University of Cape Town

Fermented foods production using isolated *Lactobacillaceae* species for the improvement of vaginal health: the case of mageu and yoghurt

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Abstract

Bacterial vaginosis (BV) is the most prevalent vaginal dysbiosis affecting women's reproductive health. The condition is characterised by the disruption of a *Lactobacillus* spp.-dominated microbiota that is associated with positive health outcomes and a shift to a microbial community that consists of diverse obligately anaerobic bacteria. Currently, antibiotics are used to treat BV and other vaginal infections. However, post-therapy recurrence and reinfection rates are high, suggesting the need for adjunctive therapy. Probiotic supplements can be used in conjunction with antibiotic treatment to re-establish the optimal vaginal microbial community. Of the probiotic products available in South Africa, only a limited number are specifically for vaginal health and none contain probiotic microorganisms found in the female genital tract. Additionally, the healthy vaginal microbiota differ with geography. Therefore, there is a need for an affordable probiotic product targeted for South African women.

In this dissertation, mageu and yoghurt are investigated for their potential as probiotic delivery vehicles. Previously isolated *Lactobacillaceae* strains from the genital tracts of healthy South African women are tested for their ability to ferment maize meal to mageu and milk to yoghurt, both as pure cultures and supplemented with traditionally-used microorganisms: *Saccharomyces cerevisiae* for mageu and *Streptococcus thermophilus* for yoghurt. Protocols were developed for the production of mageu and yoghurt with the bacterial strains. During production, fermentation was monitored by measuring pH until the end points of 3.5 and 4.5 were reached for mageu and yoghurt, respectively. After fermentation, the products were analysed by measuring titratable acidity, lactic acid and ethanol concentrations, total solids content, viable cell counts, qualitative analysis and shelf-life. Four mageu samples were then analysed for their consumer acceptability by an untrained consumer panel.

The *Lactobacillaceae* isolates, both as pure strains and supplemented with *S. cerevisiae*, were able to ferment maize meal to mageu, producing a product with a final pH of around 3.5, which qualitatively resembled mageu. The presence of pH-lowering metabolites including lactic acid and other acids was also measured using titratable acidity. The addition of *S. cerevisiae* significantly reduced the fermentation time and increased the titratable acidity of all mixed culture samples compared to the pure culture mageu samples. All mageu samples produced using the *Lactobacillaceae* isolates met the ethanol (maximum 2.5 g/L) and total solids content (minimum 8% (m/m)) production requirements set by the South African National Standards. After one week at room temperature or refrigerated, none of the mageu samples had pH values below 3, which is acceptable for mageu. However, the mixed culture mageu samples saw a larger drop in pH over the week than the pure culture samples. The sensory analysis investigated the consumer acceptability of four probiotic mageu samples, a positive control produced with flour as the inoculum source, and a commercial sample. The commercial sample was generally preferred, and was scored highest by the consumer panel, while the other samples received mixed results. The spread of results was attributed to the influence of individual preference due to the small cohort size.

The pure *Lactobacillaceae* strains were unable to ferment milk to yoghurt. By hour 12, the pH values were still between 6.32 and 6.37, and curdling of the product had occurred by hour 24. However, when supplemented with *S. thermophilus,* the mixed cultures were able to produce a product with a final pH of around 4.5 that qualitatively resembled traditional yoghurt. However, it is not known if the improved fermentation was due to *S. thermophilus* activity alone. The titratable acidity results also confirmed that pH-lowering metabolites were produced and all yoghurt samples had titratable acidity values greater than 0.6% (m/m), the minimum requirement for yoghurt. Little difference was observed between the samples for the qualitative assessment; however, the positive control had a thicker consistency than the probiotic samples.

These results show that the *Lactobacillaceae* isolates when supplemented with traditional fermentation microorganisms are able to produce mageu and yoghurt. During this project protocols for mageu and yoghurt production using the *Lactobacillaceae* isolates specific to South African women were designed. This project serves as the first step towards investigating the use of fermented foods as affordable probiotic delivery vehicles for the improvement of vaginal health in South African women.

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Glossary of Terms

Aerobic growth	Growth requiring oxygen
Anaerobic growth	Growth requiring the absence of oxygen
Autochthonous	Indigenous to an area
Bacterial vaginosis	A vaginal dysbiosis characterized by a disruption of the optimal *Lactobacillus* spp.-dominated microbiota to a diverse community of obligately anaerobic bacteria
Commensalism relationship	A relationship where one party benefits and the other neither benefits nor is harmed
Exopolysaccharides	Substances secreted by microorganisms
Fermented foods	Foods produced by microorganisms that perform desired enzymatic conversions of food compounds
Heterofermenter	A microorganism producing multiple end products during fermentation
Homofermenter	A microorganism which produces a single end product during fermentation
Inoculate	To introduce a microorganism culture into growth media
Mageu/Mahewu	A fermented maize-meal beverage
Mutualistic relationship	A relationship where both parties benefit
Pathogenic	Adjective to describe microorganisms which cause disease
Pre-reduced plates	Agar plates are placed in a container with an anaerobic sachet to remove any oxygen from the growth environment before microorganisms are grown on the plate
Postacidification	The process where lactic acid continues to be produced during storage or after fermentation
Probiotics	Live microorganisms that, when administered in adequate amounts, confer a health benefit to the host
Proteolytic	Ability to hydrolyse caseins to polypeptides, peptides and amino acids
Protocooperation	Mutual promotion of microbial growth by exchanging nutrients and growth factors

Acronyms and Abbreviations

BV	Bacterial vaginosis
BHI	Brain heart Infusion
CeBER	Centre for Bioprocess Engineering Research
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FGT	Female genital tract
GBS	Group B *Streptococcus*
GIT	Gastrointestinal tract
GRAS	Generally regarded as safe
HPLC	High Performance Liquid Chromatography
IQR	Interquartile range
ISAPP	International Scientific Association for Probiotics and Prebiotics
LAB	Lactic acid bacteria
MRS	De Man Rogosa Sharpe
NCBI	National Center for Biotechnology
OD	Optical density
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
QPS	Qualified Presumption of Safety
SABS	South African Bureau of Standards
SADC	Southern African Development Community
Subsp.	Subspecies
Spp.	Species
STI	Sexually transmitted infections
TA	Titratable acidity
TTA	Total titratable acidity
UCT	University of Cape Town
UHT	Ultra-high temperature
WHO	World Health Organization
YPD	Yeast extract-peptone-dextrose

1 Introduction

1.1 Project background and context

Currently, antibiotics are used to treat vaginal infections such as bacterial vaginosis (BV). BV is a common vaginal dysbiosis characterised by a disruption of the optimal *Lactobacillus* spp. -dominated microbiota to a diverse community of obligately anaerobic bacteria, including *Gardnerella vaginalis*. BV increases the risk of contracting HIV and has been linked with adverse reproductive outcomes, even in asymptomatic women (Anukam et al., 2006; Borgdorff et al., 2014; Donders et al., 2009; Gosmann et al., 2017; Guo et al., 2012; Koumans et al., 2007; Nelson et al., 2014; Van De Wijgert et al., 2008). Current BV treatments include orally or topically administered antibiotics. However, pathogenic microorganisms associated with BV commonly develop antibiotic resistance and are; therefore, not always completely cleared during therapy. Additionally, antibiotics alone are insufficient to re-establish the protective *Lactobacillus* spp.-dominated microbiota. These two factors contribute to treatment failure and relatively high rates of reinfection and relapse (Xiao et al., 2016). Further, antibiotics are costly, and so cheaper alternatives are needed for BV treatment. Probiotics compromising of beneficial lactobacilli have consequently become of interest as an alternative or adjunctive treatment option. Probiotics are live microorganisms that have strain-specific health benefits when supplied in sufficient quantities (Hill et al., 2014). Probiotics administered orally (without encapsulation) can reach the female genital tract (FGT) through the ano-vaginal transfer of bacteria (Reid et al., 2001). However, none of the commercial probiotic products available contain bacterial species commonly found in the FGT, let alone those specific to South African women (Happel et al., 2017). Fermented foods containing beneficial strains can act as probiotic delivery vehicles and are more cost-effective than currently commercialised products.

Lactobacillaceae strains were previously isolated by Happel et al. (2020) from the female genital tracts (FGTs) of healthy, HIV-negative South African women. Of the 57 isolates identified, Happel et al. (2020) selected ten isolates for potential probiotic use for BV treatment based on characteristics that are desirable in a vaginally-targeted product. The selection criteria included the ability to inhibit BV-associated pathogenic microbes and lactic acid production. It is now desired to design probiotic treatments using these isolates.

A potential low-cost delivery mechanism of the probiotic lactobacilli is via fermented foods consumption. Fermented foods are produced by microorganisms that perform desired enzymatic conversions of food compounds (Marco et al., 2021). The metabolites produced by the microbes affect the nutritional quality and sensory characteristics of the food. The most common bacteria used in fermented food production are lactic acid bacteria (LAB), including certain lactobacilli. They have fast growth rates, high acid production rates, and are non-pathogenic (Sieuwerts, 2009).

Two ubiquitously consumed fermented foods in South Africa are mageu and yoghurt. Mageu is a non-alcoholic fermented cereal beverage commonly consumed in Southern Africa. It is produced by the fermentation of maize meal with inoculum sources of wheat flour, brown sugar or millet malt, depending on the country of origin. The main microorganisms identified in traditionally produced mageu are yeasts (such as *Saccharomyces cerevisiae*) and LAB. Yoghurt is produced most commonly with two LAB: *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, which have *a* mutualistic relationship. The LAB ferment milk by producing lactic acid until a pH of 4.5 is reached. This report will examine the potential use of these two fermented foods as delivery vehicles for locally isolated probiotic *Lactobacillaceae* strains.

1.2 Potential of *Lactobacillaceae* isolates to produce fermented foods

This project aims to determine whether previously isolated *Lactobacillaceae* strains can ferment a maize meal-water mixture to mageu and/or milk to yoghurt. The *Lactobacillaceae* strains are a selection of those previously isolated from healthy South African women by Happel et al. (2020). To determine if the fermented maize meal and milk can be classified as mageu and yoghurt, respectively; they will have to meet the technical production requirements and sensory requirements of these fermented foods.

1.3 Scope and constraints

The *Lactobacillaceae* strains to be used in the production of the fermented foods have already been isolated from the genital tracts of South African women in a study by Happel et al. (2020). These isolates are available to the Centre for Bioprocess Engineering Research (CeBER) at the University of Cape Town (UCT). Therefore, no novel microorganisms will be isolated during this project.

This project will only focus on the production of the fermented foods, mageu and yoghurt, and whether the *Lactobacillaceae* isolates can ferment milk or maize meal. A sensory analysis of the mageu samples will be used to determine the consumer acceptability of the samples produced. Due to time constraints, consumer acceptability tests in the form of a sensory analysis will only be conducted for the mageu samples. Clinical trials using mageu as a probiotic delivery vehicle for the treatment of bacterial vaginosis will follow this project; therefore, the acceptability of the mageu products were of primary importance. Trials following the production of the probiotic foods to investigate BV treatment efficacy were not included as part of this project.

1.4 Structure of dissertation

This report begins with a literature review covering current bacterial vaginosis treatments, the role of functional foods, production of mageu and yoghurt, and fermented foods produced with *Lactobacillaceae* isolates. The review of functional foods will include an investigation of the safety requirements for producing probiotic foods for consumption. This is vital for ensuring the mageu samples produced are safe for taste testing. From the literature review, the problem statement and hypotheses are formulated. Following this, the methodology for mageu and yoghurt production and product analysis is described. Mageu analysis of the final product includes qualitative analysis, measuring titratable acidity, total solids content, lactic acid and ethanol concentrations, cell counts, and shelf-life. Yoghurt product analysis includes qualitative analysis and measuring titratable acidity. Of the mageu products, five laboratory-produced samples are tested for their consumer acceptance. The report concludes with determining whether the *Lactobacillaceae* isolates can successfully ferment maize meal to mageu and/or milk to yoghurt. Thereafter, suggestions for further research are presented.

2 Literature Review

This literature review intends to provide a background discussion on BV treatment with bacterial isolates from the female genital tract and use of fermented foods as vehicles for probiotic delivery. The first section provides a discussion on BV, followed by an overview of the existing literature on the main bacterial isolates in South African women. The second section focuses on functional foods, specifically mageu and yoghurt. The final section looks at the interplay between the two.

2.1 Bacterial vaginosis

The optimal vaginal microbial community is dominated by *Lactobacillus* species (spp.), in particular *Lactobacillus crispatus*, *Lactobacillus gasseri*, and *Lactobacillus jensenii* (Aroutcheva et al., 2001; Gajer et al., 2012; Ravel et al., 2011). These species contribute to the production of lactic acid which helps to maintain a vaginal pH of 4.5 or lower; and their presence is inversely correlated with non-optimal species such as *Gardnerella vaginalis* and *Prevotella bivia*. BV is the most prevalent global vaginal dysbiosis (Rossi et al., 2010). It is characterised by the disruption of the optimal *Lactobacillus* spp.-dominated microbial community and a transition to a relatively diverse community consisting primarily of obligately anaerobic bacterial species associated with negative sequelae (Marrazzo et al., 2010). It is characterised by a high pH, vaginal discharge and inflammation, although between 84% and 86% of women infected with BV are asymptomatic (Anukam et al., 2006; Koumans et al., 2007).

Diagnosis of BV traditionally uses either the Amsel criteria or Nugent scoring (Amsel et al., 1983; Nugent et al., 1991). The Amsel criteria uses symptoms for diagnosis. Three or more criteria fulfilled classifies as BV positive (Amsel et al., 1983). Nugent scoring, on the other hand, uses bacterial smears to quantify bacteria. A Nugent score of three or less is categorised as normal, but seven and above is classified as BV positive. An advantage of Nugent scoring is that both symptomatic and asymptomatic BV can be identified (Nugent et al., 1991). More recently, molecular methods including detection of increased sialidase activity and polymerase chain reaction (PCR)-based identification of BV-associated bacteria have also been investigated (Fredricks et al., 2007; Myziuk et al., 2003).

BV increases the infection rates of sexually transmitted infections (STIs), most notably HIV, and adverse reproductive outcomes (Borgdorff et al., 2014; Donders et al., 2009; Gosmann et al., 2017; Guo et al., 2012; Nelson et al., 2014; Van De Wijgert et al., 2008). Some of the adverse effects of BV, even in asymptomatic women, include increased risk of spontaneous abortion, preterm delivery, and increased susceptibility to viral and bacterial vaginal infections (Borgdorff et al., 2014; Donders et al., 2009; Guo et al., 2012; Nelson et al., 2014).

2.1.1 Current treatments

Current antibiotic treatments for BV in South Africa include metronidazole (oral) and clindamycin (both oral and topical) (South African Department of Health, 2015). In the United States, the current treatment for BV is oral or topical delivery of metronidazole, or oral clindamycin (Centers for Disease Control and Prevention, 2015). BV-associated microorganisms, particularly *Prevotella* spp. and *G. vaginalis*, develop metronidazole resistance and are a possible reason for the high BV reinfection rates (Nagaraja, 2008; Petrina et al., 2017). The pathogenic microbes could also be protected from antibiotics by biofilms, contributing to the high recurrence rates (Rossi et al., 2010). Treatment with antibiotics is associated with BV reinfection rates of 30% in three months, and 50% in six months (Barrons and Tassone, 2008).

Antibiotic treatment for BV can also be followed by a probiotic course to assist with the establishment of an optimal vaginal microbiota. According to The International Scientific Association for Probiotics and Prebiotics (ISAPP), probiotics are “live microorganisms that, when administered in adequate amounts, confer a health benefit to the host” (Hill et al., 2014). This definition is important as it emphasises the impact of probiotics on health. Petricevic and Witt (2008) conducted research on the effects of

administering *Lacticaseibacillus rhamnosus* Lcr35 after antibiotic treatment for BV. The study used Nugent scoring to identify participants with BV, and their subsequent scores after treatment. The participants given the probiotics following antibiotic treatment saw marked improvement in their Nugent scores in comparison to the control group. More women in the control group (55%) had intermediate vaginal microbiota (Nugent score between 4 and 6) compared to the probiotic group (11%) after treatment. This indicates that women in the control group were more at risk for developing BV than the probiotic group, as 50% of women with intermediate vaginal microbiota develop BV.

Whilst antibiotics are designed to destroy pathogenic bacteria, they are insufficient alone to re-establish the optimal bacterial communities that are associated with positive health outcomes (Reid, 2018). Therefore, researchers have begun to explore potential adjunctive options such as the use of probiotic bacteria. Members of the *Lactobacillaceae* family including *Lactobacillus* spp. are able to create conditions which are not conducive to the growth of pathogenic microbes and represent attractive probiotic candidates. Additionally, their ability to disrupt and/or prevent the establishment of biofilms by pathogenic bacteria may contribute toward the establishment of a lactobacilli-dominated vaginal microbiota (McMillan et al., 2011).

Given that bacterial strains are usually adapted to their particular environmental niche, it is reasonable to expect that probiotic candidates derived from the FGT would be more appropriate for products targeting vaginal health (Pan et al., 2020). Happel et al. (2017) conducted research on the available probiotics in South Africa for vaginal and gastrointestinal health care. Of the 104 products identified, only four were specifically for vaginal care, but all contained bacterial species found in the gastrointestinal tract (GIT) rather than the FGT. The cost price of a single course of vaginal probiotics was between 1.6% and 4.9% of the average South African monthly income (Happel et al., 2017).

