salt after 7 h of exposure (Table 4.6). Eight strains survived at > 6 $\log_{10}$ cfu/mL while the survival level of the remaining 6 strains was < 5 $\log_{10}$ cfu/mL. The most tolerant strains to acidic and bile stress were *L. plantarum* FS2 and *P. pentosaceus* D39 while *L. plantarum* D30 and *P. pentosaceus* FS5 were the most sensitive strains with 28 and 25% survival respectively, after exposure to low pH and bile salt.

4.3.4.2 Hydrophobicity (MATH) of the LAB strains

The microbial adhesion to hydrocarbon (MATH) assay was performed based on the results of the *L. plantarum* and *P. pentosaceus* strains with > 6 $\log_{10}$ cfu/mL survival after exposure to low pH and bile salt. The hydrophobicity percentage of the LAB strains tested ranged between 9 and 74% (Fig. 4.7). *P. pentosaceus* D39 and *L. plantarum* FS2 strains showed more than 40% adhesion to all the hydrocarbons while *L. plantarum* D33, *L. plantarum* FS12 and *P. pentosaceus* FS27 strains showed the least affinity towards all the solvents. Among the strains with > 40% hydrophobicity, *L. plantarum* FS2 strains were characterised by higher affinity to xylene and chloroform than ethyl acetate. Though *L. plantarum* FS1, *L. plantarum* D31 and *P. pentosaceus* FS4 strains had 40% affinity towards chloroform, they adhered poorly to xylene and ethyl acetate.

4.3.4.3 Autoaggregation and coaggregation of the LAB strains

The autoaggregation and coaggregation were based on the results of the strains that exhibited > 40% hydrophobicity. Autoaggregation was higher in *L. plantarum* FS2 strains than *P. pentosaceus* D39 strain (Fig. 4.8). Autoaggregation was lower for the cells that were suspended in PBS when compared with the control in optimum growth condition in MRS broth. The coaggregation of *L. plantarum* FS2 and *P. pentosaceus* D39 with the non-O157 STEC strains and *E. coli* ATCC 25922 ranged between 42 and 60% (Fig. 4.9). Coaggregation was highest for the *L. plantarum* FS2 with MPU(W)5(2) non-O157 STEC strains and lowest for the *P. pentosaceus* D39 strain with *E. coli* ATCC 25922.

Table 4.6: Survival of LAB strains during incubation in MRS broth for 18 h at pH 4.5 (acidified with lactic acid) and for 2 h at pH 2.5 (acidified with 1.0 N HCl) followed by incubation for 5 h at pH 6.5 in presence of 0.3% bile salts

LAB strain	LAB count (Log_{10} cfu/mL)							* Survival after exposure to low pH and bile salt (%)
	at pH 4.5	at pH 2.5			Growth in 0.3% bile salt at pH 6.5			
		0 h	1 h	2 h	3 h	5 h	7 h	
L. plantarum FS1	$8.72^{a} \pm 0.07$[1]	$8.69^{a} \pm 0.08$	$7.45^{bc} \pm 1.04$	$7.25^{ef} \pm 1.00$	$7.88^{g} \pm 0.61$	$7.41^{k} \pm 0.12$	$6.14^{f} \pm 0.35$	71
L. plantarum D31	$8.96^{a} \pm 0.20$	$8.74^{a} \pm 0.18$	$7.45^{bc} \pm 0.97$	$5.71^{bcde} \pm 1.00$	$5.44^{de} \pm 0.95$	$5.41^{h} \pm 0.01$	$6.38^{f} \pm 0.10$	73
L. plantarum FS2	$8.94^{a} \pm 0.09$	$8.86^{a} \pm 0.06$	$8.37^{c} \pm 0.94$	$7.53^{f} \pm 0.98$	$8.42^{g} \pm 0.80$	$8.32^{l} \pm 0.05$	$8.28^{h} \pm 0.62$	93
L. plantarum FS11	$8.66^{a} \pm 0.05$	$8.54^{a} \pm 0.12$	$5.31^{abc} \pm 1.10$	$4.85^{abcd} \pm 1.00$	$4.36^{bcd} \pm 0.64$	$4.22^{e} \pm 0.05$	$4.01^{de} \pm 0.48$	47
L. plantarum D35	$9.12^{b} \pm 0.09$	$8.89^{a} \pm 0.10$	$6.44^{abc} \pm 1.03$	$4.57^{ab} \pm 0.98$	$4.26^{bcd} \pm 0.97$	$3.97^{d} \pm 0.03$	$3.34^{c} \pm 0.10$	38
L. plantarum D33	$9.08^{b} \pm 0.01$	$8.88^{a} \pm 0.04$	$6.63^{abc} \pm 1.00$	$6.44^{def} \pm 1.02$	$4.80^{bcd} \pm 0.40$	$5.22^{g} \pm 0.02$	$6.41^{f} \pm 0.06$	72
L. plantarum FS0	$8.70^{a} \pm 0.01$	$8.70^{a} \pm 0.04$	$7.59^{bc} \pm 0.79$	$5.83^{bcde} \pm 0.97$	$5.66^{def} \pm 0.96$	$4.76^{f} \pm 0.12$	$4.20^{e} \pm 0.14$	48
L. plantarum D30	$8.62^{a} \pm 0.12$	$8.68^{a} \pm 0.08$	$4.78^{ab} \pm 0.98$	$4.33^{ab} \pm 1.05$	$2.83^{a} \pm 0.65$	$2.53^{a} \pm 0.03$	$2.43^{ab} \pm 0.13$	28
L. plantarum FS12	$8.80^{b} \pm 0.03$	$8.74^{a} \pm 0.02$	$6.22^{abc} \pm 0.99$	$6.22^{cdef} \pm 1.02$	$5.47^{de} \pm 0.96$	$5.64^{i} \pm 0.02$	$6.41^{f} \pm 0.12$	73
P. pentosaceus D39	$8.88^{a} \pm 0.18$	$8.62^{a} \pm 0.22$	$7.06^{abc} \pm 0.85$	$6.51^{ef} \pm 1.00$	$6.84^{efg} \pm 0.96$	$7.10^{j} \pm 0.10$	$7.39^{g} \pm 0.21$	86
P. pentosaceus FS7	$9.06^{b} \pm 0.23$	$8.98^{a} \pm 0.14$	$5.45^{abc} \pm 0.97$	$3.80^{a} \pm 0.70$	$3.46^{ab} \pm 0.64$	$3.43^{b} \pm 0.06$	$3.58^{cd} \pm 0.26$	40
P. pentosaceus FS4	$8.74^{a} \pm 0.03$	$8.72^{a} \pm 0.26$	$6.41^{abc} \pm 1.00$	$4.61^{abc} \pm 0.50$	$5.25^{cde} \pm 0.91$	$5.36^{h} \pm 0.05$	$6.30^{f} \pm 0.20$	72
P. pentosaceus FS5	$8.65^{a} \pm 0.06$	$8.80^{a} \pm 0.07$	$4.21^{a} \pm 1.00$	$3.25^{a} \pm 0.48$	$3.84^{abc} \pm 0.63$	$3.68^{c} \pm 0.10$	$2.17^{a} \pm 0.33$	25
P. pentosaceus FS27	$8.82^{a} \pm 0.11$	$8.78^{a} \pm 0.04$	$6.30^{abc} \pm 1.11$	$4.43^{ab} \pm 0.50$	$5.30^{cde} \pm 0.95$	$5.40^{h} \pm 0.05$	$6.55^{f} \pm 0.12$	75

[1]Means and standard deviations n =3. Values with different superscript in the same column are significantly different at $p \leq 0.05$.
* % Survival = (Log10 cfu/mL at 7 h / Log_{10} cfu/mL at 0 h) ×100

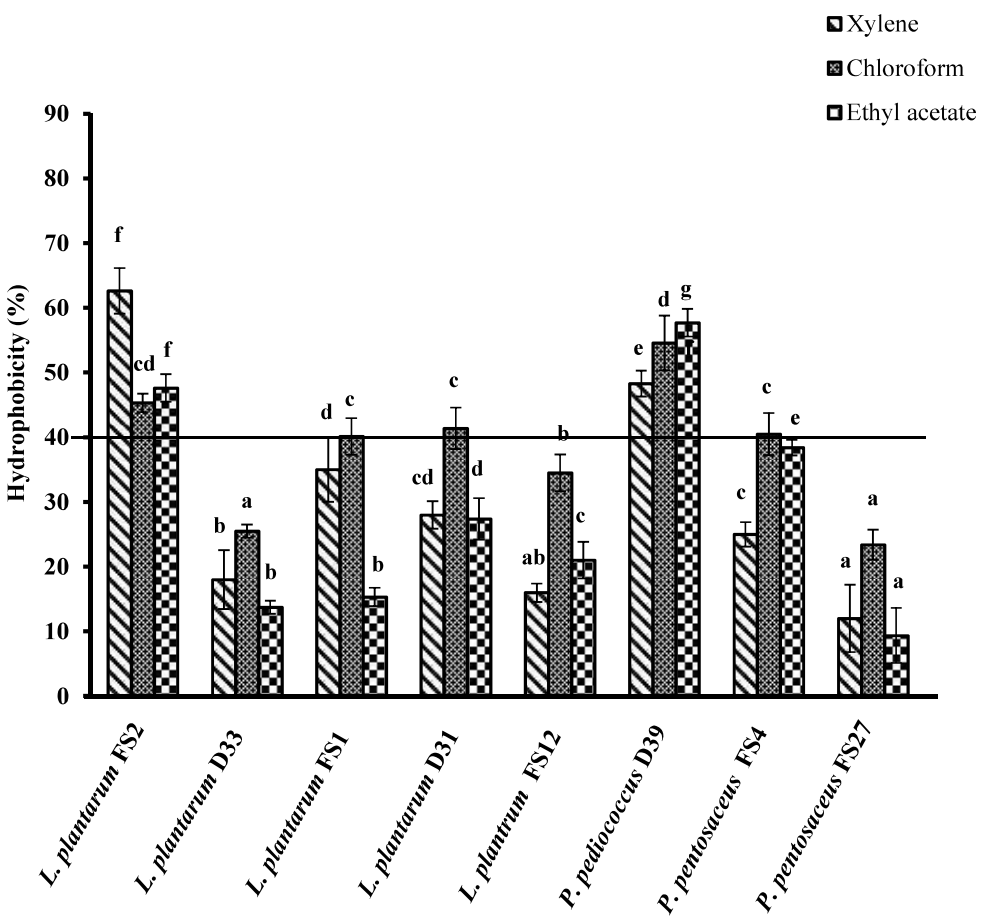

Figure 4.7: Cell surface hydrophobicity of the LAB strains as measured by microbial adhesion to hydrocarbons (MATH). The horizontal line represents 40% MATH which was taken as the least percentage that represents the strain with hydrophobic cell surface (Boris et al., 2008; Abdulla et al., 2014). Results are expressed as mean ± standard deviation (n = 3). *Same bar graph pattern with different superscript are significantly different at $p \leq 0.05$*