Van de Wijgert and Verwijs (2020) reviewed 13 studies investigating BV, lactobacilli as probiotics, and the vaginal microbiota. Seven of the 13 studies conducted used randomised control trials to investigate BV cure fractions. While most of the studies showed positive outcomes for the treatment of BV with probiotics, one showed a non-significant improvement, and two showed no increases in the BV cure fraction. Eight studies investigated BV recurrence, of which six showed significant reductions in recurrence. Overall, van de Wijgert and Verwijs (2020) concluded that lactobacilli as probiotics for BV treatment show promise. Various treatment paths were included in the studies such as probiotic treatment, probiotics in conjunction with antibiotics treatment, and probiotics as maintenance treatment to prevent recurrent BV. However, since the review did not compare different treatment strategies, no single treatment path could be suggested.

2.1.2 Bacterial isolates for probiotic treatment of bacterial vaginosis in South African women

Earlier research conducted by Happel et al. (2020) aimed to identify the top vaginal bacterial isolates in South African women that could be used for probiotic treatment of BV. As mentioned above, probiotics have an associated health benefit. Therefore, individual strains usually require conclusive evidence of providing a health benefit before they can be considered probiotics. However, in some countries such as Canada, *Lactobacillus* and *Bifidobacterium* species are already accepted as providing a health benefit and do not require strain-specific evidence due to their long history of use (Health Canada, 2009).

Bacterial isolates were collected from healthy, HIV-negative South African women between the age of 16 and 22 years old, 12 of whom were BV negative, four had intermediate Nugent scores, and 10 were BV positive. The 57 strains identified from the women were 10 *L. crispatus*, nine *L. gasseri*, 18 *L. jensenii*, eight *Limosilactobacillus vaginalis*, and 12 *Limosilactobacillus mucosae*. Notably, *L. crispatus* strains lowered the pH and tolerated a lower pH better than the other species. Additionally, the *L. crispatus* strains inhibited *G. vaginalis* the most successfully. *L. gasseri* strains were the least sensitive to clindamycin and penicillin. All bacterial strains were metronidazole resistant, an intrinsic feature of *Lactobacillus* spp. (Happel et al., 2020).

The dominating bacterial species in the FGT differ with geographic location. In North America, for example, the most common bacterial species dominating the vaginal tract are *L. crispatus*, *L. gasseri*, *L. jensenii*, *Lactobacillus iners* and *L. vaginalis* (Ravel et al., 2011). In South Africa, on the other hand, the most common species found in the FGT is *L. iners* (Klatt et al., 2017). The role of *L. iners* in FGT health is currently unclear, although the species tends to be associated with a transitional state (primarily transition from an optimal to non-optimal vaginal microbiota) and it is, therefore, not usually regarded as a probiotic candidate (Zheng et al., 2021). Importantly, *L. crispatus*-dominated bacterial communities in both regions have been associated with positive outcomes (Gosmann et al., 2017). Happel et al. (2017) highlighted the need for the development of probiotics targeted for vaginal health in the South African population, which would be affordable for those who need it most. The study by Happel et al. (2020) identifies which bacterial isolates, specific to South African women, can be used in the development of cost-effective probiotics, and provides the foundation for this research.

Happel et al. (2020) compared and identified the top performing strains for development of a probiotic by using the following criteria: (1) autochthonous to the FGT, (2) ability to adhere to FGT cells, (3) inhibition of BV-associated species, (4) ability to tolerate low pH, (5) ability to lower the pH, (6) L-lactate and D-lactate production (anti-microbial metabolites), and (7) tolerance of antibiotics (specifically those used for BV treatment). Additionally, the ability of bacterial isolates to produce biofilms *in vivo* is useful as this enhances their ability to inhibit pathogen growth, although this is not commonly tested when evaluating probiotic candidates.

Using a point scoring system incorporating the above seven criteria, 36 of the 57 strains were ranked relative to one another and placed into quartiles. To account for interdependency of certain criteria, production of lactic acid and lowering of pH, for example, a Spearman rank correlation co-efficient was used. Four models were tested for the scoring system: Model A included all factors; Model B excluded hydrogen peroxide (H_2O_2) production; Model C excluded H_2O_2 production and Group B *Streptococcus* (GBS) inhibition; and Model D excluded H_2O_2 production and included GBS inhibition, *G. vaginalis* and *Prevotella bivia* inhibition as a single category. A comparison of the four models and the top 10 strains is shown in Figure 2-1.

Top 10 Isolates	Model A	Model B	Model C	Model D
1	*L. mucosae* 90.13 PA	*L. crispatus* 70.6 PA	*L. crispatus* 70.6 PA	*L. jensenii* 92.27 PA
2	*L. crispatus* 70.6 PA	*L. mucosae* 90.13 PA	*L. mucosae* 90.13 PA	*L. mucosae* 90.13 PA
3	*L. crispatus* 100.16 a	*L. crispatus* 100.16 a	*L. jensenii* 88.33 PA	*L. jensenii* 88.33 PA
4	*L. vaginalis* 80.23 b	*L. jensenii* 88.33 PA	*L. crispatus* 100.16 a	*L. crispatus* 70.6 PA
5	*L. jensenii* 88.33 PA	*L. jensenii* 92.27 PA	*L. jensenii* 92.27 PA	*L. crispatus* 100.16 a
6	*L. jensenii* 92.27 PA	*L. vaginalis* 80.23 b	*L. vaginalis* 80.23 b	*L. crispatus* 96.9 PB
7	*L. vaginalis* 80.3 b	*L. vaginalis* 80.3 b	*L. crispatus* 96.9 PB	*L. vaginalis* 80.23 b
8	*L. crispatus* 96.9 PB	*L. crispatus* 96.9 PB	*L. crispatus* 73.55 a	*L. jensenii* 95.1 PA
9	*L. crispatus* 73.55 a	*L. crispatus* 73.55 a	*L. vaginalis* 80.3 b	*L. gasseri* 94.98 PB
10	*L. jensenii* 95.1 PA	*L. mucosae* 80.23 a	*L. jensenii* 92.1 PA	*L. gasseri* 117.73 PA

L. vaginalis strains
L. jensenii strains
L. crispatus strains
L. mucosae strains
L. gasseri strains

Figure 2-1 Comparison of the top 10 performing *Lactobacillaceae* species for each model (adapted from Happel et al., 2020). Squares are coloured by bacterial species.

2.2 Oral administration of probiotics for vaginal health

The first clinical experiments confirming translocation of *Lactobacillus* species to the FGT following oral intake were performed by Reid et al. (2001). The *Lactobacillus* species were orally administered in skim milk in cell counts greater than 10^9, and not encapsulated. This is an important consideration for this project as it shows that the microorganisms survived the low pH and bile of the stomach, the journey through the intestine, and finally reached the vagina, without cryoprotectant or immobilization. Additionally, no side effects were reported. In the study, two members of the *Lactobacillaceae* family, namely *L. rhamnosus* GR-1 and *Limosilactobacillus fermentum* RC-14, were administered to study

participants. However, the two isolates colonized the FGT of the participants differently. Some participants were colonized by *L. rhamnosus*, others by *L. fermentum*, and some by both. Reid et al. (2001) used these findings to support the use of multiple organisms in probiotic products, preferably those with combined advantages.

Subsequently, Reid et al. (2003) used the above findings to test whether administration of the same strains could provide health benefits to healthy women. This differs from the former investigation where participants were women with a history of urogenital infections. The probiotic lactobacilli were orally administered in capsules and an increase in the vaginal population of lactobacilli was observed. Most importantly, no side effects were recorded by the participants given the probiotic bacteria.

Reid (2017) discussed the idea behind oral delivery of probiotics, i.e. that urogenital infections are caused by pathogens which translocate from the rectum to the vagina, and from there ascend to the bladder. If pathogens can do this, then probiotic bacteria have the potential to do this too. This movement of microbes from the rectum to the vagina is called ano-vaginal transfer. This is possible due to the anatomical proximity of the anal canal and vagina (Reid, 2018). Orally administered probiotics have the potential to inhibit the ano-vaginal transfer of yeast and pathogenic bacteria by inducing microbial competition, whilst simultaneously colonising the FGT (Reid et al., 2001). However, oral administration delivers fewer bacteria than topical (vaginal) administration since the microbes compete with the species present in the GIT (Reid, 2018). Successful colonization by vaginal bacteria is aided by the ability to adhere to epithelial cells along with bacteriocin and biosurfactant production, and is associated with a lowering of the vaginal pH, and further inhibition of pathogenic bacteria growth (Happel et al., 2017). Furthermore, oral administration of probiotics is more beneficial than topical application of probiotics for pregnant women (Reid et al., 2001). Prebiotics are substances used by host microorganisms and provide a health benefit (Gibson et al., 2017). They are important for the oral delivery of probiotic bacteria as they improve the survivability of the beneficial bacteria (Corcoran et al., 2005).

Reid (2018) reports that topical administration of probiotics, while able to deliver the probiotics directly to the problem area and act faster on pathogens, is more costly. In developing countries, the cost of probiotic foods and dietary supplements are also too high for many households (Reid, 2017). Therefore, the use of fermented foods to deliver the desired probiotic bacteria, which are commonly produced and consumed in developing countries, could be a solution. However, the testing requirements for oral and topical treatments differ. Probiotic treatments delivered in foodstuffs, may have to meet additional requirements. This will impact the product cost and time to market. The regulations for the use of probiotic bacteria in foods, specifically their safety, are analysed further in Section 2.3.3.

2.3 Functional foods

2.3.1 Fermented foods

The ISAPP define fermented foods and beverages as those made using microorganisms which perform enzymatic conversions of food compounds (Marco et al., 2021). This definition excludes conversions which cause food spoilage as these are unwanted. Fermented foods do not require the microbes used in production to be living or present in the foods upon consumption. Examples of these foods include bread and wine, which have either killed the fermenting microbes or removed them, respectively. During fermentation, the microorganisms produce metabolites which contribute to the sensory appeal (taste, appearance, aroma) and nutritional quality of the fermented food. Microorganisms responsible for fermentation generally out-compete pathogenic organisms which cause spoilage. Fermented foods can also reduce the risk of disease (Marco et al., 2021). Importantly, research has found that consumers will generally consume food products based on taste rather than the health benefits (Nyanzi et al., 2010).

Bacteria, yeasts, and filamentous fungi are most commonly used to produce fermented foods. The most common bacteria are acetic acid bacteria and LAB, of which LAB are the most common (Marco et al., 2021). LAB will be the microorganisms of focus in this study. They are characterised by fast growth,

acid production, volatile compounds production, and exopolysaccharide production. LAB have adapted to optimize their growth (high growth rates) and acid (primarily lactic acid) production rates, instead of their yields. High acid production rates and acid tolerance (ability to tolerate low pHs) make LAB ideal for fermented food production (Sieuwerts, 2009). Members of the LAB are gram-positive, non-spore forming, acid-tolerant and anaerobic, differing in oxygen sensitivity.

2.3.2 Probiotic foods

Probiotic foods are foods that have evidence of providing strain-specific, but not species- or genus-specific, health benefits with the presence of the live non-pathogenic microorganism(s) in sufficient quantities (Figueroa-Gonzalez et al., 2011). Fermented foods which do not satisfy the above conditions (undefined quantities of microbes and/or no confirmed health benefit), are labelled as containing "live and active cultures" (Marco et al., 2021). If the microbes have been removed prior to consumption, then the foods have been produced by fermentation and are labelled as such (Marco et al., 2021). The use of probiotics in fermented foods has multiple benefits, including an increase in the nutritional properties of the food, reduction in diarrhoea and other gastrointestinal ailments, and extending the shelf-life of food with controlled fermentations (Kort et al., 2015).

2.3.3 Safety requirements

Food additive and safety requirements

In the United States, GRAS status is provided to food additives and substances and is granted by the Food and Drug Administration (FDA) (Mattia and Merker, 2008). If any substance is to be used in food, including probiotics, then GRAS status is required. GRAS status is time-dependent and is either granted or not based on the scientific evidence available at the time of the application. GRAS status is granted for the intended use of the substance only and is not licensed; therefore, GRAS information can be used by people other than the applicant if the substance is used for the same purpose. The FDA analyses the following aspects before granting GRAS status: taxonomy, pathogenic properties, toxin production, antibiotic resistance genes, history of use in food, reports of adverse effects, environmental presence, and other relevant information (Mattia and Merker, 2008).

Qualified Presumption of Safety (QPS) risk assessment is the European equivalent of GRAS, and is similarly only granted for the specific use (Herody et al., 2010). The requirements for QPS status to be granted are well-defined taxonomic identity, sufficient available knowledge to confirm its safety, established lack of pathogenic properties, and intended use (EFSA, 2005). If a taxonomic group does not have any safety concerns, the entire group can be granted QPS status. If a microorganism falls within that taxonomic group, no further safety assessment is required (Bourdichon et al., 2019). The QPS list is updated regularly.

In South Africa, the Food Control Division of the Department of Health is responsible for food legislations regarding safety of food additives. This division is also the point of contact with the joint FAO/WHO Codex Alimentarius Commission and the International Food Safety Authorities Network. The Department of Health requires that all foods comply with the Foodstuffs, Cosmetics and Disinfectants Act of 1972 to ensure consumer safety (Sikuka, 2017). The regulations referring to food additives follows the Codex Alimentarius Commissions' General Standard for Food Additives, which was adopted by South Africa in 2017 (Sannuto, 2016; South African Department of Health, 2016). In 2017, during the annual Codex Committee on Nutrition and Foods for Special Dietary Uses meeting, it was agreed that guidelines of probiotic use in foods would be placed on the agenda for the following meeting (Vinderola, 2020). The first draft was presented in 2018, and the committee agreed that the document required further refining and clarification at both that meeting and the subsequent one in 2019. The meeting in 2020 was postponed to November 2021 due to the COVID-19 pandemic. However, at the November 2021 meeting, probiotics were not placed on the agenda with no reasoning provided (CODEX Alimentarius Commission, 2021). Therefore, there is currently no Codex Alimentarius regulatory guidelines for the use of probiotics in foods (Vinderola, 2020).

Probiotic Safety Requirements

For a food to be considered having a probiotic effect, the following requirements need to be met (Joint FAO/WHO Working Group, 2002):

- The taxonomic identity of the microorganism must be clear since health benefits are strain specific. However, the exception is made for *S. thermophilus* and *Lactobacillus delbrueckii* subsp*. bulgaricus* for which there is substantial evidence for the health benefits of these microorganisms not being strain specific. Both phenotypic and genotypic tests (such as 16S rRNA gene sequencing) are advised for microorganism speciation.
- *In vitro* tests to determine the mechanism of the probiotic effect as well as efficacy. The main tests used in establishing probiotic mechanisms are resistance to gastric acidity, bile acid resistance, adherence to human epithelial cells, antimicrobial activity against pathogenic bacteria, ability to reduce pathogenic adhesion to surfaces, bile salt hydrolase activity (if for oral consumption/gastrointestinal use), and spermicide resistance (if the probiotic is intended for vaginal use).
- Proof that the probiotic strain is safe and not contaminated in its delivery form. Probiotic microorganisms should be examined for any side-effects.
- Human *in vivo* studies tested using double-blind, randomized, placebo-controlled human trials to show benefits of the probiotic.
- Determination of the minimum viable numbers required for probiotic effect.

From point 3, even if the group of bacteria have GRAS status, the Joint FAO/WHO Working Group recommends the following tests still be used to establish probiotic safety:

- Determine presence of antibiotic resistance patterns
- Analyse metabolic activities such as lactic acid production
- Analyse adverse effects during human studies and in consumers
- Test for toxin production if the strain belongs to a species known for toxic properties
- Test for haemolytic activity if the strain belongs to a species with known haemolytic potential

Lactobacillus Safety

L. delbrueckii subsp. *bulgaricus* and *S. thermophilus*, used in yoghurt production, have GRAS status (U.S. Food & Drug Administration, 2018). This same sentiment is shared by the European Food Safety Authority (EFSA), that food cultures which have a history of safe use as traditional food ingredients are legally permitted for use (Herody et al., 2010). The World Health Organisation (WHO), in conjunction with the Food and Agriculture Organization of the United Nations (FAO), states that the best proof of the safety of *Lactobacillus* species in foods is their prolonged use as probiotics without any established risks to humans. *Lactobacillus* species have established safety for use in foods and supplements, supported by their natural occurrence in the human microbiomes (Joint FAO/WHO Working Group, 2002). Furthermore, no virulence nor pathogenic properties have been associated with lactobacilli (Joint FAO/WHO Expert Consultation, 2006). However, several species of lactobacilli have not been granted QPS status. This is not because of a safety concern, but these strains have not been submitted for review to date. This shows that the QPS list is not exhaustive (Joint FAO/WHO Expert Consultation, 2006). As of March 2020, the 12th update of the QPS list indicated that the status of the *Lactobacillus* species examined remains unchanged (Pot, 2020).

2.4 Fermented foods and beverages

Mageu is an ubiquitously consumed non-alcoholic fermented beverage in Southern Africa, whereas yoghurt is consumed globally (Mashau et al., 2020; Reid et al., 2001). This literature review will focus on these two fermented foods and beverages.