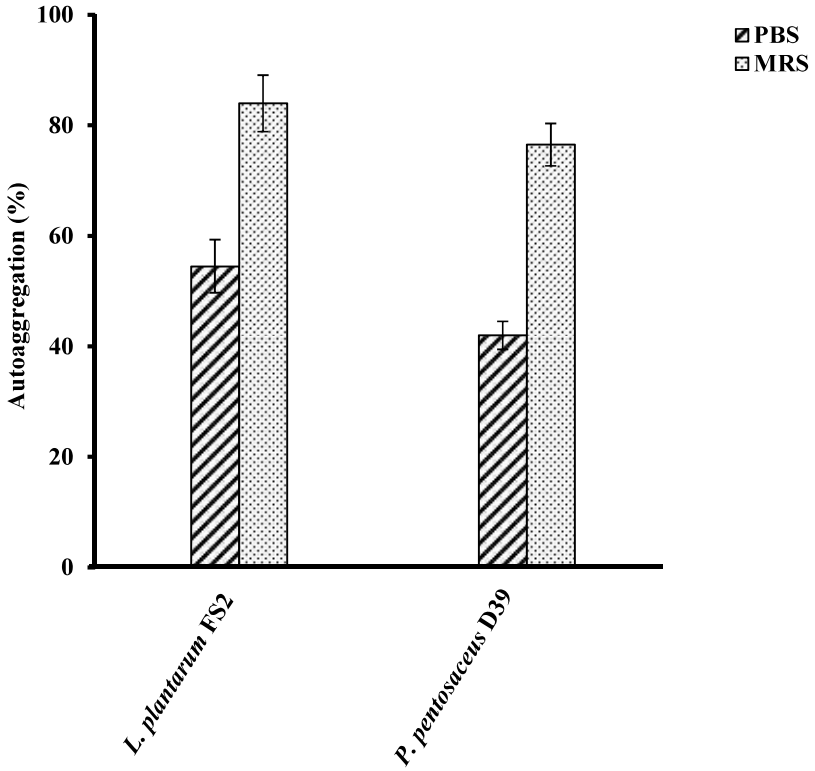

Figure 4.8: Autoaggregation of *Lactobacillus plantarum* FS2 and *Pediococcus pentosaceus* D39 after 5 h incubation at 37 ^{o}C in MRS broth and sterile phosphate buffered saline (PBS). Results are expressed as mean ± standard deviation (n = 3). *Same bar graph pattern with different superscript are significantly different at $p < 0.05$.*

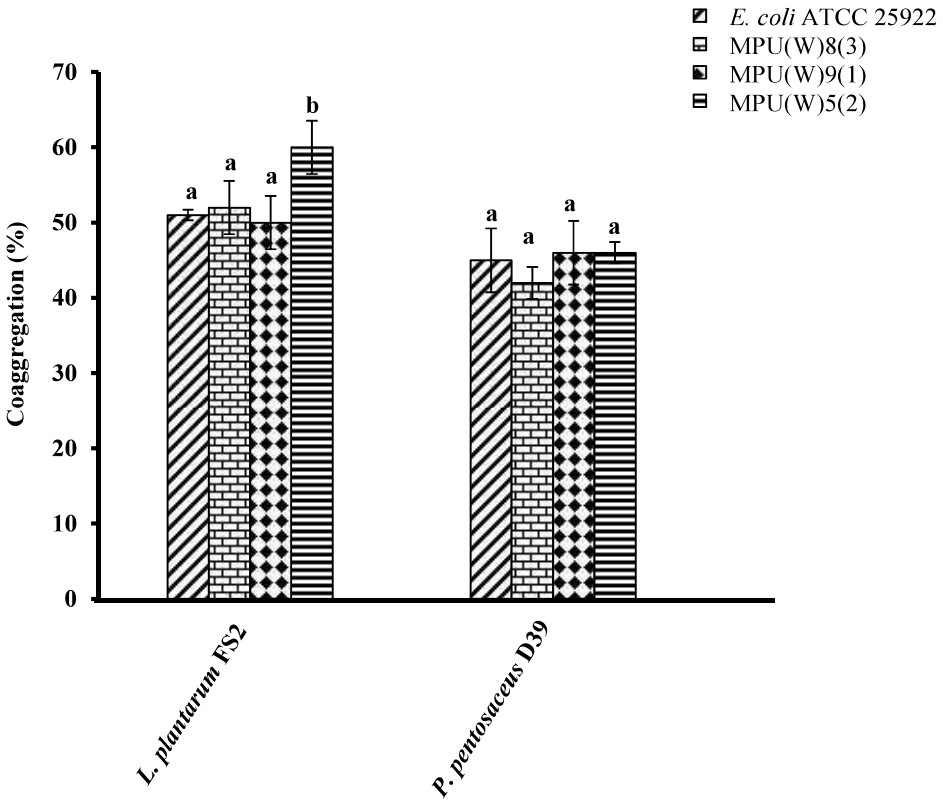

Figure 4.9: Coaggregation of *Lactobacillus plantarum* FS2 and *Pediococcus pentosaceus* D39 with non-O157 STEC strains [MPU(W)8(3), MPU(W)9(1) and MPU(W)5(2)] and *E. coli* ATCC 25922 in sterile phosphate buffered saline (PBS) after incubation for 5 h at 37 ^{o}C. Results are expressed as mean ± standard deviation (n = 3). *Same bar graph pattern with different superscript are significantly different at $p < 0.05$.*

4.3.4.4 Antimicrobial activity and adhesion to Caco-2 cells

The growth of all the *E. coli* strains was inhibited by the neutralized CFS of the *L. plantarum* FS2 and *P. pentosaceus* D39 strains (Table 4.7). The non-O157 STEC strains were the most susceptible to inhibition by the CFS of the LAB strains while *E. coli* ATCC 25922 was the least affected. *L. plantarum* FS2 showed higher antimicrobial activity against the *E. coli* strains than the *P. pentosaceus* D39. The selected LAB strains showed *in vitro* adherence to the enterocyte-like Caco-2 cells (Fig. 4.10). The adhesion varied considerably between the two LAB strains. The *L. plantarum* FS2 strongly adhered to Caco-2 cells while the *P. pentosaceus* D39 strain showed moderate adhesion ability. Adhesion of the LAB strains to Caco-2 cells is shown in Figure 4.11

4.3.4.1 16S rDNA sequencing

The 16S rDNA gene sequencing analysis and the NCBI BLAST search (www.ncbi.nlm.nih.gov) confirmed the identity of FS2 and D39 strains as *L. plantarum* and *P. pentosaceus* respectively. The neighbor-joining tree based on the 16S rDNA gene sequences showed the phylogenetic relationships between *L. plantarum* strain FS2 and *P. pentosaceus* strain D39 with genus *Lactobacillus* and *Pediococcus* respectively (Fig. 4.12). The *L. plantarum* strain FS2 was closely related with other strains of *L. plantarum, L. arizonensis* and also shared high sequence homology with *L. pentosus* strain ZU21. The potential probiotic *P. pentosaceus* strain ZZU 64, VMCU101F and PC35 from the GenBank were the closest evolutionary relatives of *P. pentosaceus* strain D39 tested in this study.

Table 4.7: Antimicrobial activities of the cell free supernant (CFS) of the *Lactobacillus plantarum* FS2 and *Pediococcus pentosaceus* D39 against non-O157 STEC strains and *E. coli* ATCC 25922

E. coli indicator strain	Inhibition zone (mm)	
	L. plantarum FS2	*P. pentosaceus* D39
MPU(W)8(3)	$28^{c} \pm 2^{1}$	$21^{b} \pm 3$
MPU(W)9(1)	$19^{b} \pm 2$	$22^{b} \pm 2$
MPU(W)5(2)	$20^{b} \pm 3$	$26^{c} \pm 2$
ATCC 25922	$13^{a} \pm 2$	$15^{a} \pm 2$

[1]Means and standard deviations n =3.
Values with different superscript are significantly different at $p \leq 0.05$.

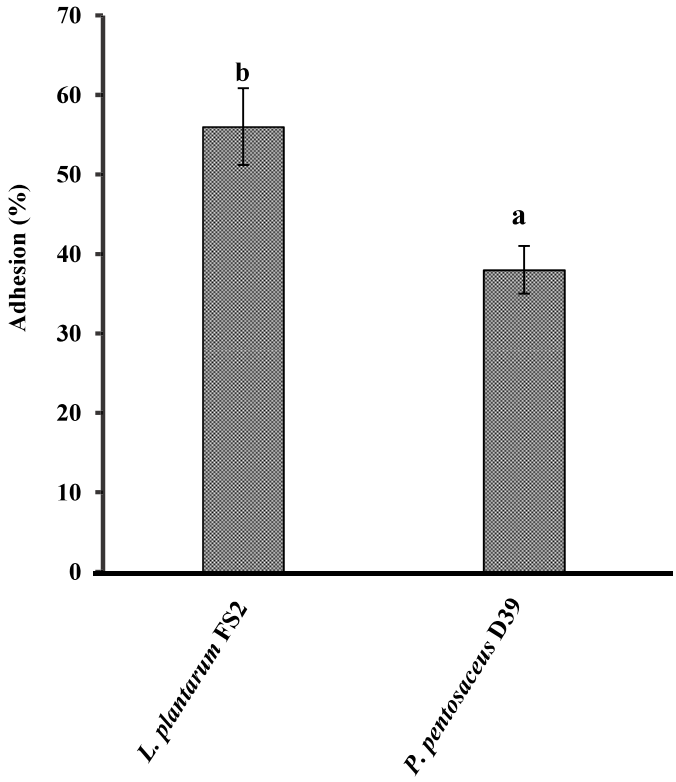

Figure 4.10: Adhesion of *Lactobacillus plantarum* FS2 and *Pediococcus pentosaceus* D39 to Caco-2 cells. Each adhesion assay was expressed as mean ± standard deviation (n = 3). *Bar graphs with different superscript are significantly different at $p < 0.05$*

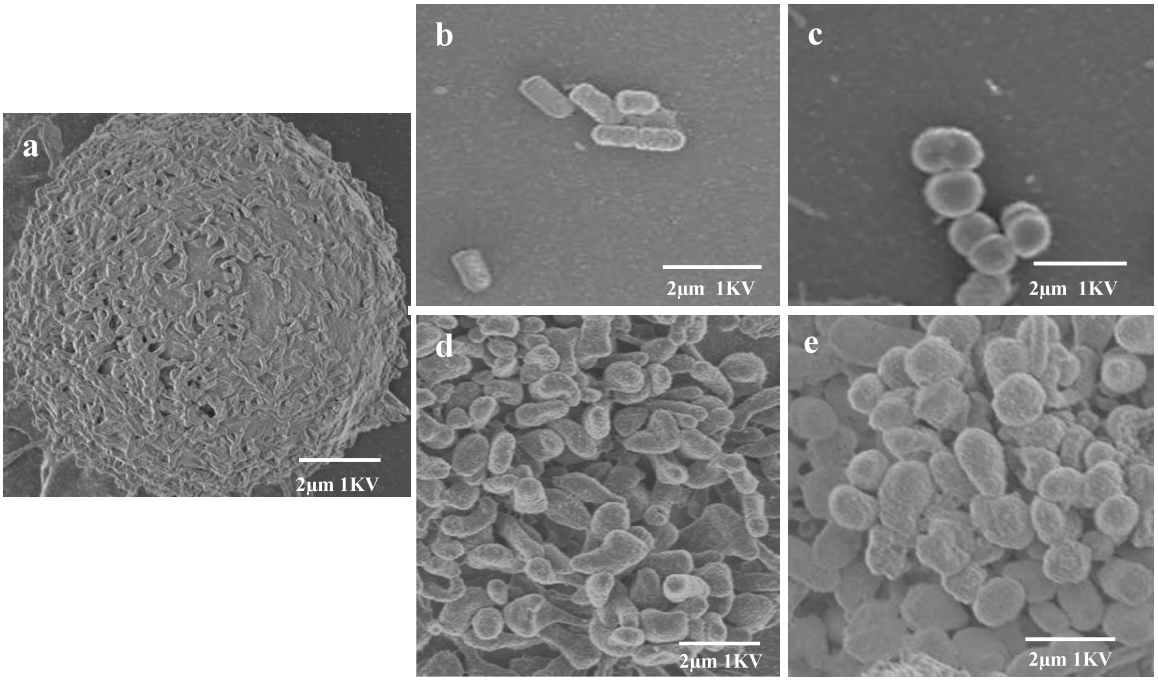

Figure 4.11: SEM showing (a) Caco-2 cells, (b) *Lactobacillus plantarum* FS2, (c) *Pediococcus pentosaceus* D39 and adherence of (d) *Lactobacillus plantarum* FS2 and (e) *Pediococcus pentosaceus* D39 to Caco-2 cells