2.4.1 Mageu

Many fermented foods in Africa use cereals. Examples include ogi and obiolor from Nigeria, ting from South Africa, ben-saalga from Burkina Faso, togwa from Tanzania, and bushera from Uganda (Pswarayi and Gänzle, 2019; Simatende, 2016). Mageu is a fermented, non-alcoholic cereal beverage commonly produced in Southern African countries including Botswana, Lesotho, Zimbabwe, Eswatini, and South Africa (CODEX Alimentarius Commission, 2019; Idowu et al., 2016). It is a cheaper alternative to fermented dairy products and is consumed by all household members, including infants (Simatende et al., 2015). It is produced through the fermentation of maize meal by LAB and yeasts. This fermented beverage goes by different names in different African countries: emahewu in Eswatini, mageu in South Africa, and mahewu in Zimbabwe (Idowu et al., 2016; Simatende, 2016). However, mageu is the most common name in the Southern African Development Community (SADC) region. Therefore, it will only be referred to as mageu hereafter (CODEX Alimentarius Commission, 2019).

Consumption of mageu in South Africa was around 146 million litres in 1984, and in 2001, 77 million litres were consumed by black miners alone (Mashau et al., 2020; McMaster et al., 2005). More recently, between July 2018 to June 2019, a total of 2.87 million litres of mageu were exported to South Africa (CODEX Alimentarius Commission, 2019). Mageu has been commercialised due to its popularity, a commonly available example of which is Mageu Number One, produced by Clover SA in multiple flavours (Fritz, 2005).

Mageu production

Two papers investigated the traditional methods of mageu production by analysing the samples produced by communities. Simatende et al. (2015) studied Swazi production of mageu and noted that a general method was followed in the various communities. Pswarayi and Gänzle (2019) investigated mageu production in three households in Zimbabwe.

The Swazi households' methods differed with their maize meal to water ratio. Some households used 20% (w/v) and others used 25% (w/v) (Simatende et al., 2015). However, the distribution of the two methods amongst the households was not mentioned. Simatende et al. (2015) compared the preparation methods of Swazi mageu to those of mageu in South Africa, which are in accordance with the methods described by Fadahunsi and Soremekun (2017). The ratio of maize meal to water differed, with mageu using typically 8% (w/v), but Nyanzi et al. (2010) used a ratio of 6% (w/v) (Fadahunsi and Soremekun, 2017). The Zimbabwean households used a ratio of 6.7% (w/v) (Pswarayi and Gänzle, 2019). Furthermore, in Zimbabwe, the water was added in two parts, with double the water volume added after the porridge had cooled from cooking (Pswarayi and Gänzle, 2019). The water used for this mageu was taken from a well, which could have been another source of microorganisms. However, this was not assessed in the study.

The inoculum sources amongst the three countries also differed. In South Africa and Zimbabwe, wheat flour, maize bran or millet malt are added as an inoculum, whereas in Eswatini some households add sugar or peeled potato (Fadahunsi and Soremekun, 2017; Idowu et al., 2016; Simatende et al., 2015). This affects the fermentation process, as without the malt, Swazi mageu does not have the enzymes needed to start the fermentation process. However, the addition of brown sugar or potato and the equipment used may contribute to the bacterial inoculum (Simatende et al., 2015). In all three countries, the maize porridge mixture was cooked to inhibit any microbial growth. Therefore, the inoculum source was added following the cooking stage, so as not to inactivate the fermentation microorganisms. While the traditional methods do not state the temperature at which the porridge is cooked, other mageu methods cook the porridge between 85°C to 90°C (Holzapfel and Taljaard, 2004; Mashau et al., 2020). Fermentation then takes place between ambient temperature and 30°C, depending on the method used (Fadahunsi and Soremekun, 2017; Holzapfel and Taljaard, 2004; Idowu et al., 2016; Mashau et al., 2020; Pswarayi and Gänzle, 2019). Lastly, the fermentation times differed. Fermentation of Swazi mageu lasts between 2 to 6 days, whereas South African and Zimbabwe mageu ferments for roughly 36 hours, as shown in Figure 2-2. This has an effect on protection from pathogens, as South African and Zimbabwean mageu is able to achieve a low pH quickly, and in doing so inhibits pathogenic growth.

In contrast, since fermentation is slower for Swazi mageu, it does not have this same level of protection as the final pH takes longer to reach (Simatende et al., 2015).

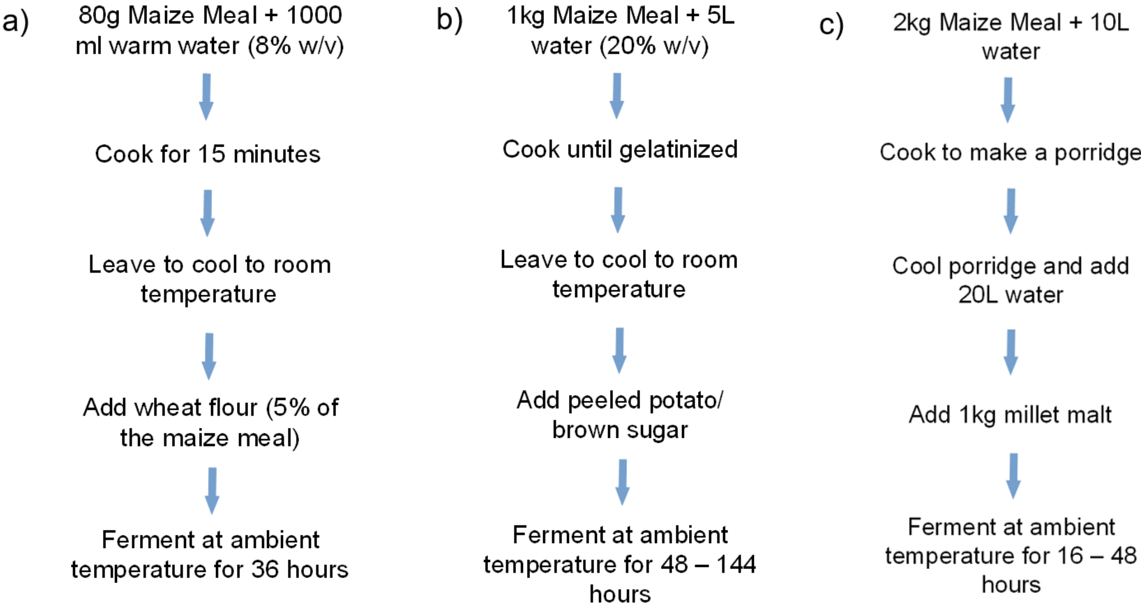

Figure 2-2 Method for mageu production in (a) South Africa (Fadahunsi and Soremekun, 2017), (b) Eswatini (Simatende et al., 2015), and (c) Zimbabwe (Pswarayi and Gänzle, 2019)

During production, Fadahunsi and Soremekun, (2017) and Haard et al. (1999) emphasize that stirring the maize porridge should only be done at the beginning of fermentation. Pswarayi and Gänzle (2019) also state that the porridge is left to ferment, implying that it is not mixed during this phase of production.

In 2017 Dupont Nutrition Biosciences ApS filed an international patent for the production of mageu using the LAB *L. delbrueckii* subsp. *lactis* (Fourcassie et al., 2017). The patent describes an industrial production method for mageu. The authors state that for industrial production a pH of 4.2 or lower must be reached within 20 hours, which is faster than traditional fermentation. The cereal to water ratio is ideally between 5 and 10% (w/w). Notably, the pH of the maize suspension prior to the addition of the microorganisms is between 5.7 and 6.3. The fermentation temperature is also different to the above traditional methods. The patent specifies a fermentation temperature between 42°C and 47°C.

The end of fermentation is assessed by measuring pH or titratable acidity. The final mageu pH differs across studies. Fadahunsi and Soremekun (2017) recorded pH values of roughly 3.30 for their samples, which were similar to values obtained by Pswarayi and Gänzle (2019). In contrast, Awobusuyi et al. (2016) and Chaves-López et al. (2020) suggest the ideal pH for mageu is between 3.5 and 3.6. The International Development Research Centre (1988) recommends a pH between 3.5 and 3.9, which is in line with the results obtained from Mashau, Jideani and Maliwichi (2020) with a pH between 3.9 and 4.0. The patented method also places the ideal final pH between 3.5 and 3.6, but a pH between 3 and 4.2 is acceptable (Fourcassie et al., 2017). The International Development Research Centre (1988) also note the ideal titratable acidity for mageu at 0.4 to 0.5% lactic acid.

According to the South African National Standard for the commercial production of mageu (SANS1199), any suitable bacterial starter culture may be used as long as the product has the characteristic lactic acid fermentation flavour and odour (South African Bureau of Standards, 2011). Other product requirements include the total solids content (minimum 8% (m/m)), alcohol content (maximum 0.25% (m/m)), and microbial requirements. However, no mention is given of the final pH or titratable acidity of the mageu product. The microbial requirements state that the mageu product must be free from *E. coli* and *Clostridium* spp. (South African Bureau of Standards, 2011).

There is currently no established Codex standard across Southern Africa for mageu production. However, Malawi, Eswatini, and South Africa all have regional standards for mageu production (CODEX Alimentarius Commission, 2019). Therefore, Eswatini proposed a regional Codex standard be established for mageu production (CODEX Alimentarius Commission, 2019). The standards should include general requirements for raw materials, hygiene practices, and methods for analysis. If national standards across African countries differ for mageu products, then there could be trade issues (CODEX Alimentarius Commission, 2019). However, no further developments have occurred since this initial proposal.

Mageu microorganisms

In South Africa, wheat flour, maize bran, sorghum or millet malt are added as inoculum sources and to initiate lactic acid fermentation (Idowu et al., 2016; Simatende et al., 2015). The fermentation processes take place at ambient temperatures, between 20 and 30°C, with *Lactococcus lactis* as the main microorganism (Mashau et al., 2020). Fadahunsi and Soremekun (2017) identified the microorganisms present during mageu fermentation and isolated *Levilactobacillus brevis, Lacticaseibacillus casei, L. lactis, Lactiplantibacillus plantarum* subsp. *plantarum, Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, indicating that LAB and yeasts are the microbes present. The two most abundant microbes were *S. cerevisiae* at 70% and *L. brevis* at 54% relative abundance. This contrasts with the results obtained by Mashau, Jideani and Maliwichi (2020), who found *L. lactis* subsp *lactis* to be the dominant bacterial species. Pswarayi and Gänzle (2019) also found that for the Zimbabwean produced mageu, the only bacteria present were LABs, with *L. fermentum* being the most abundant. The main microbes isolated from Swazi mageu were *L. plantarum* subsp. *Plantarum*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *L. fermentum*, *L. brevis*, *Weissella confusa, Lactobacillus acidophilus*, and *L. lactis* (Simatende, 2016).

The first step of mageu production is cooking the maize porridge, which kills the microorganisms present in the maize meal. The millet or sorghum is then added afterwards to provide the fermentation microbes (Pswarayi and Gänzle, 2019). The time of year and equipment used also have an impact on the microorganisms present in mageu (Pswarayi and Gänzle, 2019). Of the yeasts, *S. cerevisiae* had a higher relative abundance in winter whereas in summer, the cell counts were on par with *Candida glabrata*. This indicated that *C. glabrata*'s growth was temperature-dependent, favouring a higher temperature (Pswarayi and Gänzle, 2019). *C. glabrata* can cause infections and is a severe risk to immunocompromised individuals (Pswarayi and Gänzle, 2019). This is important as it suggests *C. glabrata* should not be used in a starter culture.

Mageu has been shown to have bacteriostatic and bactericidal properties, thereby limiting growth of pathogenic bacteria (Fadahunsi and Soremekun, 2017). Fadahunsi and Soremekun (2017) found that during mageu production the total titratable acidity (TTA) increases as the pH drops, thereby giving an indication of acidification and fermentation. They reported that the TTA increased from 0.09% to 1.35% at the beginning and end of fermentation, respectively. The change in TTA and pH occurred rapidly, showing that fermentation took place at a good rate. Fadahunsi and Soremekun (2017) highlight that spontaneous fermentation using microorganisms already present in the raw material greatly affects the quality of the end-product. Therefore, for consistency in taste, aroma, appearance, and overall acceptability, a standard starter culture may be required.

Idowu, Fadahunsi and Onabiyi (2016) also investigated the microorganisms found in mageu to establish a standard starter culture. They identified 13 LAB and 10 yeasts species from their experiments. The 23 microbes were compared for their production of lactic acid, diacetyl, and hydrogen peroxide, as well as their growth at different pHs. From the comparisons, *L. brevis* and *S. cerevisiae* were selected for starter cultures; to compare both pure cultures, the mixed culture and the control which used wheat flour as the inoculum. The moisture, protein, ash, crude fibre, fat, and carbohydrate content of the final product all differed for the starter cultures used. The mixed culture had the highest mineral (calcium, magnesium, sodium, potassium, iron, and zinc) and protein content of the four samples. An increase in protein content increases the nutritional value of the final product. Most notably, the mixed culture had a calcium content of 11.45 ± 5.0 mg/100 g compared to 9.70 ± 1.5 mg/100 g for the control. Similarly, the potassium content was 277 ± 15.5 mg/100 g and 180 ± 10.0 mg/100 g for the mixed culture and

control, respectively. Overall, the *S. cerevisiae*-produced mageu had higher mineral contents than the mageu produced using *L. brevis*, although the *L. brevis*-produced product had higher fat, ash, crude fibre and carbohydrate contents. From the above results, Idowu, Fadahunsi and Onabiyi (2016) recommended the mixed culture of *L. brevis* and *S. cerevisiae* as a mageu starter culture.

McMaster et al. (2005) investigated mageu as a vehicle for delivery of the probiotic *Bifidobacterium animalis* subsp. *lactis* DSM 10140. They tested both free and immobilized cells and determined their effect on the taste of mageu and the surviving fraction of cells. The encapsulated cells had greater surviving fractions of cells compared to the free cells, and only affected the taste of mageu after 14 days in comparison to two days with free cells. The trained taste panel did not detect a difference in the texture of mageu with the encapsulated cells compared to the control.

Armistice and Tafadzwa (2021) investigated mageu as a probiotic carrier for *L. rhamnosus* yoba. An increase in the cell count of *L. rhamnosus* yoba was observed during fermentation over 36 hours. By the end of the 36 hours the cell counts had increased from 5.8 $\log_{10}$ cfu/mL to 7 $\log_{10}$ cfu/mL. Similarly, Mukisa, Byakika and Meeme (2019) investigated the use of obushera, an Ugandan sorghum beverage, as the probiotic carrier for *L. rhamnosus* yoba. During fermentation the *L. rhamnosus* yoba cell counts increased from 7.2 $\log_{10}$ cfu/mL to 8.8 $\log_{10}$ cfu/mL in 24 hours. During storage over eight weeks, the *L. rhamnosus* yoba cell counts remained above 8 $\log_{10}$ cfu/mL. Both these cases show the potential for traditional fermented foods to act as probiotic carriers.

Sensory evaluation of mageu produced with different starter cultures

Mageu has the potential to be used as a vector to deliver probiotic bacteria as it is consumed by a large portion of the South African population and has positive health benefits. Nyanzi et al. (2010) used *L. delbrueckii* subsp. *lactis* as the control bacterium for the mageu starter culture, as it is the most common bacterium used in the industrial production of mageu. The probiotic strains tested were *Lacticaseibacillus paracasei* BGP1, *L. casei* BGP93, *L. rhamnosus* LRB, and *L. acidophilus* PRO.

Sucrose, dextrose, and inulin were added to the mageu porridge as prebiotics (Nyanzi et al., 2010). The probiotic bacteria were added once the porridge had been bottled and cooled. The probiotic porridge was incubated in water baths until a pH of between 3.5 and 3.7 was reached, indicating good growth of the bacteria. The trained sensory panel detected a sourness in the probiotic samples which was caused by the bacteria converting carbohydrates into lactic acid. The control sample had the sweetest flavour, followed by the *L. acidophilus* sample. One probiotic bacterium, *L. paracasei,* gave an off-flavour aroma to the porridge and was thus excluded from further consumer (untrained panel) acceptability tests. While the trained panel had undergone training to prepare for the sensory tests, the consumer panel did not. The trained panel preferred the *L. acidophilus* and *L. rhamnosus* probiotics for the porridge, whereas the consumer panel did not notice any marked differences between the probiotic samples and the control (Nyanzi et al., 2010).

2.4.2 Yoghurt

Milk is fermented by LAB to produce yoghurt. *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* are the LAB most commonly used (Sieuwerts, 2009). In some countries, the term ‘yoghurt’ is only given to products containing these two species. In South Africa a yoghurt culture is classified as the above two species or another similar culture which produces yoghurt (South African Department of Agriculture Forestry and Fisheries, 2019). Many yoghurts on the market contain probiotic bacterial strains, but these strains do not necessarily contribute to fermentation. During fermentation of milk, lactose is converted to lactic acid which aids in preservation of the food and contributes to the sensory characteristics of the product (Caplice and Fitzgerald, 1999). In 2020 the South African dairy sector had a market value of EUR 1.6 billion, of which yoghurt contributed EUR 700 million (European Commission, 2020).