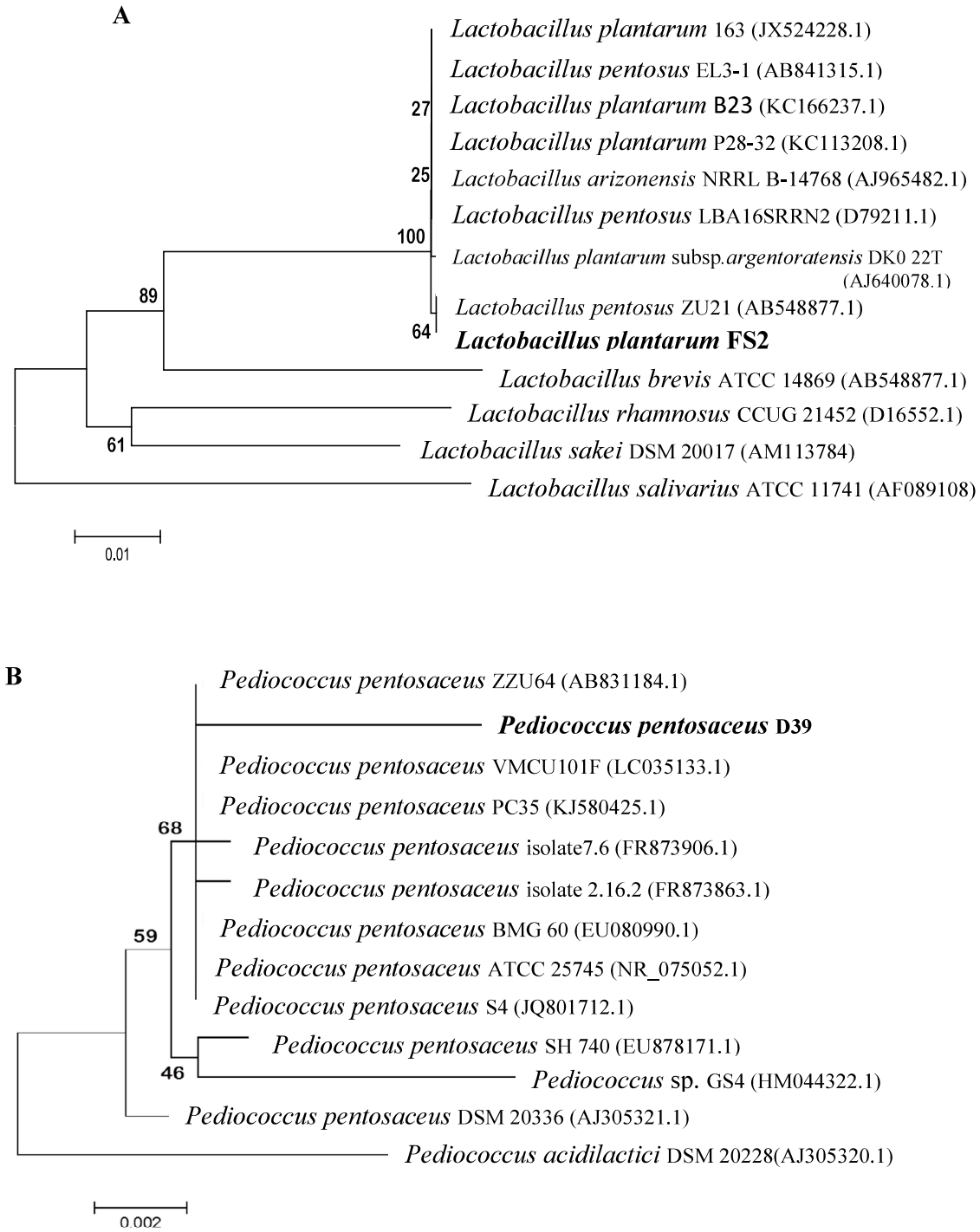

Figure 4.12: Phylogenetic tree highlighting the position of (A) *L. plantarum* FS2 and (B) *P. pentosaceus* D39 relative to the representative potential probiotic strains. The tree was constructed by the neighbor-joining method based on alignments of 16S rDNA gene sequences. Corresponding NCBI accession numbers are shown in parentheses. Numbers at the nodes indicate support values obtained from 1,000 bootstrap replications.

4.3.5 Discussion

The *L. plantarum* and *P. pentosaceus* strains with high levels of survival (> 6 $\log_{10}$ cfu/mL) after exposure to low pH and bile salt in this study could survive passage through the harsh environment of the upper part of the gastrointestinal tract and exert their possible potential probiotic action on the host (Schillinger et al., 2005; Orlowski and Bielecka, 2006). *In vitro* survival at low pH 2.5 and bile concentrations of 0.1-0.3% are considered as the standard for acid and bile tolerance of any potential probiotic cultures for any potential probiotic bacteria that will survive the harsh acidic conditions of the stomach (Pereira and Gibson, 2002). Tolerance to the low pH of the stomach and the bile content of the upper parts of the intestines are crucial for the colonization of the GIT by probiotic bacteria (Sim et al., 2015). Further, the level of tolerance of some of the *L. plantarum* and *P. pentosaceus* strains was similar to those reported for *L. acidophilus* Lac and *L. rhamnosus* GG (Mirlohi et al., 2009) and *P. acidilactici* P2, and *P. pentosaceus* FF which were regarded as potential probiotic strains (Erkkila and Petaja, 2000).

The selection of the LAB strains with > 6 $\log_{10}$ cfu/mL for the MATH assay was based on the recommendation that the number of probiotics that reach the GIT should be more than 6 log cfu/mL in order to exert the beneficial effects to human health (Gomes and Malcata, 1999; Oliveira et al., 2001; Boylston et al., 2004). The *L. plantarum* FS2 and *P. pentosaceus* D39 strains with more than 40% MATH could possibly be adherent bacteria strains with potential to adhere to the intestinal epithelial (Kos et al., 2003; Abdulla et al., 2014). This is because among several mechanisms that are involved in the adhesion of probiotic bacteria to intestinal epithelial, hydrophobic nature of the bacterial cell surface is the major determinant in the adhesion to the intestinal epithelial (Schillinger et al., 2005; Senthong et al., 2012; Abdulla et al., 2014). Further, the hydrophobicity demonstrated by *L. plantarum* FS2 and *P. pentosaceus* D39 strains is in agreement with those that were reported for probiotic strains such as *L. rhamnosus* GG, *L. rhamnosus* LC-705 and *L. plantarum* Lp-115 strains (Collado et al., 2007).

The ability of the *L. plantarum* FS2 and *P. pentosaceus* D39 strains to autoaggregate is an indication that these two LAB strains could form a barrier that will prevents colonization of the gut by pathogenic bacteria which is a crucial attributes of any potential probiotic bacteria (Collado et al., 2007). The co-aggregative ability of the *L. plantarum* FS2 and *P. pentosaceus*

D39 strains suggests that these strains can co-aggregate with pathogens, entrap them and mask the receptor sites in the intestine thereby preventing the colonization of the gut by invading pathogens (Xu et al., 2009; Collado et al., 2008). According to Ogunremi et al. (2015), co-aggregation provides an alternative mechanism for probiotic bacteria to mechanically prevent the pathogenic bacteria from attaching to the intestine epithelial cells. Aggregative ability of probiotic bacteria enhances their colonization of the intestinal epithelium due to their aggregative abilities (Del Re et al., 2000; Abdulla et al., 2014).

The antimicrobial activity of the filtered and neutralized cell free supernatant of *L. plantarum* FS2 and *P. pentosaceus* D39 strains against pathogenic *E. coli* strains indicates production of bacteriocins, as the antimicrobial activity was not lost after treatment with catalase or adjustment of pH to 6.5. The secretion of antimicrobial substances by the *L. plantarum* FS2 and *P. pentosaceus* D39 strains suggests their potential application to prevent the invasion and colonization of the gut by pathogenic bacteria (Suskovic et al., 2010). According to Fuller (1989), production of antimicrobial compounds such as bacteriocins is an important functional property to characterize probiotic bacteria. This is because the ability of probiotic bacteria to produce different antimicrobial compounds is crucial for effective competitive exclusion of pathogen from the GIT (Salminen et al., 1998).

The *in vitro* adhesion of the *L. plantarum* FS2 and *P. pentosaceus* D39 strains to Caco-2 cells suggests a possible *in vivo* colonization of the gastrointestinal tract. The relatively binding rate of LAB strains to Caco-2 cells is an indication of possible adherence of such strains to intestinal wall and to prevent pathogen adherence when used as probiotics (Sim et al., 2014). The ability of the potential probiotic LAB to adhere to mucosal surfaces will prevent their rapid removal by gut contraction and subsequent peristaltic flow of digesta, and could also confer a competitive advantage. Further, *L. plantarum* FS2 and *P. pentosaceus* D39 strains could possibly be adherent bacteria as their level of attachment to Caco-2 cells were higher than those reported for adherent probiotic strains such as *L. rhamnosus* GG, *L. johnsonii* LA1 and *L. casei* Shirota (Tuomola and Salminen, 1998; Del Re et al., 2003; Pinto et al., 2007). Therefore, the *L. plantarum* FS2 and *P. pentosaceus* D39 have the potential to competitively exclude the pathogen in the GIT by limiting the surface area available for the pathogen adhesion (Lv et al., 2014).

The comparative genomic (phylogeny) analysis revealed that the *L. plantarum* strain FS2 and *P. pentosaceus* strain D39 were closely related to other lactobacilli and pediococci that have been reported to possess probiotic characteristics (Turpin et al., 2011; Senthong et al., 2012; Thamacharoensuk et al., 2013; Zheng et al., 2013; Lv et al., 2014). The genes encoding for putative probiotic functions involved in adhesion (such as collagen-binding proteins, exopolysaccharides, lipoteichoic acids), resistance to stress (such as mannose phosphotransferase systems, bile salt hydrolases), and bacteriocin production have been identified in some of the strains with high genomic similarities to the *L. plantarum* strain FS2 and *P. pentosaceus* strain D39 tested in this study (Lv et al., 2014). This could be an indication of the possible presence of these genes in the *L. plantarum* strain FS2 and *P. pentosaceus* strain D39 tested in this study. The genetic similarity between *L. plantarum* FS2, *L. pentosus* and *L. arizonensis* is in agreement with other studies on comparative genomic analysis which identified *L. pentosus* and *L. arizonensis* as the closest genome to *L. plantarum* (Kostinek et al., 2005; Sanni et al., 2013).

4.3.6 Conclusions

Certain LAB strains that are associated with the traditional African fermented maize gruel possess desirable *in vitro* probiotic attributes. The two selected strains which exhibited high tolerance to low pH and bile-salt, aggregative characteristic coupled with antimicrobial activities against different pathogenic *E. coli* strains as well as ability to adhere to epithelial cells could be potential probiotic candidates. Further, the phylogeny analysis provided a framework for understanding the genetic similarities and probiotic functions of these strains in comparison with other potential probiotic bacteria. These two strains could be exploited as starter culture to improve the safety and health benefits of traditional cereal-based fermented foods. However, future studies could focus on detection of bacteriocins and identifying the presence of genes encoding for putative probiotic functions. Further i*n vivo* studies are also necessary to validate the colonization ability and immune stimulatory properties in order to elucidate the potential health benefits and possible application of these LAB strains in the food industry as novel probiotic bacteria.

4.4 Research Chapter 4: Effects of *Lactobacillus plantarum* and *Pediococcus pentosaceus* as potential probiotic bacteria from traditional fermented maize gruel on the survival of non-O157 STEC strains in traditional non-alcoholic fermented sorghum beverage (motoho)

4.4.1 Abstract

The effect of potential probiotic bacteria from maize gruel on the survival of environmental non-O157 STEC strains was investigated in fermented sorghum motoho. The sorghum motoho was fermented spontaneously and with *Lactobacillus plantarum* FS2 or *Pediococcus pentosaceus* D39 as well as with the combination of the two potential probiotic bacteria. Each treatment was inoculated with acid adapted (AA) or non-acid adapted (NAA) non-O157:H7 Shiga toxin producing *E. coli* (STEC) strains and their survival was monitor for 24 h at 42 ^{o}C. AA non-O157 STEC strains were inhibited more than NAA non-O157 STEC strains in all the fermented sorghum motoho. However, highest significant log reduction of 2.9 occurred in AA non-O157 STEC strains inoculated in the sorghum motoho fermented with the combination of the *Lactobacillus plantarum* FS2 or *Pediococcus pentosaceus* D39 after fermentation. Potential probiotic bacteria from traditional fermented cereals could be used as starter culture or combined with spontaneous fermentation to improve the microbiological safety of traditional fermented sorghum beverage.

4.4.2 Introduction

Shiga toxin producing *Escherichia coli* (STEC) represents a hazardous public health problem worldwide causing various human gastrointestinal tract diseases, including watery or bloody diarrhoea, and might develop a life-threatening disease (Elhadidy and Mohammed, 2013). Most STEC outbreaks, irrespective of serotype, have been caused by contaminated food and several serotypes have been linked to foodborne illness (Mathusa et al., 2010; Werber et al., 2012). The ability of STEC to cause serious disease in humans is related mainly to their capacity to produce Shiga toxins (Farrokh et al., 2013). Prevalence studies on STEC have focused primarily on *E. coli* O157:H7, because of its initial predominance in human clinical infections (Monaghan et al., 2011). However, the implication of non-O157 STEC serotypes in foodborne outbreaks and individual cases of severe illness is an indication that non-O157 STEC infections are as prevalent as, or even more than *E. coli* O157:H7 infections (Preubel et al., 2013). Non-O157 STEC serotypes are now been considered as a major contributor to human disease and frequent cause of diarrhoea especially in infants and children (Bettelheim et al., 2014). Most survival studies of STEC in food have been undertaken using *E. coli* O157:H7 strains and little is known about the survival of non-O157 STEC serotypes in food (Farrokh et al., 2013).

The acid tolerance response of STEC is an important aspect of its virulence and it has been shown that certain non-O157 STEC serotypes can survive significantly better than *E. coli* O157:H7 under acidic stress of traditionally fermented foods (Elhadidy and Mohammed, 2013). There is no consensus regarding the treatment of STEC infections (Morhins et al., 2015) and use of antimicrobial agents in the treatment of STEC associated infections is controversial because of reported increase in Shiga toxin release after antibiotic exposure (Rund et al., 2013).

The use of fermented foods containing probiotics is one of the approaches to minimise the risk of foodborne infections especially in developing countries (Franz et al., 2014). Probiotic bacteria have been shown to limit STEC infections by inhibiting both the growth and reducing the pathogenicity of non-O157 STEC strains (Rund et al., 2013; Mohsin et al., 2015). Certain *Lactobacillus* and *Pediococcus* strains from *Borde,* an Ethiopian fermented cereal food product have been shown to significantly inhibit the growth of *E. coli* O157:H7 (Tadesse et al., 2005). However, the possible role of probiotic bacteria on the survival of non-

O157 STEC serotypes in traditional fermented cereals such as sorghum has rarely been studied. Therefore this study aim to investigating the effect of processing steps and potential probiotic bacteria from fermented maize gruel on the survival of non-O157 STEC strains in traditional non-alcoholic fermented sorghum beverage known as motoho in Southern Africa.

4.4.3 Materials and methods

4.4.3.1 Sources of sorghum flour and probiotic strains

The red, fine non-tannin sorghum flour (Super Mabela, Food Corp, Randfontein, South Africa) was used for this study. The lactic acid bacteria (LAB) strains (*Lactobacillus plantarum* FS2 and *Pediococcus pentosaceus* D39) used for the fermentation were previously isolated from ogi (fermented maize gruel) and were identified using Matrix Assisted Laser Desorption/Ionization Time-of-Flight mass spectrometry (MALDI-TOF MS) (Brucker Daltonics, Bremen, Germany). The probiotic characteristics of these LAB strains had been previously determined in research chapter 3.

4.4.3.2 Inoculation of the sorghum motoho with probiotic bacteria and non-O157 STEC strains

The potential probiotic *L. plantarum* strain FS2 and *P. pentosaceus* strain D39 were activated in MRS broth (De Man et al., 1960) incubated at 37 °C for 18 h to obtain stationary phase cells. The 18 h cultures were then centrifuged at 5000 × g for 15 min at 4 °C and standardised (BioMerieux, Marcy l'Etoile, France) before suspending in the heated and cooled sorghum gruel. The three non-O157 STEC strains selected were subjected to acid adaptation as described in Research Chapter 1 (4.1.3.2) to obtain AA and NAA non-O157 STEC cells. After acid adaptation for 18 h, the resulting cell suspensions were also centrifuged at 5000 × g for 15 min at 4 °C and suspended in 0.1% BPW. A cocktail of the three non-O157 STEC strains was then prepared and standardised using McFarland Standard ampules (BioMerieux) before suspending in heated and cooled sorghum gruel. All inoculations were done to obtain cells at final inoculum level 10^6 cfu/mL in the sorghum motoho.

4.4.3.3 Processing and fermentation of motoho

Sorghum flour (70 g dry weight basis) was mixed with 1 L distilled water. The gruel was then cooked on electric hotplate for 30 min, with continuous stirring to prevent lump formation. The cooked gruel was cooled down to ambient temperature (24 ºC). The first portion of the cooked sorghum gruel was inoculated with standardised *L. plantarum* FS2 and the second portion with *P. pentosaceus* D39 while the third portion was inoculated with the combination of *L. plantarum* FS2 and *P. pentosaceus* D39. The forth portion was left to undergo spontaneous fermentation. Each treatment was also inoculated with a cocktail of either AA or NAA non-O157 STEC strains and incubated at 42 ºC for 24 h. Samples were drawn aseptically before and after cooking as well as after 24 h fermentation to determine the changes in the pH, titratable acidity (TA) and microbial population during sorghum motoho fermentation and processing.

4.4.3.4 Enumeration of microorganisms and detection of AA and NAA non-O157 STEC in the fermented sorghum motoho product

The changes in the microbial population (cfu/g) of the total aerobic bacteria, LAB, Enterobacteriaceae, yeasts and moulds and non-O157 STEC strains were determined using nutrient agar (NA) (Merck), MRS agar (De Man et al., 1960), M17 agar (Oxoid), violet red bile glucose (VRBG) agar (Oxoid), acidified (using 10% w/v tartaric acid to pH 3.5) Potatoes Dextrose Agar (PDA) (Merck) and Sorbitol MacConkey (SMAC) agar (Oxoid) respectively. Samples were enumerated by homogenizing the fermenting sorghum motoho with BPW and appropriate dilutions were made, spread plated and incubated at required temperatures. The NA, VRBG and SMAC agar plates were incubated at 37 ºC for 24 h while yeast and mould plates were incubated at 25 ºC for 3-5 days and MRS agar plates were incubated anaerobically using anaerobic jar together with anaerocult system (Merck) at 37 ºC for 48 h.

4.4.3.5 Statistical analysis

All experiments were performed three times and results were analysed using multifactor analysis of variance (ANOVA) to determine whether factors such as fermentation treatment, acid adaptation and time affected the survival of non-O157 STEC strains. Fisher's Least Significant Difference Test (LSD) was used to determine significant differences between the treatments.

4.4.4 Results and Discussion

4.4.4.1 Effect of processing steps on the microbial profile of fermented sorghum motoho in the presence of acid adapted (AA) and non-acid adapted (NAA) non-O157 STEC strains.

The presence of acid adapted non-O157 STEC strains had no substantial effect on the microbial populations in all the fermented sorghum motoho after 24 h fermentation (Table 4.8). However, the processing steps had a highly significant ($p < 0.001$) effect on the microbial profile in the sorghum motoho fermented spontaneously and with probiotic probiotic bacteria. The microbial populations in all the sorghum motoho increased significantly after 24 h fermentation. The LAB counts were lower in the spontaneously fermented sorghum motoho when compared with those fermented with single or combined probiotic bacteria (Table 4.9). There was no notable difference in the microbial population of the sorghum motoho fermented with *L. plantarum* FS2 or *P. pentosaceus* D39 after 24 h fermentation (Table 4.10 and 4.11). The sorghum motoho fermented with the combination of the two probiotic bacteria culture had lower Enterobacteriaceae counts and rapid reduction in pH than those fermented spontaneously or with single potential probiotic strain (Table 4.12). There was no notable difference in the pH and TA of all the fermented sorghum motoho inoculated with AA or NAA non-O157 STEC strains after fermentation.

The higher counts of LAB in the sorghum motoho fermented with the *L. plantarum* FS2 and *P. pentosaceus* D39 starter culture than the spontaneously fermented sorghum motoho is an indication that they have possibly dominated the fermentation process. According to Hammes et al. (1990), the use of LAB starter culture for the fermentation of cereal-based foods will ensure its dominance during the fermentation process. Further, the growth of the potential probiotic bacteria could have possibly be stimulated by the proliferation of yeasts in the fermentation which provided growth factors, such as, vitamins and soluble nitrogen compounds for the probiotic starter culture (Nout, 1991; Mugula et al., 2003). Stable co-metabolism between LAB and yeasts in fermented foods enables the utilization of substrates such as starch that are otherwise non-fermentable by LAB and thus increasing the microbial adaptability to the fermentation ecosystems (Stolz et al., 1995; Gobbetti and Corsetti, 1997). Obinna et al. (2014) attributed the dominance of *L. plantarum* starter culture in a fermented maize gruel to its ability to co-exist with the yeasts and carry out metabolic activities.