Yoghurt production

The standard method for yoghurt production begins with the pasteurisation and homogenisation of raw milk (Dave and Shah, 1997). Thereafter, the milk undergoes heat treatment (South African Department

of Agriculture Forestry and Fisheries, 2019). Heat treatment ensures that microbial activity driving the milk fermentation is controlled by the added microorganisms and not microorganisms naturally occurring in the milk. Heat treatment also denatures the whey proteins in the milk, creating the characteristic texture of yoghurt (Lucey, 2004). After fermentation, the yoghurt must not undergo further heat treatment. The literature differs with what temperature the milk should be heated. Both Capela, Hay and Shah (2006) and Hekmat and Reid (2006) treated the milk between 85°C and 87°C for 30 minutes. By contrast, Dave and Shah (1998) and Marinaki et al. (2016) heated the milk to between 40°C and 45°C for an unspecified period. However, following heating of the milk, Dave and Shah (1998), similarly to Donkor et al. (2007), added 2% skim milk powder before reheating the milk to between 85°C and 90°C for 30 minutes.

Following heat treatment, the milk is cooled to the incubation temperature which ranged from 37°C to 43°C (Capela et al., 2006; Hekmat and Reid, 2006). At this incubation temperature, the starter culture is added (Dave and Shah, 1998). Both Capela, Hay and Shah (2006) and Hekmat and Reid (2006) added probiotic bacteria at the same time as their traditional starter cultures. Thereafter, fermentation takes place until a pH of 4.5 is reached, after which the samples are stored at 4°C (Capela et al., 2006; Dave and Shah, 1998; Donkor et al., 2007). The bacteria ferment the milk by converting the sugars to lactic acid which coagulates the milk to give yoghurt its characteristic texture (Sieuwerts, 2009).

Yoghurt microorganisms

While both *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* can separately ferment milk, both species have better growth as a mixed culture than as pure cultures (Sieuwerts et al., 2008). LAB ferment sugars – for example, lactose from dairy products – and produce lactic acid which aids in decreasing the pH of fermented foods (Caplice and Fitzgerald, 1999). These two LAB also produce exopolysaccharides which give yoghurt its texture and flavour (Caplice and Fitzgerald, 1999). The type of relationship between two species in a mixed culture for yoghurt production is dependent on the bacterial strains, the type of milk, milk heating method, and milk fermentation temperature (Courtin and Rul, 2004). *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* have a mutualistic relationship, whereby their presence benefits one another (Sieuwerts, 2009).

Grown together, *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* promote each other's growth by exchanging nutrients and growth factors, known as protocooperation (Fredrickson, 1977). *S. thermophilus* has an optimal growth temperature between 40°C and 45°C, whereas *L. delbrueckii* subsp. *bulgaricus* is between 45°C and 50°C (Courtin and Rul, 2004). *L. delbrueckii* subsp. *bulgaricus* is proteolytic, while the *S. thermophilus* strains most commonly used in yoghurt production are not (Sieuwerts, 2009). Proteolysis results in the hydrolysis of caseins to polypeptides, peptides, and amino acids (Donkor et al., 2007). Both *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* use the peptides to further break down and produce amino acids using intracellular peptidases. *S. thermophilus* produces formic acid, folic acid and pyruvic acid, all of which are beneficial for *L. delbrueckii* subsp. *bulgaricus* (Courtin and Rul, 2004). *L. delbrueckii* subsp. *bulgaricus* lacks a pyruvate-formate lyase and so requires an external source of formic acid (Courtin and Rul, 2004). *S. thermophilus* consumes oxygen and produces carbon dioxide. *L. delbrueckii* subsp. *bulgaricus* grows best in anaerobic conditions, which are generated by the consumption of oxygen by *S. thermophilus* (Sieuwerts et al., 2008).

This mutualistic relationship is evident in the three growth phases that take place. In the first phase, *S. thermophilus* grows exponentially while *L. delbrueckii* subsp. *bulgaricus* experiences no growth. In the second phase, the growth rate of *S. thermophilus* decreases while *L. delbrueckii* subsp. *bulgaricus* grows exponentially, initiating protease expression. In the final phase, the peptides released from caseins are a source of amino acids and stimulate a second exponential phase of growth for *S. thermophilus* while *L. delbrueckii* subsp. *bulgaricus* continues to grow (Sieuwerts et al., 2008).

Protocooperation between *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* appears to be strain specific. Courtin and Rul (2004) investigated the growth of two strains of *L. delbrueckii* subsp. *bulgaricus* and one *S. thermophilus* to compare both mixed and pure cultures and their ability to ferment milk and produce yoghurt. The *S. thermophilus* pure culture achieved better results than the two *L. delbrueckii* subsp. *bulgaricus* strains as a lower pH was achieved, indicating better acidification of milk. For the

mixed cultures, the first strain of *L. delbrueckii* subsp*. bulgaricus* (397) had a negative effect on the growth of *S. thermophilus*, as evident in a decrease in *S. thermophilus* cell counts compared to the pure culture. In contrast, the second strain, *L. delbrueckii* subsp*. bulgaricus* (1038) had a positive effect on the final bacterial count of *S. thermophilus* and resulted in a two-fold increase in growth of the *S. thermophilus* compared to the pure *S. thermophilus* culture. It is suggested that *S. thermophilus* grew better than *L. delbrueckii* subsp*. bulgaricus* because less formic acid was produced by *S. thermophilus* which may have limited the growth of *L. delbrueckii* subsp*. bulgaricus*. Formic levels are higher at higher temperatures, and therefore temperature may have also played a role in favouring *S. thermophilus* growth over *L. delbrueckii* subsp*. bulgaricus*.

Probiotic yoghurt

Probiotic bacteria lacking proteolytic activity grow slowly in milk (Dave and Shah, 1998). Therefore, *L. delbrueckii* subsp*. bulgaricus* and *S. thermophilus* are added to speed up fermentation. However, *L. delbrueckii* subsp*. bulgaricus* continues to produce lactic acid during storage, a process known as postacidification (Dave and Shah, 1998). Lowering of the pH (increase in acidity) decreases the viability of probiotic bacteria. Therefore, instead of a mixed culture of *L. delbrueckii* subsp*. bulgaricus* and probiotic bacteria, other yoghurt bacteria are used. These include *L. acidophilus*, bifidobacteria and *S. thermophilus* (Dave and Shah, 1998). Typical probiotic cultures do not favour low pH, as seen in the study by Dave and Shah (1998) above; however, Happel et al. (2020) selected the best vaginal isolates with one of the criteria being the ability to lower pH.

Probiotic bacteria originating from the GIT are often unable to grow in food matrices due to deficiency of their metabolic capabilities (Kort et al., 2015). Therefore, similarly to the bacteria lacking proteolytic activity, a second strain of bacteria which is known to ferment food products is added. The specific strain of *S. thermophilus* used by Kort et al. (2015) was able to synthesize all essential amino acids needed for growth in milk, as identified through genomics.

While probiotic yoghurt has many advantages, it must still be preferred by the consumer, a decision which is generally based on acceptability of flavour, texture, and appearance. Hekmat and Reid (2006) compared the above qualities for regular yoghurt and probiotic yoghurt, using freeze-dried *L. reuteri* RC-14 and *L. rhamnosus* GR-1 as probiotics. Overall, the regular yoghurt with a higher fat content (3.25%) had the best feedback from the panellists. Notably, the three remaining samples – 1% fat with yeast extract, 1% fat with inulin, and 1% fat without prebiotics – received similar scores with no significant differences noted. This may be due to the species of *Lactobacillus* used which did not inhibit the yoghurt bacteria nor produce excess lactic acid. Some LAB can spoil milk by producing too much lactic acid, resulting in an unpleasant flavour and odour. Therefore, probiotic yoghurt could compete with standard yoghurt for consumer preference.

The case of Yoba-for-Life

Kort et al. (2015) designed a dried starter culture sachet containing probiotic bacteria and yoghurt bacteria for easy production of the drinking yoghurt, Yoba-for-Life. The probiotic bacterium selected was *L. rhamnosus* GG, which was renamed to *L. rhamnosus* yoba*. L. rhamnosus* yoba is extensively studied and reduces bouts of diarrhoea and promotes gastrointestinal wellbeing (Szajewska et al., 2011)*. L. rhamnosus* yoba cannot solely grow in dairy products due to its non-proteolytic properties and inability to convert lactose into lactic acid. To solve this problem, glucose, proteolytic enzymes or partially hydrolysed nitrogen sources (yeast extract) can be added to the milk. Alternatively, a proteolytic strain of *S. thermophilus* can be used to produce lactic acid and degrade casein (Kort et al., 2015). While *L. delbrueckii* subsp*. bulgaricus* is normally used for milk fermentation, it could interfere with the probiotic activity of *L. rhamnosus* (Reid, 2017). The addition of *S. thermophilus* aided in the growth of *L. rhamnosus* yoba, which reached 10^9 cfu and met the daily recommended probiotic dose. Most importantly, the mixed culture allowed for *L. rhamnosus* yoba to retain its pili, which are needed to adhere to the intestinal epithelium (Kort et al., 2015).

The two bacteria potentially have a commensalism relationship, in which *S. thermophilus* produces desired compounds for *L. rhamnosus* yoba but *L. rhamnosus* yoba does not produce the same for *S.*

thermophilus, which is unaffected. *S. thermophilus* produces succinate, glycerol and galactose, all of which *L. rhamnosus* yoba can consume instead of lactose.

Production of the Yoba-for-Life sachet, from isolating the starter culture to the final yoghurt product, is shown in Table 2-1 below. To produce yoghurt using the Yoba-for-Life sachet, the following materials are needed: milk, saucepan, thermometer, lactometer, heat source, and the sachet. The quality of the milk is first tested using a lactometer. The milk is then heated to 85°C, with the addition of sugar to sweeten the yoghurt, if desired, at 60°C. Once at 85°C, the milk is maintained at this temperature for 15 minutes, followed by cooling to 45°C. A vacuum flask is used to maintain this temperature, after which the starter culture is added. After 12 hours, one litre of yoghurt has formed. If a larger quantity of yoghurt is required, a fresh batch of milk is heated again to 85°C for 15 minutes and is then again cooled to 45°C. The one litre of Yoba-for-Life yoghurt is then added to the milk as the starter culture, after which it is maintained at 45°C for 12 hours (Yoba for Life Foundation, n.d.). This process differs from that of the standard yoghurt production. The Yoba-for-Life production requires three cycles of heating between 45°C, 60°C and 85°C, whereas the standard production uses one or two heat cycles. However, both processes inoculate the milk at 45°C.

Table 2-1 Yoba-for-Life starter culture and yoghurt production process (adapted from Kort et al., 2015)

Step	What	How	Details	Who
1	Production of frozen biomass	Fermentation, concentration, and freezing	Target: 1-5×10^{10} cfu/mL	Starter culture companies
2	Production of freeze-dried culture power	Freeze drying	Target: 1-3×10^{11} cfu/g	Starter culture companies
3	Standardization with a dried carrier	Mixing	Target: 5×10^{9} cfu/g by diluting with dry inert carrier, e.g. skimmed milk power, maltodextrin, starch	Starter culture companies
4	Filling and Packaging	Stick pack filling machines	Use moisture proof seal	Filing and packaging company
Seed cultures checked for absence of pathogenic and spoilage organisms				
5	Preparation of fresh starter	Inoculation of food raw material (e.g. milk or cereals) with seed culture	Add one gram seed culture to one litre of food raw material; assure acidification until pH 4.3	Local communities
6	Preparation of probiotic fermented food	Inoculation of food raw material with fresh starter	Add one litre into maximal 100 litre of food raw material; assure acidification until pH 4.3	Local communities

2.5 Identified vaginal bacterial isolates for fermented food production

Several studies have investigated the potential of *Lactobacillus* spp. typically found in the vaginal tract to be delivered as consumed live cultures and their ability to produce fermented milk products such as yoghurt and cheese. This includes four of the species identified by Happel et al. (2020) as potential probiotic candidates, namely *L. crispatus*, *L. jensenii, L. vaginalis* and *L. gasseri*.

In one study, Laue et al. (2018) investigated *L. crispatus, L. gasseri, L. jensenii*, and *L. rhamnosus* strains as probiotics for the treatment of BV following antibiotic administration. This study was important as it looked at administering the cultures relevant to this project through a fermented drink. The common

yoghurt bacteria (*L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus)* were used to ferment the milk, after which the probiotic strains were supplemented. The control group were given chemically acidified milk without any added bacteria. After four weeks of treatment, none of the 17 women given the probiotic yoghurt had BV, as determined using the Amsel criteria. In contrast, the control group had six reports (of 17 participants) of BV following four weeks of treatment. Additionally, the intervention group saw a bigger difference in vaginal pH decrease than the control group. The paper concluded that the identified strains could be used to produce yoghurt by fermentation, although no evidence supported this conclusion as the bacteria were supplied as live cultures following fermentation by *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*.

Strain compatibility is important when considering the use of probiotic strains in fermented foods. For example, in yoghurt production, if a non-proteolytic *S. thermophilus* strain is used, a mutual relationship is only achievable if *L. delbrueckii* subsp. *bulgaricus* is proteolytic. However, as exhibited with Yoba-for-Life, a proteolytic *S. thermophilus* strain exhibits a commensalism relationship with *L. rhamnosus yoba* (Kort et al., 2015). Virtanen et al. (2006) investigated the antioxidant activity of LAB, and the connection between proteolysis and antioxidant behaviour. Twenty-five LAB strains were analysed including *L. jensenii*, which had the second highest proteolytic activity of the strains investigated. A second study by de Moraes et al. (2017) focused on *L. mucosae* strains, and investigated proteolytic activity and ability to acidify milk. The three *L. mucosae* strains exhibited varying levels of proteolytic activity and were able to reduce the pH of milk from 6.20 to 5.52 on average.

In another study, the ability of *L. crispatus* to produce Squacquerone cheese as a functional food for female genital health was investigated (Patrignani et al., 2019). The *L. crispatus* strain used maintained viability throughout the refrigerated storage period of 13 days. Several strains of *L. crispatus* have reported protective effects against spoilage and pathogenic microbes. Patrignani et al. (2019) also noted that some *L. crispatus* strains have good fermentation kinetics which can be used in production of fermented dairy products from milk.

Viability of the probiotic strains during storage is another important aspect. In the above study, *S. thermophilus* starter culture was used for milk inoculation, and following heating, the strain of *L. crispatus* was added. The addition of *L. crispatus* had a positive effect on the viability of *S. thermophilus*, which saw only a decrease in cell count from 7.4 $\log_{10}$ cfu/g to 5.7 $\log_{10}$ cfu/g for the test cheese over the 13 days of storage. In contrast, the control cheese (with *S. thermophilus* but not *L. crispatus*) saw a decrease from 6.1 $\log_{10}$ cfu/g to 5.0 $\log_{10}$ cfu/g. A sensory analysis was also performed for comparative purposes. The panel scored the test cheese higher for overall acceptability, with significant differences in scores noted at day four. After 13 days of storage, the difference in scores of the two cheeses was less, however the test cheese was still preferred. Experiments were also performed on the ability of *L. crispatus* to survive the conditions of the stomach and upper intestine. *L. crispatus* was significantly affected by low pHs, used to stimulate the stomach, but was unaffected by bile salts and pancreatic juices. Patrignani et al. (2019) suggest that soft cheeses, Squacquerone and Crescenza are preferable as vehicles of probiotic delivery since their protective proteins, neutral pH, and buffering capacity are able to protect the bacteria during passage through the gastrointestinal tract. Patrignani et al. (2019) report that their research is novel in that previously *L. crispatus* had only been used in pharmaceutical formulations (capsules) for treatment. However, their study used *L. crispatus* in production of a functional food, targeted for women.

2.6 Literature review summary

To summarize the identified research, current treatment of BV with antibiotics are costly and are associated with high recurrence rates. Therefore, additional probiotic treatments are desirable to re-establish a lactobacilli-dominated vaginal microbiota. Importantly, oral administration of lactobacilli without encapsulation can reach the FGT and no side-effects were reported during administration to healthy women. Of the currently available probiotic products in South Africa, none contain bacterial species specific to the FGT. Previously, bacterial strains collected from HIV-negative South African women were ranked and evaluated for potential probiotic development. The ranking criteria included

the ability to both tolerate low pH and to lower medium pH through metabolic activity, which are necessary characteristics for the production of fermented foods.

LAB are most commonly used in food fermentations and lactobacilli are safe for consumption due to their long history of probiotic use. Mageu, a ubiquitously consumed fermented food in Southern Africa, has been researched as a probiotic delivery vehicle. Whilst traditional mageu production methods differ, the yeast with the highest relative abundance in the traditionally-produced samples was *S. cerevisiae*. For yoghurt production, *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* are most commonly used. Yoghurt has also been investigated as a probiotic delivery vehicle and was comparable to standard yoghurt samples from a consumer acceptability perspective. Both yoghurt and cheese were produced using lactobacilli as probiotic foods and yielded promising results for the improvement of vaginal health. However, the bacterial isolates specific to South African women (the FGT microbiota differs with geographical location) are not yet known to produce mageu and yoghurt as probiotic foods for vaginal health.

2.7 Defining the research project

2.7.1 Problem statement and objectives

Antibiotic treatments for bacterial vaginosis are expensive and may contribute to the recurrence of infection. While probiotics can be used in conjunction with antibiotic treatment, there are currently no probiotic products designed with bacterial strains commonly found in the vaginal tract of South African women. A low-cost probiotic product targeted at the improvement of vaginal health is therefore needed. To address this gap, this project aims to investigate the production of fermented foods commonly consumed in South Africa using selected probiotic *Lactobacillaceae* strains isolated by Happel et al. (2020).

The research project objectives are thus:

- Determine whether the selected probiotic *Lactobacillaceae* strains can ferment maize meal to mageu and/or ferment milk to yoghurt by measuring fermentation factors.
- Determine the consumer acceptance of the probiotic mageu samples by performing a sensory analysis.