Table 4.8: Multifactor ANOVA of the microbial population in sorghum motoho fermented spontaneously and with potential probiotic bacteria (*Lactobacillus plantarum* FS2 and *Pediococcus pentosaceus* D39) for 24 h at 42 °C in the presence of acid adapted (AA) and non-acid adapted (NAA) non-O157 Shiga toxin producing *E. coli* (STEC)

Type of fermented sorghum motoho	Degrees of freedom	*P* value				
		Total aerobic bacteria	Lactic acid bacteria	*Streptococcus* and *Lactococcus*	Enterobacteriaceae	Yeasts and moulds
Spontaneously fermented sorghum motoho						
Presence of acid adapted cells	1	0.700	0.580	0.639	0.232	0.944
Processing steps (3 stages)	2	0.000	0.000	0.000	0.000	0.000
Processing × acid adaptation	2	0.485	0.181	0.503	0.128	0.209
L. plantarum FS2 fermented sorghum motoho						
Presence of acid adapted cells	1	0.400	0.082	0.124	0.090	0.790
Processing steps (3 stages)	2	0.000	0.000	0.000	0.000	0.000
Processing × acid adaptation	2	0.971	0.326	0.139	0.543	0.571
P. pentosaceus D39 fermented sorghum motoho						
Presence of acid adapted cells	1	0.769	0.140	0.091	0.485	0.890
Processing steps (3 stages)	2	0.000	0.000	0.000	0.000	0.000
Processing × acid adaptation	2	0.040	0.610	0.025	0.250	0.437
L. plantarum FS2 and *P. pentosaceus* D39 fermented sorghum motoho						
Presence of acid adapted cells	1	0.565	0.736	0.219	0.752	0.803
Processing steps (3 stages)	2	0.000	0.000	0.000	0.000	0.000
Processing × acid adaptation	2	0.068	0.251	0.703	0.507	0.344

Table 4.9: Effects of cooking and fermenting sorghum spontaneously for 24 h at 42 °C in the presence of acid adapted (AA) and non-acid adapted (NAA) non-O157 Shiga toxin producing *E. coli* (STEC) on the microbiological profile of the motoho product

Stage of motoho processing	Inoculated non-O157 STEC	pH	Titratable acidity (%)	Total aerobic bacteria (Log_{10} cfu/mL)	Lactic acid bacteria (Log_{10} cfu/mL)	*Streptococcus* and *Lactococcus* (Log_{10} cfu/mL)	Enterobacteriaceae (Log_{10} cfu/mL)	Yeasts and moulds (Log_{10} cfu/mL)
After water addition to the sorghum flour	AA	$6.40^{b} \pm 0.04$[1]	$0.023^{b} \pm 0.002$	$2.70^{b} \pm 0.07$	$2.49^{b} \pm 0.08$	$2.45^{a} \pm 0.06$	$1.47^{b} \pm 0.05$	$2.00^{b} \pm 0.02$
	NAA	$6.30^{b} \pm 0.03$	$0.024^{b} \pm 0.002$	$2.22^{b} \pm 0.05$	$2.25^{b} \pm 0.18$	$2.34^{a} \pm 0.04$	$1.27^{b} \pm 0.07$	$2.23^{b} \pm 0.06$
After cooking and inoculation with probiotic bacteria and non-O157 STEC	AA	$6.98^{c} \pm 0.08$	$0.004^{a} \pm 0.001$	$0.83^{a} \pm 0.01$	$1.21^{a} \pm 0.02$	$1.48^{a} \pm 0.03$	$0.34^{a} \pm 0.03$	$0.62^{a} \pm 0.05$
	NAA	$6.84^{c} \pm 0.05$	$0.004^{a} \pm 0.002$	$0.95^{a} \pm 0.03$	$1.19^{a} \pm 0.01$	$1.40^{a} \pm 0.04$	$0.43^{a} \pm 0.03$	$0.83^{a} \pm 0.01$
After 24 h incubation	AA	$4.83^{a} \pm 0.04$	$0.068^{c} \pm 0.004$	$7.14^{c} \pm 0.30$	$7.44^{c} \pm 0.04$	$6.89^{b} \pm 0.50$	$5.60^{c} \pm 0.36$	$6.86^{c} \pm 0.29$
	NAA	$4.69^{a} \pm 0.12$	$0.083^{d} \pm 0.004$	$7.23^{c} \pm 0.30$	$7.65^{c} \pm 0.01$	$7.46^{b} \pm 0.20$	$5.42^{d} \pm 0.01$	$6.46^{c} \pm 0.06$

[1]Means and standard deviations n =3. Values with different superscript in the same column are significantly different at $p \leq 0.05$.

Table 4.10: Effects of cooking and fermenting sorghum with potential probiotic *Lactobacillus plantarum* strain FS2 for 24 h at 42 °C in the presence of acid adapted (AA) and non-acid adapted (NAA) non-O157 Shiga toxin producing *E. coli* (STEC) on the microbiological profile of the motoho product

Stage of motoho processing	Inoculated non-O157 STEC	pH	Titratable acidity (%)	Total aerobic bacteria (Log_{10} cfu/mL)	Lactic acid bacteria (Log_{10} cfu/mL)	*Streptococcus* and *Lactococcus* (Log_{10} cfu/mL)	Enterobacteriaceae (Log_{10} cfu/mL)	Yeasts and moulds (Log_{10} cfu/mL)
After water addition to the sorghum flour	AA	$6.53^{b} \pm 0.08$[1]	$0.025^{b} \pm 0.001$	$2.68^{b} \pm 0.12$	$2.59^{a} \pm 0.02$	$2.57^{b} \pm 0.12$	$1.60^{a} \pm 0.08$	$2.00^{a} \pm 0.06$
	NAA	$6.58^{b} \pm 0.05$	$0.024^{b} \pm 0.001$	$2.54^{b} \pm 0.20$	$2.32^{a} \pm 0.06$	$2.34^{b} \pm 0.06$	$1.41^{a} \pm 0.02$	$2.23^{a} \pm 0.01$
After cooking and inoculation with probiotic bacteria and non-O157 STEC	AA	$6.92^{c} \pm 0.06$	$0.005^{a} \pm 0.001$	$0.83^{a} \pm 0.02$	$1.96^{ab} \pm 0.11$	$1.16^{a} \pm 0.06$	$0.35^{a} \pm 0.07$	$1.00^{a} \pm 0.03$
	NAA	$6.88^{c} \pm 0.06$	$0.005^{a} \pm 0.001$	$0.72^{a} \pm 0.03$	$1.63^{b} \pm 0.04$	$1.11^{a} \pm 0.10$	$0.43^{a} \pm 0.06$	$1.44^{a} \pm 0.08$
After 24 h incubation	AA	$4.26^{a} \pm 0.06$	$0.110^{c} \pm 0.014$	$7.88^{c} \pm 0.67$	$8.45^{c} \pm 0.67$	$8.84^{c} \pm 0.09$	$4.35^{b} \pm 0.52$	$6.81^{b} \pm 0.97$
	NAA	$4.27^{a} \pm 0.04$	$0.121^{c} \pm 0.014$	$7.67^{c} \pm 0.10$	$7.42^{c} \pm 0.09$	$8.83^{c} \pm 0.30$	$4.67^{b} \pm 0.52$	$6.42^{b} \pm 0.40$

[1]Means and standard deviations n =3. Values with different superscript in the same column are significantly different at $p \leq 0.05$.

Table 4.11: Effects of cooking and fermenting sorghum with potential probiotic *Pediococcus pentosaceus* strain D39 for 24 h at 42 °C in the presence of acid adapted (AA) and non-acid adapted (NAA) non-O157 Shiga toxin producing *E. coli* (STEC) on the microbiological profile of the motoho product

Stage of motoho processing	Inoculated non-O157 STEC	pH	Titratable acidity (%)	Total aerobic bacteria (Log_{10} cfu/mL)	Lactic acid bacteria (Log_{10} cfu/mL)	*Streptococcus* and *Lactococcus* (Log_{10} cfu/mL)	Enterobacteriaceae (Log_{10} cfu/mL)	Yeasts and moulds (Log_{10} cfu/mL)
After water addition to the sorghum flour	AA	$6.50^{a} \pm 0.03$[1]	$0.026^{b} \pm 0.003$	$2.59^{c} \pm 0.16$	$2.54^{d} \pm 0.08$	$2.62^{d} \pm 0.01$	$1.15^{a} \pm 0.03$	$2.00^{b} \pm 0.06$
	NAA	$6.48^{a} \pm 0.03$	$0.028^{b} \pm 0.002$	$3.47^{d} \pm 0.05$	$2.25^{c} \pm 0.10$	$2.34^{c} \pm 0.01$	$1.20^{a} \pm 0.12$	$2.18^{b} \pm 0.07$
After cooking and inoculation with probiotic bacteria and non-O157 STEC	AA	$6.78^{b} \pm 0.11$	$0.006^{a} \pm 0.001$	$0.87^{a} \pm 0.04$	$1.48^{a} \pm 0.09$	$1.28^{a} \pm 0.03$	$0.57^{ab} \pm 0.04$	$1.17^{a} \pm 0.05$
	NAA	$6.76^{b} \pm 0.04$	$0.005^{a} \pm 0.001$	$0.65^{a} \pm 0.07$	$1.30^{a} \pm 0.03$	$1.19^{a} \pm 0.06$	$0.35^{b} \pm 0.07$	$1.04^{a} \pm 0.06$
After 24 h incubation	AA	$4.47^{c} \pm 0.01$	$0.100^{c} \pm 0.001$	$8.73^{b} \pm 0.02$	$8.80^{b} \pm 0.01$	$8.57^{b} \pm 0.08$	$4.60^{c} \pm 0.73$	$7.53^{c} \pm 0.22$
	NAA	$4.46^{c} \pm 0.03$	$0.094^{c} \pm 0.002$	$8.23^{b} \pm 0.72$	$8.66^{b} \pm 0.19$	$8.49^{b} \pm 0.06$	$5.17^{c} \pm 0.08$	$7.43^{c} \pm 0.34$

[1]Means and standard deviations n =3. Values with different superscript in the same column are significantly different at $p \leq 0.05$.

Table 4.12: Effects of cooking and fermenting sorghum with the combination of potential probiotic bacteria (*Lactobacillus plantarum* FS2 and *Pediococcus pentosaceus* D39) for 24 h at 42 °C in the presence of acid adapted (AA) and non-acid adapted (NAA) non-O157 Shiga toxin producing *E. coli* (STEC) on the microbiological profile of the motoho product

Stage of motoho processing	Inoculated non-O157 STEC	pH	Titratable acidity (%)	Total aerobic bacteria (Log_{10} cfu/mL)	Lactic acid bacteria (Log_{10} cfu/mL)	*Streptococcus* and *Lactococcus* (Log_{10} cfu/mL)	Enterobacteriaceae (Log_{10} cfu/mL)	Yeasts and moulds (Log_{10} cfu/mL)
After water addition to the sorghum flour	AA	$6.55^{c} \pm 0.04$[1]	$0.019^{b} \pm .001$	$2.64^{b} \pm 0.06$	$2.42^{b} \pm 0.13$	$2.49^{b} \pm 0.03$	$1.41^{b} \pm 0.04$	$2.34^{a} \pm 0.14$
	NAA	$6.52^{c} \pm 0.03$	$0.021^{b} \pm .001$	$3.32^{b} \pm 0.24$	$2.21^{b} \pm 0.16$	$2.34^{b} \pm 0.24$	$1.34^{b} \pm 0.06$	$2.24^{a} \pm 0.01$
After cooking and inoculation with probiotic bacteria and non-O157 STEC	AA	$6.76^{b} \pm 0.01$	$0.008^{a} \pm .001$	$1.10^{a} \pm 0.08$	$1.08^{a} \pm 0.05$	$1.28^{a} \pm 0.14$	$0.44^{c} \pm 0.06$	$1.65^{ab} \pm 0.03$
	NAA	$6.78^{b} \pm 0.04$	$0.008^{a} \pm .002$	$1.23^{a} \pm 0.04$	$1.17^{a} \pm 0.08$	$1.13^{a} \pm 0.07$	$0.51^{c} \pm 0.05$	$1.45^{b} \pm 0.06$
After 24 h incubation	AA	$3.94^{a} \pm 0.01$	$0.125^{c} \pm 0.01$	$8.62^{c} \pm 0.08$	$8.65^{c} \pm 0.11$	$8.72^{c} \pm 0.06$	$3.86^{c} \pm 0.02$	$7.24^{c} \pm 0.71$
	NAA	$3.90^{a} \pm 0.01$	$0.151^{d} \pm 0.01$	$8.12^{c} \pm 0.63$	$8.69^{c} \pm 0.17$	$8.22^{c} \pm 0.76$	$3.95^{c} \pm 0.01$	$7.67^{c} \pm 0.11$

[1]Means and standard deviations n =3. Values with different superscript in the same column are significantly different at $p \leq 0.05$.

The level of Enterobacteriaceae in all the fermented sorghum motoho suggests that certain pathogenic bacteria can proliferate in the product and develop adaptation to the changing pH as fermentation progresses (Gadaga et al., 2013). However, the lower Enterobacteriaceae counts in the sorghum motoho fermented with the combination of the *L. plantarum* FS2 and *P. pentosaceus* D39 in comparison with the spontaneously or single strain fermented sorghum motoho corresponded with the rapid decrease in the pH during fermentation. This could be attributed to the fact that the combination of the potential probiotic bacteria possibly resulted in accumulation of antimicrobial substances such as bacteriocins which enhanced the inhibition of pathogenic bacteria during the fermentation process (Gadaga et al., 2004; Obinna-Echem et al., 2014). According to Kingamkono et al. (1994) and Willumsen et al. (1997), combination of starter culture for cereal fermentation will ensure that a desirable pH that will inhibit the growth and toxin production by foodborne enteropathogens is rapidly achieved. Thus, the *L. plantarum* FS2 and *P. pentosaceus* D39 could be combined as starter culture for the fermentation and production of a safe cereal-based traditional fermented product.

4.4.4.1 Effect of *L. plantarum* FS2 and *P. pentosaceus* D39 on the survival of AA and NAA non-O157 STEC strains in fermented sorghum motoho

Adaptation to acid and fermentation process had a highly significant ($p < 0.001$) effect on the survival of non-O157 STEC strains in the fermented sorghum motoho (Table 4.13). Fermentation with potential probiotic bacteria also had a significant ($p < 0.01$) effect on the survival of non-O157 STEC strains. NAA non-O157 STEC strains survived substantially higher than AA non-O157 STEC strains in all the fermented sorghum motoho after 24 h of inoculation and fermentation (Table 4.14). A substantial highest log reduction of 2.9 occurred in AA non-O157 STEC strains inoculated in the sorghum motoho fermented with the combination of the *L. plantarum* FS2 and *P. pentosaceus* D39 after fermentation. There was no substantial difference in the survival of NAA non-O157 STEC strains in sorghum motoho fermented either spontaneously or with a single potential probiotic strain.

Table 4.13: Multifactor ANOVA of the survival of acid adapted (AA) and non-acid adapted (NAA) non-O157 Shiga toxin producing *E. coli* (STEC) inoculated in sorghum motoho incubated spontaneously and with the potential probiotic bacteria (*Lactobacillus plantarum* FS2 and *Pediococcus pentosaceus* D39) for 24 h at 42 ^{o}C.

Treatment	Degrees of freedom	*P* value
Potential probiotic	3	0.006
Acid adaptation	1	0.000
Fermentation process	1	0.000
Potential probiotic × Processing stage	3	0.000
Acid-adaptation × Processing stage	1	0.000
Potential probiotic × acid adaptation	3	0.004
Potential probiotic×acid-adaptation×Processing stage	3	0.034

Table 4.14: Effects of fermenting sorghum spontaneously and with potential probiotic bacteria (*Lactobacillus plantarum* FS2 and *Pediococcus pentosaceus* D39) for 24 h at 42 °C on the survival of acid adapted (AA) and non-acid adapted (NAA) non-O157 Shiga toxin producing *E. coli* (STEC) in the motoho product

Stage of motoho processing	Inoculated non-O157 STEC	Spontaneously fermented sorghum motoho	*Lactobacillus plantarum* FS2 fermented sorghum motoho (Log_{10} cfu/mL)	*Pediococcus pentosaceus* D39 fermented sorghum motoho (Log_{10} cfu/mL)	*Lactobacillus plantarum* FS2 and *Pediococcus pentosaceus* D39 fermented sorghum motoho (Log_{10} cfu/mL)
After water addition to the sorghum flour	AA	-	-	-	-
	NAA	-	-	-	-
After cooking and inoculation with probiotic bacteria and non-O157 STEC	AA	$4.52^{g} \pm 0.02$[1]	$4.42^{g} \pm 0.06$	$4.49^{g} \pm 0.02$	$4.58^{g} \pm 0.02$
	NAA	$4.48^{g} \pm 0.04$	$4.40^{g} \pm 0.07$	$4.46^{g} \pm 0.01$	$4.57^{g} \pm 0.05$
After 24 h incubation	AA	$2.70^{d} \pm 0.01$	$2.16^{b} \pm 0.09$	$2.43^{c} \pm 0.26$	$1.67^{a} \pm 0.39$
	NAA	$3.78^{f} \pm 0.04$	$3.95^{f} \pm 0.02$	$3.98^{f} \pm 0.02$	$3.22^{e} \pm 0.05$

[1]Means and standard deviations n =3. Values with different superscript are significantly different at $p \leq 0.05$.
- not determined

Since there is no published data on the survival of non-O157:H7 STEC strains in fermented cereal products, reference will be made to data concerning *E. coli* O157:H7 strain and other *E. coli* pathotypes in the discussion of the results. The higher survival of NAA than AA non-O157 STEC strains in all the fermented sorghum motoho could be attributed to the acid tolerance ability of the non-O157 STEC strains used for this study. It has been reported that the extent to which STEC such as *E. coli* O157:H7 is inhibited by low pH of cereal fermented products depends on the acid tolerance of the strain concerned (Gadaga et al., 2004). Buchanan and Edelson (1996) also reported that acid adaptation did not enhance acid tolerance in acid tolerant strains of *E. coli* O157:H7. This was attributed to the weakening effects of cellular damage during acid adaptation which exceeded the protective effect of acid shock proteins or other protective mechanisms induced at the low pH in acid tolerant strains. Tsai and Ingham (1997) reported that acid adaptation did not enhance tolerance of *E. coli* O157:H7 to the acidic stress in food products such mustard and sweet pickle relish.

The higher survival of NAA than AA non-O157 STEC strains may be due to the fact that the NAA non-O157 STEC strains acquired acid adaptation during fermentation while the AA non-O157 STEC strain, which had previously been adapted to lower pH could not undergo the physiological changes required to adapt again to higher pH of the fermented sorghum motoho (Dlamini and Buys, 2009; Ryu and Buchant, 1998).

Adaptation of non-O157 STEC strains with lactic acid could have also enhanced the susceptibility of AA non-O157 STEC strains to inhibition by other organic acids produced during the fermentation of the sorghum motoho. This is because the response of acid adapted cells to inhibition in acidic food depends on the type of acidulant used to induce acid adaptation (Ryu and Buchant, 1998). Semanchek and Golden (1998) reported that adaptation of *E. coli* O157:H7 with lactic acid resulted in injured adapted cells which reduced their survival in further exposure to acidic foods.

Further, the greater inhibition of AA non-O157 STEC strains in the sorghum motoho fermented with the combination of the potential probiotic bacteria than those fermented spontaneously or with single strain potential probiotic culture may be due to the accumulation of antimicrobial substances coupled with the effect of acid adaptation which increased the vulnerability of the acid adapted cells to inhibition (Hsin-Yi and Chou, 2001). Fermentation of a similar product known as *Hussuwa*, a Sudanese fermented sorghum porridge with

combined starter culture of *Pediococci* and *Lactobacilli* has been reported to result in accumulation of antimicrobial substances and subsequent reductionin the level of pathogenic bacteria (Yousif et al., 2010). Combination of starter culture of lactobacilli for natural fermentation of *togwa*, a Tanzanian fermented maize gruel, has also been reported to bring about a rapid reduction in pH which resulted in inhibition of pathogenic bacteria than the use of a single strain culture or spontaneous fermentation (Mugula et al., 2003).

4.4.5 Conclusions

Environmental non-O157 STEC strains may develop acid adaption in fermented sorghum motoho and could pose a threat to public health as their growth may not be eliminated during fermentation. However, application of probiotic starter culture coupled with prior adaptation to acid such as backslopping could be a better means of controlling the growth of non-O157 STEC strains in fermented sorghum beverage than generally use spontaneous fermentation.

5. GENERAL DISCUSSION

This general discussion will critically review some of the methodologies applied in this research, discuss the findings on the effects of acid adaptation and potential probiotic bacteria on the survival of non-O157 STEC strains in traditional African fermented foods. Lastly, future research including *in vivo* studies on the potential probiotic bacteria isolated and characterised from ogi will be proposed.

5.1 Methodological considerations

In the study, the method described by Buchanan and Edelson (1999) was used to prepare acid adapted (AA) and non-acid adapted (NAA) non-O157 STEC strains. Buchanan and Eldeson (1999) determined acid tolerance of stationary phase acid adapted *E. coli* O157:H7 cells in Brain Heart Infusion (BHI) broth acidified with HCl acid to pH 2.5 and pH 3.0. This method was used in the current study because it has been shown to enhance maximal acid tolerance in *E. coli* at low pH (Alvarez-Ordonez et al., 2009; Dlamini and Buys, 2009; Parry-Hanson et al., 2010). Though no particular method has been reported involving acid adaptation in non-O157 STEC serotypes, it has been hypothesized that non-O157 STEC serotypes may behave similar to *E. coli* O157:H7 (Mathusa et al., 2010), hence, the choice of this method may be justified.

Acid adaptation was performed in Tryptone Soy Broth (TSB) because this medium mimics conditions likely to be encountered in food system and it also enhances the recovery of injured cells (Buchanan and Edelson, 1996). The incubation temperature of 37 ^{o}C for 18 h was chosen because it is the optimum growth temperature of most non-O157 STEC serotypes. The underlying principle for 18 h culturing is that non-O157 STEC strains will ferment the available glucose into acid, which subsequently will result in a gradual reduction in pH of the fermenting medium. The gradual decrease in the pH of the medium will trigger physiological and biochemical changes that may induce acid tolerance in subsequent exposure to lethal acidic conditions (Leyer et al., 1995; Bearson et al., 1997). In addition, the 18 h incubation period was also to ensure that cells had reached stationary phase and that acid adaptation was fully activated at the end of the incubation period. The Morpholino propanesulphonic acid (MOPS) was incorporated to prevent any change in pH of the medium as the NAA cells can metabolize the small quantities of glucose (0.25%) in TSB to acid thereby inducing acid adaptation in the process.