2.7.2 Research hypotheses and key questions

The proposed research hypotheses are as follows:

1. Probiotic mageu can be produced by fermenting maize meal using *Saccharomyces cerevisiae* and *Lactobacillaceae* strains that were among the top ten isolates for probiotic development identified by Happel et al. (2020). This is because the isolates are known lactic acid producers and are able to tolerate low pH. Successful fermentation will be characterised by the final product displaying qualitative characteristics of mageu and the strains being able to ferment maize meal to the pH of around 3.5 that is characteristic of mageu. Since lactic acid is the main flavour compound for mageu, the sensory profile of the probiotic mageu will not differ significantly from the control mageu.

2. Probiotic yoghurt can be produced by fermenting milk using *Streptococcus thermophilus* and *Lactobacillaceae* strains identified by Happel et al. (2020) as most promising for treatment or prevention of bacterial vaginosis, in replacement of the traditional *L. delbrueckii* subsp. *bulgaricus* yoghurt bacterium. Like *L. delbrueckii* subsp. *bulgaricus*, the *Lactobacillaceae* isolates produce lactic acid and are able to tolerate low pH. This will enable the mixed culture of *S. thermophilus* and the *Lactobacillaceae* strains to ferment milk to the requisite pH of 4.5 for yoghurt.

The *Lactobacillaceae* strains used to produce the fermented foods are among the top ten isolates identified by Happel et al. (2020) (see Figure 2-1). These isolates have been assessed for their ability to tolerate low pH levels, the ability to lower pH, and lactate production. These are three characteristics important for the production of the fermented foods yoghurt and mageu. Previous studies investigated the production of fermented foods using a selection of the same species of lactobacilli that are used in this study, namely *L. crispatus*, *L. jensenii, L. vaginalis* and *L. gasseri.* However, the specific strains isolated from the vaginal tracts of South African women have not been tested for their ability to produce fermented foods. This study specifically tests the ability of these strains to produce mageu and yoghurt. This project also investigates the consumer acceptability of the probiotic mageu products. Since traditional methods use spontaneous fermentation for mageu production, the consumer acceptability tests will determine if the probiotic bacteria influence the sensory characteristics of mageu. Consumer acceptability is important to consider for potential commercialisation of the products.

The product requirements for mageu include a minimum 8.0% (m/m) total solids content, maximum 0.25% (m/m) alcohol content, and characteristic lactic acid fermentation flavour (sour) (South African Bureau of Standards, 2011). Yoghurt requirements include a minimum 0.6% (m/m) titratable acidity and that the product be characteristically thicker than milk (CODEX Alimentarius, 2018).

The key research questions that will be addressed in this dissertation are consequently:

- Can pure *Lactobacillaceae* cultures, identified by Happel et al. (2020), ferment milk to yoghurt or ferment maize meal to mageu, as seen by the production of pH-lowering metabolites, and the final formation of products meeting the respective product requirements?
- Can supplementing isolated *Lactobacillaceae* isolates with *Saccharomyces cerevisiae* for mageu production (maize meal fermentation) positively affect the fermentation process with respect to the fermentation time and the final pH?
- Can supplementing isolated *Lactobacillaceae* strains with *Streptococcus thermophilus* positively affect yoghurt production (milk fermentation) with respect to the fermentation time and the final pH?
- What is the consumer acceptability of the mageu probiotic products?

3 Approach to Project and Methodology

3.1 Research methodology

The research methodology applied in this study is shown by Figure 3-1. There were two key components of the research: the production of the two fermented foods, mageu and yoghurt, and a sensory analysis of a selection of the mageu products. The production of mageu and yoghurt further consisted of three parts: producing the controls using traditionally-used microorganisms, using the *Lactobacillaceae* strains as single cultures, and co-culturing the *Lactobacillaceae* strains with traditional microorganisms.

Production of the food products adhered to the South African regulations for yoghurt and mageu product specifications. The regulations implemented in South Africa include those from the South African Bureau of Standards (SABS) and Codex Alimentarius Commission Guidelines, set out by the Joint FAO/WHO Food Standard Programmes (Food Advisory Consumer Service, 2017). South Africa is one of the 150 countries which subscribes to the Codex guidelines. In South Africa, any food additives have to adhere to the Foodstuff, Cosmetics and Disinfectants Act of 1972 (Food Advisory Consumer Service, 2017).

The control mageu and yoghurt were produced using traditional starter cultures, while the experimental mageu and yoghurt were produced using combinations of the *Lactobacillaceae* isolates of interest in this study and traditional starter microorganisms. Aside from the inocula, all ingredients used in the fermented foods production were foodstuffs. During production, fermentation was monitored by measuring pH. Following the production of the fermented foods, the products were analysed by investigating titratable acidity, total solids content, lactic acid and ethanol concentrations, cell counts, shelf-life, and sensory analysis.

After analysis, a sub-selection of four mageu probiotic products, along with the positive control produced with wheat flour, and a commercial mageu product were analysed for their consumer acceptability using an untrained consumer panel. The mageu samples for the taste testing were selected by identifying which bacterial isolates are currently gaining traction for probiotic use in vaginal health applications, and which isolates would have the most distinctive tastes due to different metabolites being produced. Therefore, one homofermentative strain and one heterofermentative strain were selected to show the broadest range in taste profiles. Additionally, the impact of yeast on the flavour profiles was also of interest. So, the mageu samples produced with the same isolates (one homofermenter and one heterofermenter) and yeast were also included.

1 Produce Mageu and Yoghurt

Control Samples
Commercial production methods with traditionally-used microorganisms

Pure *Lactobacillaceae* Isolates
Pure *Lactobacillaceae* strains

Mixed cultures
Traditional microorganisms + *Lactobacillaceae* strains

Monitor Fermentation
Measure pH

Analyse Fermentation Products
- Titratable acidity
- Total solids content
- Lactic acid concentration
- Ethanol concentration
- Bacteria and yeast cell counts
- Shelf life

2 Conduct Sensory Analysis for Mageu Samples

- Untrained consumer panel consisting of UCT students
- Requirements: regular consumers of mageu
- Once-off participation

Monitor Fermentation
- 9-Point hedonic scale
- Statistical analysis

Figure 3-1 Experimental approach for fermented food production and analysis

3.2 Culture collection and maintenance

3.2.1 *Lactobacillaceae* strains

Culture maintenance and growth

Of the top ten *Lactobacillaceae* isolates identified by Happel et al. (2020), five were used to produce the fermented foods. The five isolates used were acquired as pure cultures in the form of glycerol stocks from the Pathology Department at the Faculty of Health Sciences at UCT. The isolates used were:

- *Lactobacillus crispatus* 70.6,
- *Lactobacillus crispatus* 73.55,
- *Limosilactobacillus mucosae* 90.13,
- *Lactobacillus gasseri* 94.98, and
- *Lactobacillus jensenii* 95.1.

The glycerol stocks were stored at -80°C until use. For *Lactobacillaceae* cultivation, the standard medium is De Man Rogosa Sharpe (MRS) medium (Merck, 69966) supplemented with L-cysteine monohydrate (Merck, C7880). For cultivation, an 80 mL mixture of MRS (50 g/L) and L-cysteine monohydrate (0.072 g/L) was prepared in serum bottles. The broth was sterilized by autoclaving at 121°C for 20 minutes. Once the sterile broth had cooled, the bottles were inoculated with the glycerol stocks of the *Lactobacillaceae* isolates using sterile syringes and needles. The inoculated serum bottles were then incubated at 37°C.

Glycerol stock preparation

To make more glycerol stocks, the following method was used. MRS with L-cysteine monohydrate agar plates were prepared in the same concentration as the broth, and bacteriological agar (Merck, A5306) at a concentration of 15 g/L was added. The agar mixture was autoclaved before being poured into sterile petri dishes. The plates were incubated at 37°C overnight in a container with an anaerobic sachet (Anaero-pack, Davies Diagnostics, MGC-PACK-10-01), which created an anaerobic environment (Collee et al., 1972). Incubating the plates overnight tested for contamination and pre-reduced the plates. A sample from the glycerol stocks was spread-plated onto the plates, placed in the anaerobic container with a fresh Anaero-pack, and incubated at 37°C for 24-48 hours. Once good growth was seen, the plates were sub-cultured onto pre-reduced MRS plates. In the anaerobic container, sterile standard broth for *Lactobacillaceae* cultivation (1 mL per cryovial) was pre-reduced overnight in sterile cryovials (2 mL). Single colonies from the sub-cultured plates were inoculated into each cryovial containing the standard broth. The cryovials were incubated anaerobically (with an Anaero-pack) at 37°C for 24-48 hours until good growth was seen. A 50% glycerol solution was prepared using equal volumes of 99.5% glycerol (Merck, G9012) and deionised water. The glycerol solution was sterilised by autoclaving at 121°C for 20 minutes. One mL of 50% glycerol was added to the 1 mL of culture in the cryovials. The stocks were vortexed before being stored in the -80°C freezer.

3.2.2 Saccharomyces cerevisiae

Culture maintenance and growth

Dried Anchor Yeast was purchased from a local retailer (Pick 'n Pay) and stored at room temperature in a dark, dry cupboard until use. The standard media for *S. cerevisiae* is Yeast Extract-Peptone-Dextrose (YPD). YPD media was prepared as 10 g/L yeast extract (Merck, Y1625), 20 g/L peptone (Merck, 70173), and 22 g/L glucose monohydrate (Merck, Y0001745). For growing *S. cerevisiae*, dried yeast was inoculated into sterile YPD media and incubated aerobically at 30°C for 24-48 hours.

For measuring cell counts, YPD plates were prepared in the same concentrations as the broth and supplemented with 15 g/L bacteriological agar (Merck, A5306). The agar was autoclaved before being poured into sterile plates. The plates were tested for contamination by being placed in the 30°C temperature control room overnight. If no contamination was seen, the yeast cultivated in the YPD broth was spread plated onto the plates. No glycerol stocks were made of yeast since mageu was produced using dried yeast only.

3.2.3 Streptococcus thermophilus

Isolation of S. thermophilus from yoghurt culture

A "Natural Yoghurt" starter culture from Crafty Cultures was purchased. This freeze-dried culture contained only *L. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. *L. delbrueckii* subsp. *bulgaricus* favours anaerobic growth (like the other lactobacilli), while *S. thermophilus* favours aerobic growth. The standard media for the two bacteria also differ. Similarly to the other *Lactobacillaceae*, the standard media for *L. delbrueckii* subsp. *bulgaricus* is MRS, whereas for *S. thermophilus* it is M17. Sterile M17 broth (Merck, 56156) was prepared at a concentration of 42 g/L (as per the manufacturer's instructions) and was sterilised through autoclaving. To isolate *S. thermophilus*, a very small mass (0.05 g) of the freeze-dried culture was inoculated into the sterile M17 broth (40 mL) and incubated aerobically at 37°C. These are the standard incubation conditions for *S. thermophilus*.

Plates of M17 were prepared by autoclaving an M17 broth and bacteriological agar mixture, before pouring the agar into sterile petri dishes. The M17 plates were incubated aerobically overnight at 37°C to test for contamination. Once good growth was observed (good turbidity seen) in the M17 media, the culture was spread-plated onto the M17 plates. The plates were incubated at standard conditions (aerobically at 37°C) for 24-48 hours. Once good growth was observed, a single colony (presumptive *S. thermophilus* isolate) was sub-cultured and spread onto a fresh M17 plate. This second plate was

again incubated at standard conditions for 24-48 hours. Single colonies from this second plate were inoculated into sterile 1 mL M17 broth in cryovials. The cryovials were incubated at standard conditions until good growth was observed. To the culture, 1 mL of sterile 50% glycerol solution was added. The stocks were vortexed before being stored at -80°C until needed. To confirm the identity of stocks, polymerase chain reaction (PCR) amplification of the 16S rRNA gene followed by Sanger sequencing was performed (see Section 3.2.4).

Culture maintenance and growth

Once the 16S rRNA PCR results confirmed speciation, the glycerol stocks were used to inoculate sterile M17 broth (2 mL of glycerol stock added to the broth). *S. thermophilus* was cultivated aerobically (in Schott bottles instead of serum bottles) at 37°C for 24-48 hours before being prepared for milk inoculation.

3.2.4 16S rRNA PCR gene sequencing

To confirm the purity of the *Lactobacillaceae* isolates and to confirm speciation of the bacteria isolated from the yoghurt culture, 16S rRNA gene PCR and sequencing were performed. The same process was followed for both *Lactobacillaceae* isolates and *S. thermophilus*.

Firstly, the culture was streaked on its selective media, MRS or M17. The presumptive *S. thermophilus* isolates were Gram stained and the results displayed Gram positive cocci (Bartholomew and Mittwer, 1952). The CeBER protocol for Gram staining was used with a Gram stain kit (Merck, 77730-1KT-F). First a colony of the culture was suspended in a drop of deionised water on a slide. The culture was then spread over the slide, allowed to dry and heat fixed by moving the slide over a flame. To carry out the staining procedure, crystal violet was added to the culture for 30 seconds before being poured off and rinsed with deionised water. Iodine solution was then added to the culture and left for 60 seconds before also being poured off and rinsed. The decolourizer (ethanol) was added to the slide and rinsed off after 5 seconds. Lastly, the counterstain safranin was added to the slide and left to stand for 60 seconds before being rinsed off. The bacterial cultures were then confirmed by colony PCR and Sanger sequencing of the 16S rRNA gene.

PCR preparation began with suspending a single colony in 100 µl colony lysis buffer containing 0.2 mg/mL proteinase K (NEB, P8107S). The lysis buffer consisted of 10 mM Tris-HCl, pH 7.4 (Sigma, T0319) and 0.1 mM EDTA (Sigma, EDS). The samples were incubated at 37°C for 30 minutes, followed by 80°C for 30 minutes to inactivate the proteinase K. The samples were then centrifuged (Eppendorf Centrifuge 5418 R) at 5,000 rpm for 3 mins. The supernatant was removed and used as the template in subsequent PCR. Each PCR mixture contained 25 µl 2× Kapa Taq ReadyMix (Roche, KK1006), 0.5 µM of each primer (27F and 1492R) (Inqaba, IB OL0002) (Weisburg et al., 1991), 4 µl of the colony PCR template, and made up to a final volume of 50 µl with nuclease free water (Sigma, W4502). The PCR cycling conditions used were: 95°C for 3 min, followed by 30 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 90 sec. A final extension step at 72°C for 7 minutes was included.

Successful amplification of 1.6 kbp products was confirmed by electrophoresis in 1% agarose gels (Seakem, 50004). A representative gel showing *S. thermophilus* products is displayed in Figure 3-2. Inqaba Biotech performed the PCR cleanup and Sanger sequencing. Finally, the nucleotide sequence results were compared to the 16S rRNA database maintained by the National Center for Biotechnology Information (NCBI) using the BLAST algorithm (Zhang et al., 2000).

Figure 3-2 Agarose gel electrophoresis analysis of 16S rRNA gene PCR products (Samples A and B) from a putative *S. thermophilus* strain isolated from yoghurt culture grown in M17 media. Mw is the DNA molecular weight marker (1Kb ladder, NEB).

3.3 Inoculum preparation

3.3.1 *Lactobacillaceae* strain inoculum preparation

The inoculum preparation of the five *Lactobacillaceae* isolates listed in Section 3.2.1 was the same for both mageu and yoghurt production. The standard broth for the *Lactobacillaceae* isolates (MRS, as specified in Section 3.2.1) was prepared in serum bottles. Glycerol stocks of the five isolates were each inoculated into a serum bottle containing 80 mL of MRS using sterile syringes and needles. The serum bottles were incubated at 37°C for an average of 18 hours. The optical density (OD) of each culture was measured at an absorbance of 600 nm using the Thermo Scientific Genesys 10S UV-Vis

spectrophotometer and VISIONlite software (Thermo Scientific, Version 5.2). The absorbance sample was diluted appropriately to ensure the measured OD value was between 0 and 0.7, as above 0.7, the concentration dependence of the absorbance readings was not linear. The volume added of the inoculum was determined using Equation 3-1:

$$C_1V_1 = C_2V_2 \quad \text{Equation 3-1}$$

where C_1 is the OD measured for the culture sample, V_1 is the volume of inoculum to add to the 500 mL maize-water mixture or milk, C_2 is the starting optical density required in the 500 mL bottle, and V_2 is the final volume of the maize-water mixture or milk plus inoculum (working liquid volume). It was assumed that V_2 is 500 mL as the inoculum volume was negligible (≤ 3% of working volume).

The desired initial cell concentration in the 500 mL bottle was approximately 10^7 cfu/mL. Using *L. jensenii* 95.1 as the basis, the optical density (OD_{600nm}) required to reach this cell concentration determined experimentally using Equation 3-2 with a known inoculation volume (V_1). The calculations used to determine this correlation are presented in Appendix C. All future inoculum volumes were therefore calculated as follows:

$$V_1[ml] = \frac{C_2V_2}{C_1} = \frac{0.0188 \times 500\ mL}{OD_{600nm}\ of\ isolate\ culture\ in\ inoculum} \quad \text{Equation 3-2}$$

Once the required inoculum volume for each isolate had been calculated, that volume was measured into sterile Eppendorf tubes in a Biological Safety Cabinet. Those tubes were then centrifuged (Eppendorf Centrifuge 5418 R) at 13,000 rpm for two minutes. Following centrifugation, the supernatant was discarded aseptically, and the pellets were washed with sterile phosphate-buffered saline (PBS) solution before being centrifuged again at 13,000 rpm for two minutes. PBS solution was prepared using the protocol in the CeBER Laboratory Methods Manual. The PBS solution was made up of 137 mM sodium chloride (Merck, S9888), 2.7 mM potassium chloride (Merck, P3911), 10 mM di-sodium hydrogen phosphate (Merck, 1.06559), and 2 mM potassium di-hydrogen phosphate (Merck, 1.05104). The cells were then aseptically resuspended in the PBS solution before being added to the maize-water mixture or milk for the mageu and yoghurt experiments, respectively. It was assumed that there were minimal losses in the number of cells during the separation and washing process.