The cells were prepared to a stationary phase because stationary phase cells are more tolerant to low pH than cells at log phase (Buchanan and Edelson, 1996; Buchanan and Doyle, 1997). The non-O157 STEC strains were cultured at pH 7.4 to determine their growth in optimum conditions while the pH 4.5 is the average pH of most traditional fermented foods. Therefore, pH 4.5 was used as a model to predict the organism's likely behaviour when exposed to acidic conditions in fermented goat's milk and traditional African fermented cereal-based foods. Since the outer membrane proteins and the fatty acids in the membrane phospholipids bilayer of *E. coli* play crucial role in the adaptation to acid stress conditions (Chang and Cronan, 1999), it would have been valuable to evaluate the alterations in the fatty acid profile of the cell membrane after adaptation. If this was done, it could have provided an insight on the impact of adaptation on the permeability and modification of cell membrane phospholipids of the AA non-O157 STEC strains which possibly influenced their acid tolerance when challenged in traditional fermented foods.

Acid tolerance of the acid adapted non-O157 STEC strains was performed in BHI broth acidified with 2 M lactic acid to pH 2.5. The cells were challenged at pH 2.5 in order to evaluate the possible survival of non-O157 STEC strains in the acidic condition of the stomach. This is crucial because for any invading pathogen to cause disease in humans it must survive the acidic conditions of the stomach as the acidity of the gastric juice provides a first line of defence against food-borne pathogens (Benjamin and Datta, 1995). Brain Heart Infusion broth was selected over simpler systems such as phosphate buffer saline (PBS) or minimal medium because it is closely resemble the composition of foods associated with the transmission of enterohaemorrhagic *E. coli* (Buchanan and Edelson, 1996). Further, the response of acid adapted cells depends on the type of acidulant used to induce acid adaptation (Ryu and Beuchat, 1998). In this study, lactic acid was used as the acidulant of choice because it is produced by LAB during fermentation of many traditional fermented foods and it has commercial application in acidifying food products. Though it would have been more appropriate to use inorganic acids such as HCl as an acidulant of choice for acid challenge to stimulate conditions similar to that encountered in the stomach, its use lacks practical application because it is rarely added to foods as acidulant (Deng et al., 1999). Further, in food systems, the acids to which pathogens are exposed are either produced intrinsically through fermentation or are added for the purpose of food preservation. This limits the practical application of HCl acid in accessing the response of enteric bacteria to low pH in

food products. The non-O157 STEC strains with acid tolerance potential were serotyped. The Somatic O and capsular K antigens were identified by the tube and slide agglutination tests respectively (Orskov et al., 1977).

5.1.1 Microbiological analyses

The changes in the microbial population of the microorganisms associated with the spontaneous fermentation of ogi were determined at 24 h interval on selective agar media. This was done in order to describe the microbial profile during spontaneous fermentation and processing of ogi as well as determining the dominant LAB during the fermentation. Presumptive identification such as cellular morphology, Gram staining, catalase reaction and motility test of the LAB isolates were performed (Collins et al., 1989). The LAB isolates were then identified using Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS). Since LAB and yeasts are the most predominant microorganisms associated with the fermentation of ogi (Teniola and Odunfa, 2002; Omemu et al., 2007), evaluation of microbial diversity and dynamics during fermentation of both ogi and sorghum motoho would have verified the specific role and contribution of background microflora such as yeast in the survival of non-O157 STEC during the fermentation process.

The dendrogram analysis was constructed in order to determine the similarity among the dominant *L. plantarum* strains at different stages of ogi spontaneous fermentation and *L. plantarum* strain B411. The comparison was done in order to predict the suitability of the *L. plantarum* strain B411 as starter culture for the fermentation and production of ogi. However, sterilisation of the maize grain before fermentation to eliminate the involvement of natural microflora would have further confirmed the ability of the *L. plantarum* strain B411 to effectively ferment ogi as a single strain starter culture.

The survival of AA and NAA non-O157 STEC strains in fermented goat's milk, ogi and sorghum motoho was determined on Sorbitol MaConkey agar (SMAC). Though SMAC is widely used for the isolation and enumeration of *E. coli* O157:H7, it was used in the current study to enumerate non-O157 STEC strains because there are no clearly identified selective media for the isolation or enumeration of non-O157 STEC serotypes (Gould et al., 2009; Kaspar and Doyle, 2010). This is probably because non-O157 STEC consists of a large

group of different serotypes and having widely accepted selective-differential media to determine the individual serotypes could be challenging. Most of the media used for the isolation or enumeration of non-O157 STEC are modified from those originally developed for *E. coli* O157:H7. Some antibiotics such as cefixime and vancomycin as well as selective agents such as tellurite have been added to SMAC (CT-SMAC) to inhibit the growth of non-STEC bacteria (Vimont et al., 2007). However, the limitation of using CT-SMAC in enumeration of non-O157 STEC is that some serotypes are sensitive to these antibiotics and selective agents (Hussein et al., 2008). The addition of novobiocin, cefixime and tellurite to SMAC has been shown to greatly inhibit some non-O157 STEC serotypes (Baylis et al., 2008 and Orden et al., 2008). Using SMAC to enumerate non-O157 STEC will result in bright pink to mauve colonies indicating sorbitol-fermenting organisms which are mostly non-O157 STEC serotypes while the Gram positive microorganisms will be inhibited on this medium by crystal violet and the bile salts mixture in the formulation (Mathusa et al., 2010). The primary carbon source which is sorbitol in SMAC will support the growth of non-O157 STEC serotypes (Atkinson et al., 2012).

5.1.2 Probiotic characterisation

The *in vitro* assessment was performed to determine the probiotic properties of the *L. plantarum* strain B411 and the dominant LAB isolated from ogi spontaneous fermentation. The LAB strains were first cultured in pH 4.5 to determine the growth response in fermented foods while incubation at pH 2.5 and subsequent exposure to 0.3% bile was performed in order to determine their resistance to gastric acidity and bile toxicity. The pH 2.5 was to mimic the acidic conditions of the stomach while the 0.3% bile was to reproduce the conditions of the small intestine. The hydrophobicity was determined as described by Kos et al. (2003). The bacteria adhesion to hydrocarbons was determined using xylene, chloroform and ethyl acetate. The xylene which is an apolar solvent was used in order to determine the bacterial cell surface hydrophobicity or hydrophilicity while the chloroform (a monopolar and acidic solvent) and ethyl acetate (a monopolar and basic solvent) were used to measure the electron donor (basic) and electron acceptor (acidic) characteristics of bacterial cell surface, respectively (Bellon-Fontaine et al., 1996). The autoaggregation of the LAB strains were determined by culturing in sterile phosphate buffered saline (PBS) and MRS broth while the co-aggregation with pathogens was determine only in sterile PBS. The PBS and the MRS broth were incubated in a shaking water bath at 37 ^{o}C for 5 h. The sterile PBS was used

because the osmolarity and ion concentrations of the solutions match those of the human body and is non-toxic to bacteria cells.

The antimicrobial activities of the Cell-free supernatant (CFS) of the LAB strains were determined against non-O157 STEC strains and *E. coli* ATCC 25922 by agar well diffusion method. The pH of the filtered supernatants was adjusted to pH 6.5 with 1 M NaOH to neutralise the effect of organic acids and the inhibitory activity from the hydrogen peroxide was also eliminated with the addition of catalase. However, purification and characterisation of the bacteriocins would have given a deeper understanding of the class and antimicrobial activities of the bacteriocins produced by the potential probiotic bacteria. Caco-2 cells were used to evaluate the *in vitro* adhesion of the LAB strains to intestinal epithelium cells. The use of Caco-2 cells was based on the fact that they are capable of forming junctional complexes which can constitute a confluence monolayer that mimics the intestinal epithelial (Cereijido et al., 1998). Though adhesion of probiotic bacteria to Caco-2 cells is a strain-specific characteristic (Chauviere et al., 1992), it would have been valuable to perform the adhesion potential of the LAB strains tested in this study in comparison with already known probiotic bacteria. The 16S rDNA sequencing technique and phylogeny analysis was used to determine genetic similarity between the *L. plantarum* FS2, *P. pentosaceus* 39D and other reported potential probiotic *L. plantarum* and *P. pentosaceus* strains respectively. This sequencing technique was used because it can determine high level of genetic similarity (up to 97% level) between bacteria of the same species (Palys et al., 1997).

5.2 Acid resistance of non-O157 STEC serotypes in the goat's milk, ogi and sorghum motoho fermented with potential probiotic bacteria

The growth of both AA and NAA non-O157 STEC strains were not inhibited in the goat's milk fermented with *L. plantarum* strain B411 alone. The final pH of the AA non-O157 STEC strains was 4.5 when inoculated in the *L. plantarum* fermented goat's milk which was at pH 5.5. Therefore, it is possible that the AA non-O157 STEC strains had developed acid tolerance during prior adaption to acid at lower pH 4.5 which subsequently enhanced the survival of AA non-O157 STEC strains in the *L. plantarum* fermented goat's milk. On the other hand, the pH of the *L. plantarum* fermented goat's milk possibly induced acid adaptaion in the NAA non-O157 STEC strains. High pH (> 5.0) has been reported to induce acid

adaptation in non-acid adapted *E. coli* cells in fermented milk products (Gran et al., 2003). This is because *E. coli* has the ability to grow within the pH range of 5.0 to 8.5 while maintaining internal pH level (Foster, 2000). According to Leyer et al. (1995) and Bearson et al. (1997), mild acidic conditions is capable of inducing acid adaptation in *E. coli* by modulating physiological changes to suit the new environment.

The goat's milk that was fermented with the combination of the yoghurt starter culture and the potential probiotic *L. plantarum* B411 had a bacteriostatic effect on the NAA non-O157 STEC strains while the AA non-O157 STEC strains were marginally inhibited after incubation for 6 h (Fig. 4.1). As established, the non-O157 STEC strains used for this study possessed acid tolerance potential in BHI broth at low pH (Table 4.1). Therefore it is possible that the low pH in goat's milk fermented with the yoghurt starter culture and in combination with the potential probiotic *L. plantarum* B411 induced acid adaptation in NAA non-O157 STEC strains. Since the potential probiotic *L. plantarum* B11 was capable of producing bacteriocins (Table 4.3), the presence of yoghurt starter culture possibly supported the growth of the *L. plantarum* B411 leading to the production and accumulation of antimicrobial substances. On the other hand, the substantial inhibition of the AA non-O157 STEC strains in the goat's milk fermented with the combination of the yoghurt starter culture and the potential probiotic *L. plantarum* B411 may be due to the fact that the process of re-adaptation by the acid adapted cells to growth in the fermenting goat's milk was stressful thereby resulting in failure of the adapted cells to acquire adaptation. According to Ryu and Buchant (1998), acid adaptation is a complicated process in which many physiological changes, including the production of protective acid stress proteins and damage to the cell membranes may occur which could influenced the survival of acid adapted cells to subsequent exposure to acidic conditions.