3.3.2 Yeast inoculum preparation

For the mageu samples with *Lactobacillaceae* strains and yeast, 0.895 g of dried Anchor Yeast was added to each 500 mL bottle. The yeast was weighed in sterile Eppendorf tubes on an analytical scale before inoculation. At a mass of 0.895 g yeast, the yeast colony count, determined through serial dilutions with spread plating and colony counting on YPD agar, was 10^6 cfu/mL. The results of the serial dilutions are presented in Appendix D.

3.3.3 S. thermophilus inoculum preparation

The cell concentration of *S. thermophilus* used was approximately 10^6 cfu/mL. Due to sluggish growth displayed by *S. thermophilus* during inoculum preparation, there was not enough culture to inoculate each milk bottle at the same concentration as the *Lactobacillaceae* isolates (10^7 cfu/mL). Therefore, the volume of *S. thermophilus* per bottle was reduced so that there was enough culture for each milk bottle. Therefore, a smaller inoculum volume was used compared to the other cultures. The equation for the volume of *S. thermophilus* added to the 500 mL was therefore adjusted to:

$$V_1[ml] = \frac{C_2V_2}{C_1} = \frac{0.0143 \times 500\ mL}{OD_{600nm}\ for\ S. thermophilus} \quad \text{Equation 3-3}$$

The same method as for the *Lactobacillaceae* isolates was used to prepare *S. thermophilus* for inoculation. The cells were centrifuged, washed with PBS, centrifuged again, and then resuspended before being added to the milk.

3.4 Yoghurt production

3.4.1 Milk type selection

Both fresh full cream milk and Ultra-high temperature (UHT) long-life full cream milk were used for yoghurt production. The fresh milk was Pick ‘n Pay brand and had been homogenised and pasteurised by the manufacturer. The UHT long-life milk was from Clover and was specifically selected due to the triple protection process the milk goes through to limit any contamination. The triple protect process includes double purification, deep cooling, and using packaging which has been ultra-cleaned (Clover, n.d.).

3.4.2 Pasteurisation and heating

The fresh (pasteurised) milk was heat treated (pasteurised again) between 85°C and 90°C in a water bath for 30 minutes in filled 500 mL Schott bottles (Capela et al., 2006; Dave and Shah, 1997; Donkor et al., 2007; Hekmat and Reid, 2006). During heating the bottles were swirled occasionally for uniform heat transfer. Thereafter, the milk was left to cool at room temperature until the milk was at a temperature between 40°C and 45°C (Capela et al., 2006; Dave and Shah, 1998; Marinaki et al., 2016). The UHT long-life milk was not pasteurised because the milk had lower chances of being contaminated by microorganisms than the fresh milk. Instead, it was only heated to between 40°C and 45°C in filled 500 mL Schott bottles placed into the water bath.

3.4.3 Inoculum addition

Following pasteurisation/warming of the milk, the inoculum was added. The OD_{600nm} of the five *Lactobacillaceae* strains in the inoculum serum bottles was measured to determine the inoculum volume to add to the 500 mL bottles using Equation 3-2. The culture was then prepared using the washing and centrifugation method described in Section 3.3.1. The cells were resuspended before being added to the heated milk. This same method was used to inoculate the milk bottles with *S. thermophilus*. Only single *Lactobacillaceae* strains were used to inoculate the milk in each experiment. Since *S. thermophilus* is the LAB traditionally used in making yoghurt, it was used to supplement the *Lactobacillaceae* strains at a concentration of 10^6 cfu/mL. No inoculum was added for the negative control yoghurt bottles. Roughly 0.1 g (‘knife-tip’) of freeze-dried yoghurt culture was added to the milk for the positive control bottles, as suggested by Crafty Cultures. A summary of the inputs for the yoghurt fermentations are presented in Table 3-1.

Table 3-1 Summary of substrate and microorganisms used for each yoghurt fermentation

Yoghurt Fermentation Inputs	
Substrate	Milk
Microorganisms used for test fermentations	*Lactobacillaceae* strains (as single cultures and mixed cultures with *S. thermophilus*)
Microorganisms used for positive control	Yoghurt culture (*S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*)
Microorganisms used for negative control	No inoculum added

3.4.4 Monitoring fermentation

Before inoculation, a sample of the heat-tempered milk was pipetted using a sterile tip into a test tube for pH measurement and recorded using a pH probe and meter (Lasec benchtop 50+ DHS pH meter), that had been calibrated using buffer solutions of pH four and pH seven. Following inoculation, a sample was taken (using a sterile pipette tip) and the pH was measured and noted as hour zero. Then, the pH was measured every hour for 12 hours. If the pH of the yoghurt had not reached 4.5 by hour 12, a final pH measured measurement was taken at hour 24 (Capela et al., 2006; Dave and Shah, 1997; Donkor et al., 2007). Due to the COVID-19 curfew lockdown regulations in South Africa, pH could not be measured between hours 12 and 24. Fermentation was terminated by placing the sample in the 4°C refrigerator until analysis (Dave and Shah, 1998).

3.5 Yoghurt analysis

3.5.1 Qualitative analysis

The first analysis conducted for the yoghurt samples was qualitative. This included performing a sensory analysis by investigating consistency and smell, to determine whether yoghurt had been produced. First, the consistency of the bottles was examined by pouring some sample into a beaker. The homogenisation was observed and any abnormalities in the form of clumping were noted. The bottles were then shaken to create a homogenous mixture before being poured once again. The consistency of the samples was then rated on a scale including thin (like milk), slightly thick, thick, very thick, and extremely thick. While all relative, a result of ‘thick’ was most similar to commercial drinking yoghurt products. The samples were then smelled and rated on a sweet-smelling scale. The sweet scale range included faintly sweet, slightly sweet, sweet, and very sweet. During this process, if any other characteristics were observed, without having to taste the samples, they were also recorded.

3.5.2 Titratable acidity

The titratable acidity was measured at the end of each fermentation experiment. After fermentation, 10 mL of yoghurt was pipetted into an Erlenmeyer flask and weighed. Since the yoghurt samples were thick and viscous, they were diluted with 5 mL of deionised water. Diluting of food samples, specifically mageu, when measuring titratable acidity was also performed by Mashau et al. (2020) and Fadahunsi and Soremekun (2017), who followed the method by Nout et al. (1989). Three drops of phenolphthalein indicator solution were then added to the mixture and mixed through swirling the flask (Fabro et al., 2006; Mashau et al., 2020). The phenolphthalein indicator solution was prepared as 1% (w/v) phenolphthalein (Merck, 105945) to 75% ethanol absolute (Merck, 1.07017) and 25% distilled water. A 0.1 M sodium hydroxide solution was poured into a burette using a funnel (Fadahunsi and Soremekun, 2017; Mashau et al., 2020). The sodium hydroxide solution was produced by adding 1 g of sodium hydroxide pellets (≥ 97%, Merck, 221465) to a 250 mL volumetric flask, and making up the rest of the volume with deionised water. Following the method described by Fabro et al. (2006) and Mashau et al. (2020), the initial sodium hydroxide volume was recorded. Next, the mixture was titrated against the 0.1 M sodium hydroxide solution until a pink colour change was observed and persisted for 30 seconds. The titratable acidity of all yoghurt samples was measured in triplicate. The titratable acidity was then recorded as a weight percentage (g lactic acid per g of sample) (Marinaki et al., 2016). Equation 3-4 used to determine titratable acidity is expressed below.

$$Lactic\ acid\ \%\ (m/m) = \frac{mL\ NaOH \times 90 \times Normality\ of\ NaOH}{Weight\ of\ yoghurt\ and\ water \times 1000} \times \frac{Weight\ of\ yoghurt\ and\ water}{Weight\ of\ yoghurt} \times 100 \quad \text{Equation 3-4}$$

where 90 is the molecular weight of lactic acid, the normality of 0.1 M NaOH is 0.1 N, and the 1000 factor accounts for the unit conversion of mL to L. To account for the addition of water to the yoghurt sample, a mass conversion factor was used of the total weight to the weight of the yoghurt alone.

3.6 Mageu preparation

3.6.1 Maize-meal preparation and heating

Before beginning fermentation, 500 mL of municipal water was added to each 500 mL Schott bottle and was autoclaved at 121°C for 20 minutes to sterilize the bottle and water. These are the standard conditions used for autoclaving throughout the experiments. The sterile water was left at room temperature (21°C) overnight.

The water bath was heated to 30°C (just above room temperature), while the maize meal-water mixture was prepared. Then, the Schott bottles containing the sterile water were heated in the microwave for 30 seconds to heat the water slightly above ambient temperature. For the South African traditional mageu method, 8% (w/v) of maize meal is added to water (Fadahunsi and Soremekun, 2017). However, at this weight percentage, the total solids content of the initial mageu products produced in this study was below the South African National Standard for the production of mageu minimum requirement of 8% (m/m) (South African Bureau of Standards, 2011). Therefore, the mass percentage of maize meal added was increased to 12% (w/v) to ensure the total solids content was above the minimum 8% (m/m). This was achieved by adding 60 g of White Star maize meal to the 500 mL water in each Schott bottle, using a sterilized stainless steel measuring cup, stainless steel spoon, and funnel.

The maize meal-water mixture was heated in the water bath, with the temperature of the water bath adjusted to 90°C. The maize meal-water mixture took roughly 50 minutes to reach 85°C. At this temperature, the mixture was stirred occasionally for 15 minutes using sterile stainless-steel spoons (Mashau et al., 2020). Heating to this temperature served to pasteurise the mixture while cooking and thickening to form a porridge (Holzapfel and Taljaard, 2004; Idowu et al., 2016; Mashau et al., 2020). After 15 minutes, the bottles were removed from the water bath and left to cool to between 35°C and 40°C before inoculating (Mashau et al., 2020).

3.6.2 Inoculum addition

Single *Lactobacillaceae* strains were added to each maize-water mixture to achieve a starting concentration of 10^7 cfu/mL. For the negative control mageu, no inoculum was added to the maize-water mixture. For the positive control, 5% (weight/weight maize meal) wheat flour was added as the inoculum (Fadahunsi and Soremekun, 2017; Haard et al., 1999; Mashau et al., 2020). During inoculation, the bottles were mixed thoroughly to ensure good distribution of the inoculum. After that, the bottles were not mixed again.

3.6.3 Monitoring fermentation

Before inoculating the maize-water mixture, a sample was used to measure pH. Sterile tips were used for pipetting a small sample of the mixture into a test tube. The pH of the test tube sample was then measured using a calibrated Lasec benchtop 50+ DHS pH meter. After inoculation, pH was measured again and noted as time 0. After that, pH was measured hourly for 12 hours from hours 12 to 24 (based on the critical times identified in a trial run with continuous pH monitoring), then every 12 hours for 144 hours or until a pH of 3.5 was reached, whichever occurred first. At the end of fermentation, the samples were placed in the fridge at 4°C until analysis (Dave and Shah, 1998).

3.7 Mageu analysis

3.7.1 Qualitative analysis

Qualitative analysis was conducted by a sensory analysis and investigating consistency and smell, and any other sensory characteristics. As for the yoghurt samples, consistency was analysed first. However, the mageu bottles were homogenised first through shaking before being poured into a beaker. Store-

bought mageu contains clumps of maize meal as this is characteristic of the maize porridge produced before fermentation. Therefore, the mageu samples were analysed rather for differences in viscosity. The consistency of the samples was then rated on a scale. The scale included very thin, thin, slightly thick, thick, very thick, and extremely thick. For samples classified as very thin, the viscosity was similar to that of water, whereas an extremely thick sample meant that the sample could not be readily poured without using force.

The samples were then smelled and rated on a sourness scale. The sourness scale ranked the samples from neutral, followed by a level of 1 and all the way to a level of 5. If a yeast smell, similar to that experienced in a brewery, was present, this was also noted. During this process, if any other characteristics were observed, without having to taste the samples, they were also recorded. For example, when opening the fermentation bottles any evidence of gas release was noted.

3.7.2 Titratable acidity

The same method was used for measuring titratable acidity for mageu as was used for yoghurt. Equation 3-5 was used to determine titratable acidity:

$$Lactic\ acid\ \%\ (m/m) = \frac{mL\ NaOH \times 90 \times Normality\ of\ NaOH}{Weight\ of\ mageu\ and\ water \times 1000} \times \frac{Weight\ of\ mageu\ and\ water}{Weight\ of\ mageu} \times 100 \qquad \text{Equation 3-5}$$

3.7.3 Total solids content

The total solids content was measured at the end of each fermentation experiment. First, the mass of an aluminium evaporating dish was measured using an analytical balance. Then, the mass of the mageu sample was recorded and added to the evaporating dish. The evaporating dish was placed in an 80°C oven overnight after which the mass of the evaporating dish containing the dried mageu sample was measured. The total solids content was measured in triplicate for each mageu product (per 500 mL bottle). The total solids content was determined using the South African National Standard's adapted equation for mageu production given by Equation 3-6 below (South African Bureau of Standards, 2011).

$$Total\ solids\ content\ \%\ (m/m) = \frac{m_1 - m_2}{m_3} \qquad \text{Equation 3-6}$$

where m_1 is the mass of the dried solids and the evaporating dish, m_2 is the mass of the evaporating dish, and m_3 is the mass of the liquid mageu sample.

3.7.4 Lactic acid and ethanol concentration

Sample preparation

Lactic acid and ethanol concentrations in the mageu products were quantified using high-performance liquid chromatography (HPLC). The method by Pswarayi and Gänzle (2019) was used to prepare the samples for analysis. First, solids were removed by centrifugation (Eppendorf centrifuge 5418 R). Since Pswarayi and Gänzle (2019) do not give conditions for the centrifugation, conditions used by Dysvik et al. (2020) for sour beer were consulted. Dysvik et al. (2020) centrifuged samples at 1,470 x g for 15 minutes. To adjust these conditions for mageu, a centrifuge step of 13,000 rpm for 15 minutes was used in the current study. At these conditions, the solids separated well from the supernatant. Following centrifugation, the supernatant was mixed in a volumetric ratio of 1:1 with 7% perchloric acid (70% Merck, 244252) (Pswarayi and Gänzle, 2019). The 70% perchloric acid was diluted ten-fold using ultrapure water to obtain a purity of 7%. Next, the mixture was vortexed before being placed in the fridge (4°C) overnight to allow the proteins to precipitate. The mixture was then centrifuged at the same conditions, and the proteins were removed. The samples were stored at -20°C until analysis.

From storage at -20°C, the mageu samples were mixed in a volumetric ratio of 1:1 with mobile phase for a dilution factor of 2. The diluted samples were then vortexed before they were filtered through a

0.22 µm syringe filter into the HPLC vials. The samples in HPLC vials prepared in advance (the day before) were stored in the 4°C fridge until analysed.

Column operating conditions

The column used to analyse organic acids and sugars was the Bio-Rad Aminex HPX-87H ion exclusion column (300 mm × 7.8 mm). The mobile phase used was 5 mM sulphuric acid (Kimix, SUL001) (Pswarayi and Gänzle, 2019). Standards of lactate and ethanol with concentrations varying from 0.1 g/L to 10 g/L were prepared using sodium lactate (Merck, L4263) and ethanol absolute (Merck, 1.07017) and diluted with the mobile phase. The operating conditions for the column were a flow rate of 0.5 mL/min and a temperature of 40°C. The organic acids and sugars were quantified through refractive index and UV detection, operating at a wavelength of 210 nm.

Calculating concentrations

The results were analysed to quantify the lactate and ethanol concentrations. The concentration standards were used to plot a standard curve against the integration area calculated by Chromeleon (version 7), the software used for HPLC analysis. The standard curves were used to convert the integration area of the unknown samples to a concentration in g/L for lactate and ethanol. The dilution factors were also accounted for when calculating the ethanol and lactate concentrations. Once an initial concentration was obtained using the standard curve, the concentration was multiplied by the perchloric acid dilution factor of two and the mobile phase dilution factor of two. These calculations are presented in Appendix D.

3.7.5 Enumerating bacteria and yeast cells

Cell counts were performed through spread plating. For enumerating *Lactobacillaceae* strains and yeasts, MRS plates and YPD plates were used, respectively. The YPD media was prepared using the method described in Section 3.3.2.

A ten-fold dilution series of the culture was performed by first pipetting 0.9 mL of sterile PBS in each Eppendorf tube. The culture sample (either mageu, *Lactobacillaceae* inoculum or yeast diluted in YPD media) was vortexed before 0.1 mL of the sample was pipetted into the first Eppendorf tube. After that, 0.1 mL of the increasingly diluted culture was transferred into each consecutive Eppendorf tube, with mixing in between. Of the serial dilutions, three appropriate dilutions were selected for plating, and 0.1 mL of diluted culture was pipetted onto the centre of an agar plate. The most common dilutions used were 10^4, 10^5 and 10^6. The L-shaped rod stored in 70% ethanol was passed through the Bunsen burner flame and allowed to cool before being used to spread the microbial sample evenly over the agar plate.

The MRS plates were then placed in a container with an Anaero-Pak (Davies Diagnostics, MGC-PACK-10-01) to create an anaerobic environment. The container was incubated at 37°C. The YPD plates were grown aerobically and incubated at 30°C. Colonies on the plates were counted after 24-48 hours, depending on the rate of growth. The final colony count per mL was determined using Equation 3-7.

$$N_T = Number\ of\ colonies\ \times D \times P_f \quad \text{Equation 3-7}$$

where N_T is the total number of viable colonies in colony forming units per mL (cfu/mL), D is the dilution factor (10^4 for example), and P_f is the plating factor (10 since 0.1 mL was plated).

3.7.6 Measuring shelf-life

On the same day fermentation was terminated (day 0), each mageu product in a 500 mL bottle was transferred into two sterile glass bottles. One bottle was placed in the 4°C fridge and the other on a shelf in the laboratory maintained at room temperature of 21°C. Post-fermentation microbial activity in the mageu samples was determined by measuring the pH (Lasec benchtop 50+ DHS) of both the refrigerated and room temperature samples after 7 days.

3.7.7 Testing for contamination

When producing the mageu samples to be used in the sensory analysis, the final products were tested for *E.coli* and *Clostridium* presence as required by the South African National Standard for mageu production (South African Bureau of Standards, 2011). A ten-fold dilution series was performed for each mageu sample using sterile PBS solution (Sigma, 806544). The diluted cultures were then plated on MacConkey agar containing crystal violet, sodium chloride, and 0.15% (w/v) bile salts (Merck, M8302) to test for the presence of *Enterobacteriaceae*. The plates were incubated aerobically at 37°C for 48 hours.

To test for contamination by anaerobic spore forming organisms, the mageu dilutions were heat-treated at 80°C for 12 mins, before being plated on brain heart infusion (BHI) (Sigma, 53286) agar plates. The BHI plates were then incubated anaerobically (85 N_2, 10% CO_2, 5% H_2) at 37°C for 48 hours in an anaerobic workstation (Concept 1000, Baker Ruskinn). Where growth on the BHI plates was observed, single colonies were sub-cultured into fresh BHI agar and incubated aerobically at 37°C for an additional 24 hours to determine susceptibility to oxygen.

3.8 Sensory analysis

The sensory analysis used an untrained consumer panel to analyse the mageu samples (Lawless and Heymann, 2010). This type of testing is classified as hedonic, where the degree of liking is investigated. The requirement for participation was either current or previous regular consumption of mageu. Regular consumption was defined as consuming mageu a minimum of once every two months. The panellists were students from the UCT, recruited via email following ethics approval by the Engineering and Built Environment Ethics Committee and approval from the Department of Student Affairs. Ethics approval documents and the email for recruiting participants are present in Appendix B. Until analysis and following fermentation, the mageu samples were stored in the fridge at 4°C.

Participants were limited to taste six samples so as not to create sensory overload. Five samples were produced as per the method described in Section 3.6. The sixth sample was a commercially available product (Mageu Number 1 cream flavour). This flavour was selected as it most closely resembled the laboratory-produced samples in terms of smell, flavour and consistency. Before the participants entered the venue, between 15 mL to 20 mL of each sample had been poured into separate cups labelled by randomised three-digit codes. The codes were changed for each tasting day so participants could not speak to one another and form biases before tasting the samples. The informed consent document, questionnaire, pen, and glass of water were also placed on the desk. Participants were also given spoons as some samples were very thick. Each participant was given the five laboratory-produced samples in a random order, with the code order corresponding to that written on their questionnaires (Lawless and Heymann, 2010). For all participants, the commercial mageu sample was placed consistently last. This is because the commercial sample contains additives, including sugar and flavouring. Therefore, the taste of sugar from the commercial sample would have skewed the tasting of the other samples which did not contain sugar.

The questionnaire used a 9-point Hedonic scale analysing degree of likeness and likelihood of purchasing a sample (Lawless and Heymann, 2010). For the degree of likeness, participants were asked to score each sample on a scale of 'like extremely' (given a score of 9), to 'dislike extremely' (given a score of 1). For the degree of likelihood to purchase, participants scored each sample from 'definitely would buy' (given a score of 5) to 'definitely would not buy' (given a score of 1). Examples of the questionnaires are presented in Appendix A.

Before the taste testing, participants were made aware that participation was voluntary and informed about the project. All participants were then required to sign an informed consent document before they began tasting. Next, an explanation for how to fill out the questionnaire and perform the taste test was given. The explanation was to taste the samples in the order they were placed from left to right. The participants were asked to taste the first sample, answer the two questions on the questionnaire for that

sample alone, and then take a sip of water before moving on to the next sample. This process was repeated for all six samples. After the tasting, each participant was given a small gift and thanked.

To ensure all South African lockdown COVID-19 protocols were adhered to; the following measures were implemented. A contact tracing register was kept for participants in the same time slot. All participants were asked to show a green UCT Health Screening Result (signifying absence of COVID-19 symptoms and low recent exposure risk) before the tasting. A maximum of ten participants were booked for one time slot. Testing stations (desks and chairs) were placed a minimum of 1.5 m from one another and sanitised before and after the tasting. Venues were aired between time slots. Participants were asked to keep their masks on at all times and only to remove them when tasting. At the end of the tasting session, all participants were asked to inform the researcher via email if they began to display COVID-19 symptoms within 48 hours after the tasting.

3.9 Statistical analysis

To statistically determine outliers from the data, the interquartile range (IQR) was used. The lower and upper quartiles were determined using the EXCEL quartile functions. The interquartile range is then determined using the equation below.

$$Interquartile\ range\ (IQR) = Upper\ quartile - Lower\ quartile \quad \text{Equation 3-8}$$

Outliers were then determined by multiplying the IQR by 1.5 and subtracting that value from the lower quartile to get the lower boundary or adding it to the upper quartile to get the upper boundary.

$$Lower\ outlier = Lower\ quartile - 1.5 \times IQR \quad \text{Equation 3-9}$$

$$Upper\ outlier = Upper\ quartile + 1.5 \times IQR \quad \text{Equation 3-10}$$

To determine statistical significance, two-tailed t-tests were used and analysed at a significance level of $p<0.05$.

4 Mageu Results and Discussion

This chapter will assess the ability of the various bacterial strains to ferment maize porridge to mageu. The bacterial strains will be evaluated as both single cultures, and mixed cultures with *S. cerevisiae* (from Anchor Yeast). The positive control was produced using wheat flour as the inoculum source, while no active inoculum was added to the negative control. The fermentations were monitored by measuring pH followed by product analysis through measuring titratable acidity, lactic acid and ethanol concentrations, total solids content, microbial cell counts, qualitative analysis, and shelf-life. Four probiotic mageu samples, a positive control and a commercial mageu sample (Mageu Number 1) were investigated for their consumer acceptability using a sensory panel. The mageu samples are evaluated on their 'degree of liking' and 'likelihood to purchase'.

4.1 Fermentation pH profiles and length

The fermentation trajectory and length, quantified as the change in pH with time, were determined for the various mageu fermentation experiments. In this section, the pH reduction profiles of the mageu produced using various bacteria are presented, followed by a comparison of the bacterial strains as mixed cultures with yeast. Lastly, the individual strains are presented, both as pure and mixed cultures, to compare how the various fermentation profiles differ with the addition of yeast.

As mentioned in Section 3.6.3, mageu fermentation was terminated at hour 144 or when a pH of 3.5 was reached, whichever occurred first. However, due to the South African COVID-19 pandemic restrictions, pH could not be measured continuously over the entire 144 hours. Therefore, fermentation was terminated between a pH of 3.43 and 3.57. All fermentations were performed in at least quadruplicate (duplicates were performed per run).

4.1.1 Single strain Lactobacillaceae fermentations

In this section the various *Lactobacillaceae* isolates as pure fermentation cultures are compared based on their ability to ferment maize meal to mageu by investigating pH over fermentation time. The pH reduction profiles for the fermentation experiments using the pure strains are presented in Figure 4.1 and the fermentation times and final pH values for the strains are presented in Table 4.1.

At the start of the fermentations, the initial pH ranged from 6.03 to 6.14. This result is within the range (pH 5.7 to 6.3) proposed by the authors of the mageu patent (Fourcassie et al., 2017). A distinctive longer lag phase was observed for the negative control. This is expected as no active inoculum was provided. However, the pH did still decrease. Therefore, heat treatment before inoculation does not remove all naturally occurring microorganisms present in the maize meal. For example, spore-forming *Bacillus* spp., which would not have been killed during heat treatment, may have been present in the maize. Alternative sources of microorganisms may have been from the water or implements used, although this is less likely as these were sterilized through autoclaving. Otherwise contamination may have occurred during sampling when the bottles were opened to obtain a small sample to test for pH. The negative control also had the largest variance among the samples, as shown by the standard deviation displayed as error bars. This is most probably because the microorganisms present in the negative controls underwent an uncontrolled and spontaneous fermentation and would have had varying initial microbial loads, compared to the *Lactobacillaceae* isolates that were added at a pre-determined concentration.

L. crispatus 70.6, *L. crispatus* 73.55, *L. mucosae* 90.13, and *L. gasseri* 94.98 fermentations displayed similar drops in pH between hours 12 and 24, with the plots closely overlapping. The positive control also achieved very similar pH values compared to these four pure strains. However, the *L. jensenii* 95.1 fermentation had noticeably higher pH values during those same hours. By hour 60, all fermentations had nearly plateaued and subsequently decreased only slightly to reach their final pH.

From hours 24 to 60, *L. crispatus* 73.55 recorded consistently lower pH values than all other pure strains and controls. However, from hour 72 until the end of fermentation, the positive control matched the pH of the *L. crispatus* system. In fact, from hour 36 until the end of fermentation, the positive control had the steepest decrease in pH, though its pH was unchanged between hours 24 and 36. The steep drop in pH relative to the negative control is indicative that the *Lactobacillaceae* strains facilitate mageu fermentation to the same degree as the positive control. After that, the pH reduction rate slowed, indicating that the metabolites causing the pH drop were no longer being produced at their maximum rate. While the bacterial strains are able to tolerate low pH and continue to lower the pH, microbial growth rate of the strains decreases as the pH decreases to pH 3.5 (Happel et al., 2020). Specifically, *L. gasseri* 94.98, *L. crispatus* 70.6 and *L. mucosae* 90.13 exhibited growth rates roughly 50% lower when the growth media pH was decreased from 4 to 3.5 (Happel et al., 2020). The final pH of 3.5 for mageu is lower than the optimal vaginal and growth pH between 4 and 4.5 for the isolated bacterial strains; and so, the lower mageu pH possibly inhibits further growth of the bacterial strains.

Figure 4-1 Plot of the decrease in pH over mageu fermentation time for the five pure *Lactobacillaceae* strains, negative control, and positive control

The average fermentation times of each of the pure strain fermentations and the controls are given in Table 4-1. *L. crispatus* 73.55 had the shortest average fermentation time of 78 hours, followed by the positive control at 87 hours. The remaining four isolates had longer fermentation times. Surprisingly, the negative control had a shorter average fermentation time than the other four isolates, despite the initial lag in the pH decrease. It would have been expected that all the *Lactobacillaceae*-produced samples would have shorter fermentation times due to the higher microbial loads in the inoculum. However, it is not known what naturally-occurring microorganisms were present in the negative control that would have been outcompeted by the added active inocula in the probiotic mageu samples. In the pure stain samples, the rapid initial fermentation may have decreased the microbial load of the naturally-occurring microorganisms present in the maize meal that were not removed through heat treatment. It is possible that the microorganisms in the negative control were still able to ferment the maize porridge by producing acidic metabolites. The results from Happel et al. (2020) showed that of the five bacterial strains, *L. gasseri* 94.98 had the highest growth rate at a pH of 3.5. However, of the five strains, *L. crispatus* 73.55 and *L. jensenii* 95.1 had very little change in their growth rates when the pH was reduced from 4 to 3.5, compared to the other strains with more noticeable decreases. Additionally, Happel et al. (2020) states that the *L. crispatus* species were able to lower the pH of the growth media the most of all the species.

Mageu produced with wheat flour by Idowu et al. (2016) achieved a pH of 3.30 in 36 hours and their pure *L. brevis* culture obtained a pH of 3.57 within 36 hours. Their fermentations were therefore shorter compared to this study's positive control which reached a pH of 3.5 at 87 hours. The method used to produce the mageu in this study was consistent with Idowu et al. (2016). However, the inoculum size used by Idowu et al. (2016) was in the magnitude of 10^8 cfu/mL, approximately 10-fold higher than that used in this study. The inoculum concentration used in this study was calculated as 10^7 cfu/mL using OD measurements collated with cell counts of the *L. jensenii* 95.1 culture as a basis. However, when performing colony counts during inoculation, the cell counts of the bacterial strains were found to be in the magnitude of 10^5 cfu/mL for *L. crispatus* 73.55 and 10^6 cfu/mL for the other four strains, including *L. jensenii* 95.1. The five strains showed different morphologies during inspection under the microscope; and so, it is not unexpected that the cell concentration of the strains would differ for the same optical density. The microscopic inspection was performed at the Faculty of Health Sciences during a previous project (Happel, 2018).

Despite having the lowest inoculum cell concentration, *L. crispatus* 73.55 still performed best of the five single strains. Since Idowu et al. (2016) obtained the ideal mageu final pH values in a shorter time with a larger inoculum size, increasing the initial cell concentration used in this study could potentially decrease fermentation time. On the other hand, Pswarayi and Gänzle (2019) used an inoculum concentration of 10^6 cfu/mL per bacterial strain, but used a mixed culture of five bacterial strains. The final pH achieved by this consortium was 3.5 within 24 hours. Therefore, using mixed cultures could also potentially decrease the fermentation time.

Table 4-1 Average fermentation time for the single strain-produced mageu, negative control and positive control

Sample	Average Fermentation Time (hour)	Final pH
Single strain *L. crispatus* 70.6	144 ± 0	3.66 ± 0.11
Single strain *L. crispatus* 73.55	78 ± 26	3.53 ± 0.01
Single strain *L. mucosae* 90.13	144 ± 0	3.61 ± 0.03
Single strain *L. gasseri* 94.98	141 ± 5	3.60 ± 0.06
Single strain *L. jensenii* 95.1	135 ± 10	3.54 ± 0.03
Negative Control	94 ± 14[1]	3.54 ± 0.02
Positive Control	87 ± 10	3.52 ± 0.02

Fermentation times are displayed as means ± standard deviations from duplicate or triplicate independent experiments performed in duplicates.

[1] The negative control had a shorter average fermentation time than the isolates, possibly due to the native microorganisms present in the maize meal that were not inactivated during heat treatment.

4.1.2 Comparison of fermentations using single strain Lactobacillaceae with and without yeast

The fermentation pH profiles using *L. crispatus* 70.6 as a single strain and with yeast are presented in Figure 4-2a. The most noticeable difference between the two fermentation pH profiles is the decrease in fermentation time with the yeast addition, and that the mixed culture sample was able to reach a pH of 3.5, while the single culture sample could not. After inoculation with the *L. crispatus* 70.6 and yeast, the pH dropped instantaneously, which did not happen for the pure *L. crispatus* 70.6 fermentation. This is investigated further in Section 4.1.3 where all the mageu samples produced by the mixed cultures are discussed. The fermentation with yeast also had a steeper drop in pH between hours 12 and 24, which ultimately allowed the final pH of 3.5 to be reached quicker than the end point of fermentation (pH of 3.66 ± 0.11) of the pure strain sample. Furthermore, the sample with the pure strain had greater variance, denoted by the standard deviation error bars, than the sample produced with *L. crispatus* and yeast.

Since pH is a measure of the concentration of hydronium ions, Figure 4-2b is complementary to the pH plot and shows the average concentration of the hydronium ions over fermentation length. A linear increase in hydronium ion activity is observed between hours 12 and 60 for the mixed culture fermentation, where hour 60 corresponds to the point at which a pH of approximately 3.5 was reached. A steeper increase in hydronium activity is observed for the mixed cultured mageu compared to the single culture fermentation. Furthermore, hydronium ion production began to slow at around hour 72 for the pure *L. crispatus* 70.6 mageu.

It is possible that the addition of yeast increased the metabolic activity of *L. crispatus* 70.6 to produce more pH-reducing metabolites, or the yeast itself produced pH-reducing metabolites, or a combination of both. Either way, the addition of yeast increased the overall hydronium ion production, which caused a faster pH reduction, thereby reducing fermentation time compared to the fermentation of the pure *L. crispatus* 70.6 culture. As mentioned in 2.4.1, achieving a lower pH more quickly is preferable as the lower pH provides protection from pathogens.