Another possible reason for the marginal inhibition of the AA than NAA non-O157 STEC strains could be the negative impact of prior adaptation to acid which enhanced the vulnerability of the adapted cells to antimicrobial compounds produced by the potential probiotic bacteria. Hsin-Yi and Chou (2001) reported that acid adaptation increased the susceptibility of *E. coli* O157:H7 to antimicrobial substances produced by LAB starter cultures in fermented milk products. Further, the low pH of the goat's milk fermented with the yoghurt starter culture in combination with potential probiotic *L. plantarum* B411 could

have enhanced the antimicrobial activities of the bacteriocins produced against the acid adapted cells. According to Zacharof and Lovitt (2012), bacteriocins produced by LAB have greater antibacterial activity at pH < 5 because their adsorption to the cell surface of pathogenic bacteria is pH dependent.

The similar survival trends observed for AA and NAA non-O157 STEC strains in ogi suggests that prior adaptation to acid did not contribute to acid tolerance in ogi fermentation and may not be necessary for the survival of non-O157 STEC strains in ogi fermentation. Though application of LAB as starter culture will ensure its dominance during fermentation process (Hammes et al., 1990), the involvement of natural microflora such as yeasts and other LAB during ogi fermentation cannot be ruled out since no sterilization treatment was applied on the maize grain before fermentation. In cereal fermentation, some lactobacilli are capable of hydrolysing the starch resulting in low pH while yeasts, in return, provide growth stimulants such as vitamins and amino acid needed for growth by the LAB (Querol and Fleet, 2006). Therefore, the presence of other natural microflora could have favoured the growth of *L. plantarum* B411during ogi fermentation thereby resulting in accumulation of antimicrobial substances.

The *in vitro* evaluation of the antimicrobial activities of the *L. plantarum* B411 and some of the LAB that dominated ogi spontaneous fermentation established production of bacteriocins. Therefore, the inhibition of both AA and NAA non-O157 STEC in the ogi may be due to the antimicrobial activities of the bacteriocins produced by both the *L. plantarum* B411 and some of the LAB that dominated the ogi fermentation. Various mechanisms have been proposed for antimicrobial action of bacteriocins against pathogenic bacteria (Konisky, 1982; Settanni and Corsetti, 2008; Stevens et al., 1991). Bacteriocins generally exert their antimicrobial action by interfering with the cell wall or the membrane of target organisms, either by inhibiting cell wall biosynthesis or causing pore formation, subsequently resulting in death (O'Sullivan et al., 2002; Settanni and Corsetti, 2008). Bacteriocins such as *plataricins* produced by *L. plantarum* are capable of creating disruption of active transport and produce leakage of ions such as potassium and magnesium ions from the target cell by forming voltage-dependent channels in phospholipid bilayer membranes and hydrolysis of ATP resulting in cell death (Konisky, 1982).

The *L. plantarum* FS2 and *P. pentosaceus* D39 isolated from ogi were used to fermented sorghum motoho. The NAA non-O157 STEC strains survived higher than the AA non-O157 STEC strains when challenged in fermented sorghum motoho which was similar to what occurred in fermented goat's milk. As earlier stated, the final pH of the acid adapted cells was 4.5 at the point of inoculation while the pH of the fermenting sorghum motoho was 6.5. This variation in pH possibly resulted in the inhibition of the AA non-O157 STEC strains as acid adaption may be lost by acid adapted cells when expose to optimum growth conditions (Jordan et al., 1999). Further, the weakening effects of cellular damage that probably occurred during acid adaptation exceeded the protective effect of acid shock proteins or other protective metabolic changes induced by the low pH thereby decreasing acid tolerance in acid adapted cells (Buchanan and Edelson, 1996). On the other, the high pH at the onset of the fermentation of the sorghum motoho could have enhanced acid adaptation in the non-adapted cells as the fermentation progressed.

The combination of the *L. plantarum* FS2 and *P. pentosaceus* D39 for the fermentation of the sorghum motoho substantially inhibited the growth of AA non-O157 STEC strains (Table 4.14). It was established in Chapter 3 that the *L. plantarum* FS2 and *P. pentosaceus* D39 strains used as starter culture for the fermentation of sorghum motoho were capable of producing bacteriocins (Table 4.7). Therefore, the ability of these potential probiotic bacteria to produce bacteriocins coupled with the negative effect of prior acid adaptation could have possibly led to the substantial inhibition of AA non-O157 STEC strains in the sorghum motoho fermented with the combination of the *L. plantarum* FS2 and *P. pentosaceus* D39 starter culture. Similar to what occurred when challenged in fermented goat's milk, the substantial inhibition of AA non-O157 STEC in the sorghum motoho with the combination with the potential probiotic bacteria may also be due to the damage effect of prior adpataion to acid on the cell membrane which subsequently enhanced the antimicrobial activities of the bacteriocins produced by the potential probiotic starter culture. Since acid adaptation was induced with lactic acid before inoculation in the fermenting sorghum motoho, the presence of other organic acids could have impacted negatively on the survival of AA non-O157 STEC strains. Regardless of prior exposure to acidic environment, STEC will again undergo physiological stress during subsequent exposure in response to other organic acids different from the acidulant used to induce acid adapation (Deng et al., 1999).

5.3 Future Research

This study isolated *Lactobacillus plantarum* and *Pediococcus pentosceus* strains with multifunctional potential that can be used as functional starter culture to produce traditional fermented probiotic products. Intensive investigation on the safety such as the production of endotoxin by these *Lactobacillus plantarum* and *Pediococcus pentosceus* strains should be performed in further study. In addition, these potential probiotic strains should also be assayed for their antibiotic resistance to prevent the undesirable transfer of resistance genes to other endogenous bacteria. Further *in vivo* studies such as clinical trials and intervention studies as well as ability to modulate immune response should be carried out as guided by the 2002 FAO/WHO guidelines for evaluation of probiotic bacteria. This is to ascertain the probiotic health benefits and possible application of these LAB strains as novel probiotic bacteria. Further studies could also focus on identifying the presence of genes encoding for putative probiotic functions for deeper knowledge of the probiotic characteristics of these strains. Also, determination of substrate preferences of the strains would be necessary for co-delivery of probiotics and prebiotics in the GIT.

Since acid adaptation did not enhanced the survival of non-O157 STEC strains both in the fermented goat's milk and in the traditional African fermented cereal-based foods, further investigation on the sensitivity of acid adapted non-O157 STEC strains used for this study will give a deeper understanding of their stress response in traditional African fermented foods. Since traditional fermented ogi is often boil before consumption, further study should evaluate acid induced cross protection in acid adapted non-O157 STEC serotypes used in this study.

The contribution of the outer membrane fatty acids to acid adaptation and subsequent acid tolerance in non-O157 STEC strains should also be determined. Finally, further studies should also be carried out to determine the levels of gene expression of acid resistance genes and outer membrane proteins genes which could be responsible for acid adaptation in the non-O157 STEC strains. This will provide an insight for better control of the growth and survival of non-acid adapted non-O157 STEC strains which have the potential to develop acid adaption during fermentation of goat's milk and traditional African fermented cereal-based foods.

6. CONCLUSIONS AND RECOMMENDATIONS

This study showed that certain LAB associated with the traditional African fermented maize gruel possess desirable *in vitro* probiotic attributes and could be potential probiotic bacteria. The LAB that were characterised in this study are likely to be a safe choice as potential probiotic bacteria as they were isolated from indigenous African fermented food. Further, these LAB strains could be good candidates as starter culture to improve the safety and health benefits of traditional African fermented foods.

This study also highlighted the response and behaviour of non-O157 STEC strains in traditional African fermented foods. Though acid adaptation enhanced acid tolerance of non-O157 STEC strains in broth at low pH, it was detrimental to the survival in traditional African fermented foods in the presence of potential probiotic bacteria. This suggests that acid adaptation of non-O157 STEC strains in broth may not reflect their response in traditional fermented foods. On the other hand, non-acid adapted non-O157 STEC could become acid-adapted in traditional fermented foods and prolong the survival in such foods.

Although acid adaption enhanced the inhibition non-O157 STEC strains in fermented goat's milk and sorghum motoho, but did not contribute to survival in fermented ogi. This indicates that long-term exposure to stress factors such as low pH in the presence of potential probiotic bacteria are capable of changing the relative stress response in non-O157 STEC strains in traditional fermented foods as apparent when challenged in ogi. While relative short exposure period could induce acid adaptation in non-acid adapted non-O157 STEC strains as evident in the fermented goat's milk and sorghum motoho in which the non-acid adapted cells were not substantially inhibited.

The use of bacteriocin producing starter culture alone may not guarantee an adequate barrier to prevent the growth of non-O157 STEC strains in traditional fermented foods unless it is combined with other additional hurdles. Therefore, a combination of prior adaptation such as practised in backslopping with bacteriocins producing starter culture may work synergistically or at least provide greater protection than a single method alone, thus improving the safety and quality of traditional African fermented foods.

In addition, prior adaptation to acid coupled with the combination of potential probiotic starter culture is the most effective way of preventing the growth of non-O157 STEC in traditional African fermented foods. Therefore it is proposed that in order to prevent the

growth of non-O157 STEC strains in traditional African fermented foods, bacteriocin producing starter culture has to be combined with other additional hurdles such as prior exposure to acid that are capable of disrupting the cell membrane.

Finally, application of bacteriocin producing starter cultures for the household and medium scale production of traditional African fermented foods and beverages are very limited. Therefore, the LAB strains characterised in this study could be developed into freeze dry starter culture for the household and medium scale production of traditional African fermented cereal-based foods. This will enhance the microbiological safety of traditional African fermented foods. Further, the microbial diversity and dynamics of ogi and sorghum motoho fermentations should be investigated in order to clarify the role of the associated microflora in the inhibition of non-O157 STEC strains during fermentation process.

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8. Publications and presentations from this work

Scientific paper:

Fayemi, O. E. and Buys, E. M. (2015). Effect of *Lactobacillus plantarum* on the survival of acid tolerant non-O157 Shiga toxin producing *E. coli* strains in fermented goat's milk. Paper accepted for publication in the International Journal of Dairy Technology on 29th October, 2015.

Conference presentations

Oral presentations:

Fayemi, O.E., Taylor, J.R.N. and Buys, E.M. Effect of probiotic bacteria on environmental Shiga toxin producing *E. coli* in fermented goat's milk. 46th Annual General Meeting and Symposium on Food Safety and security: A dairy perspective. South African Society of Dairy Technology, 17th – 19th April, 2013, Khaya Ibhubesi, Parys Free State, South Africa.

Fayemi, O.E., Taylor, J.R.N. and Buys, E.M. Effect of Probiotic on the Survival of Non-O157 Shiga Toxin-Producing *E. coli* (STEC) Strains in African Fermented Weaning Food Products. Annual conference of International Association for Food Protection (July 25th-28th, 2015). Portland Oregon, U.S.A.

Poster presentations:

Fayemi, O.E., Taylor, J.R.N. and Buys, E.M. Survival of Shiga toxin *E. coli* in 'ogi' (Maize gruel) and goat's milk fermented with probiotic bacteria. 20th South African Association for Food Science and Technology (SAAFoST) Biennial International Congress and Exhibition (7th-9th October, 2013) CSIR International Convention Centre, Pretoria, South Africa.

Fayemi, O.E., Taylor, J.R.N. and Buys, E.M. 2014. Effect of probiotic bacteria on acid adapted (AA) and Non-acid adapted (NAA) Environmental Shiga toxin *E. coli* strains in fermented goat's milk and ogi (maize gruel). 24th International ICFMH conference, Food Micro (1-4 September 2014), Nantes, France.