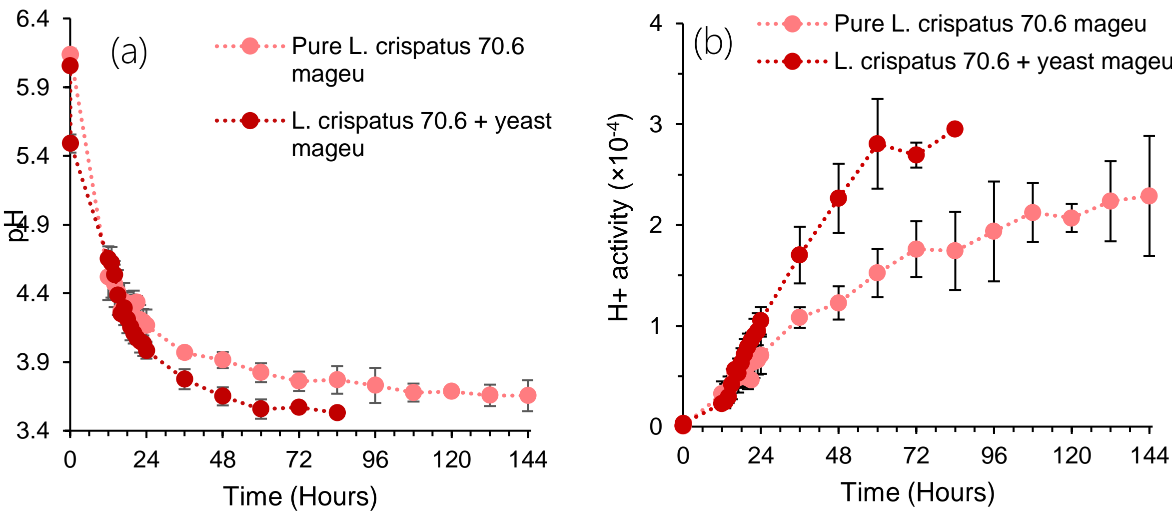

Figure 4-2 Plots of (a) pH over fermentation time and (b) hydronium ion activity over fermentation time for mageu produced using *L. crispatus* 70.6, both for the pure strain and with yeast

The fermentation profiles of mageu produced by *L. crispatus* 73.55 as a pure culture and with yeast are presented in Figure 4-3a. Unlike in the case of *L. crispatus* 70.6 (Figure 4-2a), the mageu fermentation using *L. crispatus* 73.55 with yeast did not have a consistently lower pH than the pure strain sample during the fermentation hours of 12 and 24. After hour 20, the two *L. crispatus* 73.55 systems' pH values overlapped. However, the sample with yeast reached a pH of 3.5 significantly earlier ($p<0.05$) than the pure sample, despite there being little difference in the pH values from hours 60 onwards. Also different

to the *L. crispatus* 70.6 fermentations, both *L. crispatus* 73.55 systems had similar variance in their pH values during fermentation (expressed as standard deviation). Inter-run differences with respect to fermentation time were observed for pure culture *L. crispatus* 73.55 fermentations. For two independent runs performed in duplicate, the mageu produced by the pure culture had an average fermentation time of 60 hours. However, for the third independent run performed in duplicate, the average fermentation time was 114 hours. By contrast, the samples produced with yeast were more consistent. Two runs performed in duplicate had an average fermentation time of 58 hours, compared to the third in duplicate of 42 hours.

The hydronium ion activity plot for the mageu samples produced using *L. crispatus* 73.55 is displayed in Figure 4-3b. Up to hour 24 the increase in hydronium ions was the same for both the mageu produced by the pure and mixed *L. crispatus* 73.55 cultures. However, between hours 24 and 48, an increase in the hydronium ion production rate was observed for the mixed culture mageu relative to the pure culture, followed by stabilizing of the hydronium ion activity. For the pure *L. crispatus* 73.55 mageu, the rate of change of hydronium ion activity began to slow at hour 72. Similarly to the *L. crispatus* 70.6 with yeast mageu, it was the sharper increase in hydronium ion activity of the *L. crispatus* 73.55 with yeast mageu which meant the final pH was reached earlier than for the pure culture mageu.

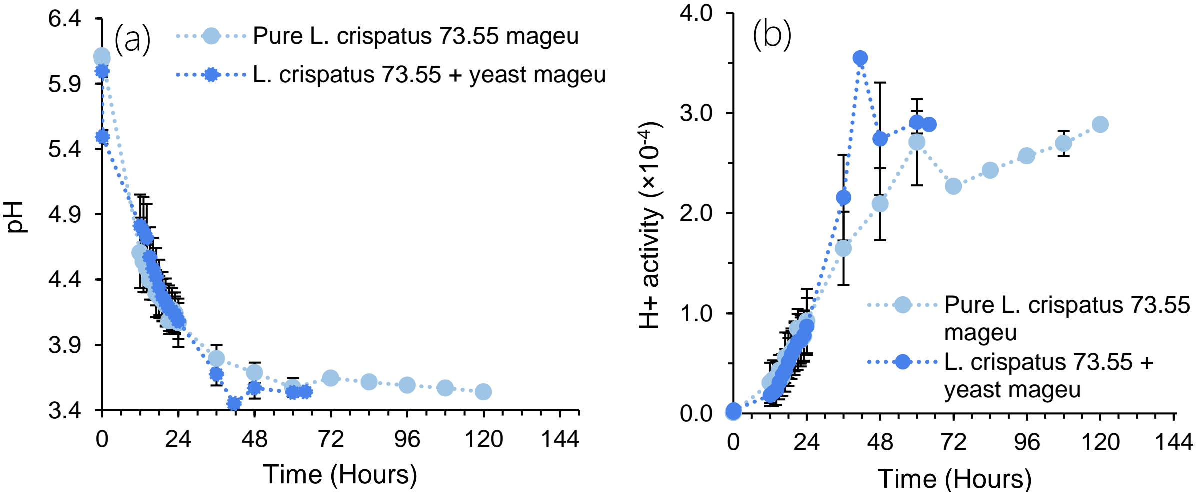

Figure 4-3 Plots of (a) pH over fermentation time and (b) hydronium ion activity over fermentation time for mageu produced using *L. crispatus* 73.55, both for the pure strain and with yeast

The fermentation pH profiles for pure strain *L. mucosae* 90.13 mageu and *L. mucosae* 90.13 with yeast mageu are displayed in Figure 4-4a. The fermentation plots of *L. mucosae* 90.13 as a pure strain and with yeast have two different patterns, unlike the two *L. crispatus* strains. The plot of the pure *L. mucosae* 90.13 fermentation, like all the other pure isolates, has a decaying exponential shape. However, the plot of the *L. mucosae* 90.13 with yeast fermentation displays a concave downward shape, opposite to the pure strain fermentation plot. Minimal variance was observed for the fermentations with yeast (small error bars) between hours 12 and 24. Therefore, the convex downward shape could be characteristic of this strain and not due to an outlying run. It is possible that the initial drop in pH may have slowed the growth of *L. mucosae* 90.13, which would have limited acid production. However, after 24 hours the *L. mucosae* 90.13 cells may have reached a particular relative abundance which would have favoured further growth and acid production, enough to drop the pH below that of the pure strain sample. Due to this concave downward plot, the pH between hours 12 and 24 for the samples with yeast was consistently higher than the pure strain fermentation. Around hour 30, the pH of the samples with yeast dropped lower than the pure sample. The sample with yeast reached a pH of 3.5 at hour 64, whereas the pure sample did not reach a pH of 3.5 within 144 hours.

The complementary plot to the plot of pH over fermentation time is the hydronium activity over time, displayed in Figure 4-4b. After hour 24, an exponential increase in hydronium ion activity was observed

for the mixed culture mageu with *L. mucosae* 90.13 and yeast. If hydronium activity were constant across all cells, then the hydronium activity could represent 'normal' exponential linear growth of the microbial cells. By contrast, more linear hydronium ion activity trends, as observed for the *L. crispatus* strains, may indicate limited microbial growth since only consistently steady growth and no accelerated growth at the beginning of fermentation was observed. Between hour 60 and 64, there was no change in hydronium activity. By contrast the pure *L. mucosae* 90.13 mageu had a steady increase in hydronium ion activity up until hour 144.

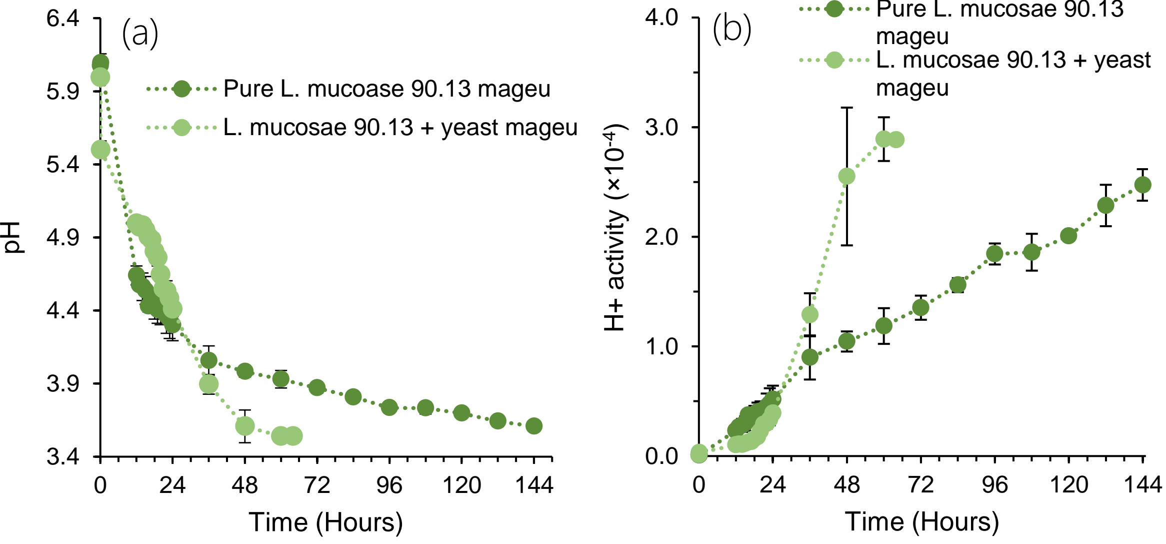

Figure 4-4 Plots of (a) pH over fermentation time and (b) hydronium ion activity over fermentation time for mageu produced using *L. mucosae* 90.13, both for the pure strain and with yeast

The plots of *L. gasseri* 94.98 as a pure strain and with yeast also show different fermentation trends, displayed in Figure 4-5a. The *L. gasseri* 94.98 with yeast mageu sample had a very steep decrease in pH, displaying a linear trend until the final fermentation time of 48 hours. By contrast, the pure *L. gasseri* 94.98 mageu sample saw a sharp initial decrease in pH between time 0 and hour 12 (even more so than the mixed mageu sample). However, the pure strain mageu sample pH reduction rate then slowed and only reached a final pH of 3.60 at hour 144.

The hydronium ion activity plots for the *L. gasseri* 94.98 mageu samples are displayed in Figure 4-5b. Similarly to the *L. mucosae* 90.13 with yeast mageu sample, an exponential trend is observed for the mixed culture while the pure culture has a two-step linear plot. The first linear step is steeper which corresponds to the steeper drop in pH observed in Figure 4-5a. The second linear step is less steep as presumably microbial growth rate and acid production rate had slowed. As mentioned above, *L. gasseri* 94.98 had the highest growth rate of the five bacterial strains at pH 3.5 from the study by Happel et al. (2020). Despite this, the growth rate of *L. gasseri* 94.98 halved when the growth media pH was decreased from 4 to 3.5. Therefore, the lowering of the mageu pH beyond 4 may have inhibited growth of the bacterial strain, causing the slower pH reduction rate from hour 36 onwards. While it appeared that the pH had stabilized for the pure strain mageu sample, there was still evidence of the hydronium ion concentration increasing.

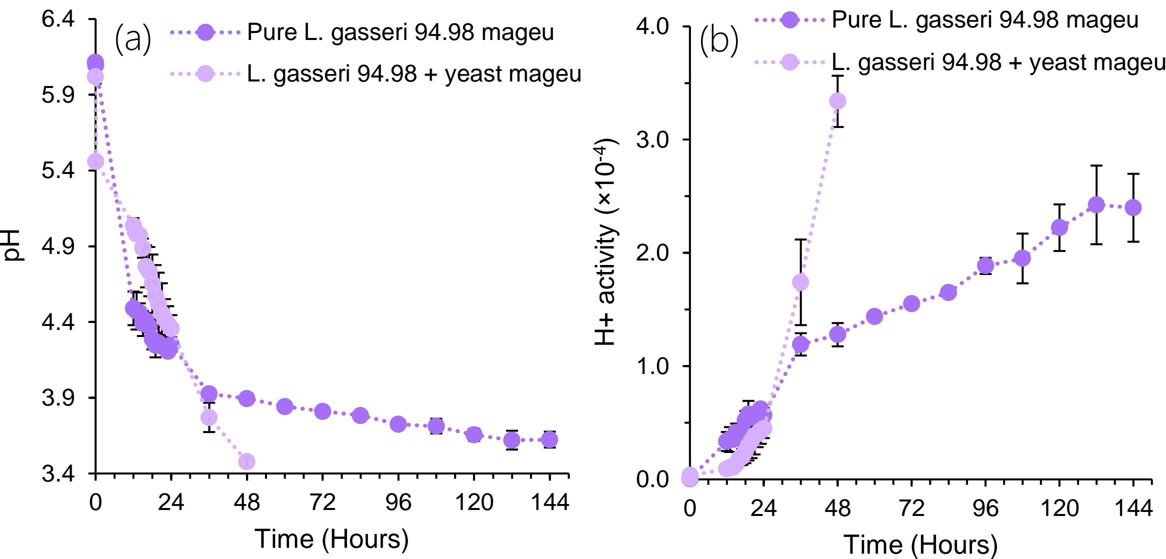

Figure 4-5 Plots of (a) pH over fermentation time and (b) hydronium ion activity over fermentation time for mageu produced using *L. gasseri 94.98*, both for the pure strain and with yeast

The pH fermentation plots for mageu produced with *L. jensenii* 95.1 as a pure culture and mixed culture with yeast are displayed in Figure 4-6a. In this case, the *L. jensenii* 95.1 fermentation pH plots of the pure samples and the samples with yeast exhibited similar kinetics, although the pH values were consistently lower for the latter sample. The *L. jensenii* 95.1 strain had varying results across the three runs performed in duplicate, causing in a relatively high variance. Despite this variance, the final fermentation time for the samples with yeast was consistent at 72 hours. By comparison, the pure *L. jensenii* 95.1 mageu had a final fermentation time of 144 hours with a pH of 3.54. However, it could be argued that the initial drop in pH caused by the addition of yeast at hour 0 is what enabled the sample with yeast to have a shorter fermentation time rather than differences during the fermentations.

The hydronium ion activity plots for the *L. jensenii* 95.1 mageu samples are displayed in Figure 4-6b. While the pure *L. jensenii* 95.1 mageu sample saw a decrease in the pH reduction rate from hour 72, the hydronium ion activity was still steadily increasing. The rate of hydronium ion activity increase for the *L. jensenii* 95.1 sample with yeast was greater than the pure sample later in the fermentation (specifically between hours 60 and 72).

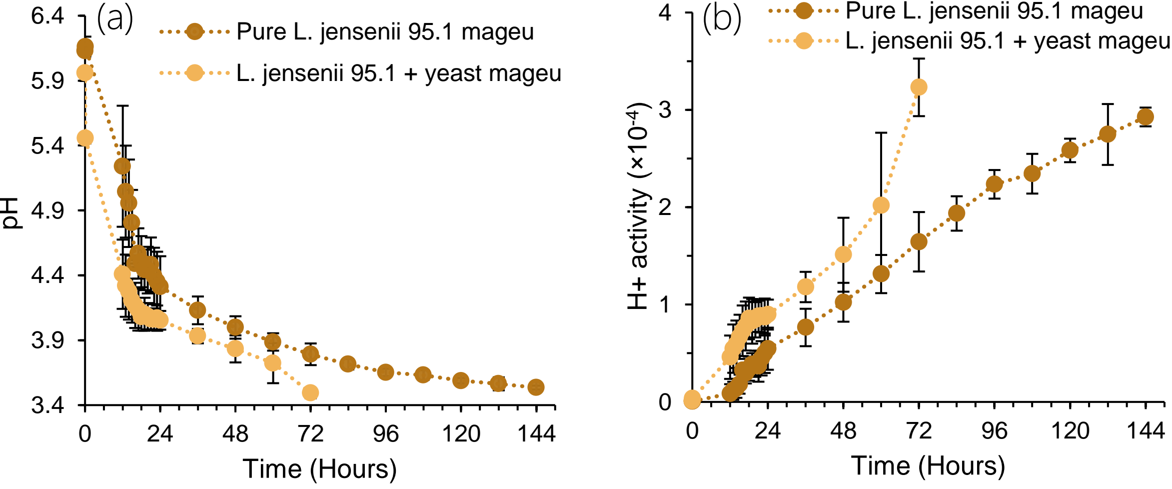

Figure 4-6 Plots of (a) pH over fermentation time and (b) hydronium ion activity over fermentation time for mageu produced using *L. jensenii 95.1*, both for the pure strain and with yeast

To summarise the results presented in Figures 4-2 to 4-6, the average fermentation lengths for all samples are displayed in Figure 4-7. The fermentation lengths across all isolates dropped significantly ($p<0.05$) with the addition of yeast compared to the pure samples. The error bars denoting standard deviation show that the largest variance was observed for the *L. crispatus* 73.55 strain. The variance in fermentation length was not consistently higher for the mageu samples produced with pure strains than those produced with the addition of yeast. This variance found across the mageu fermentations could be attributed to the natural variances that occur when working with live microorganisms and the additional natural inoculums present in the maize meal.

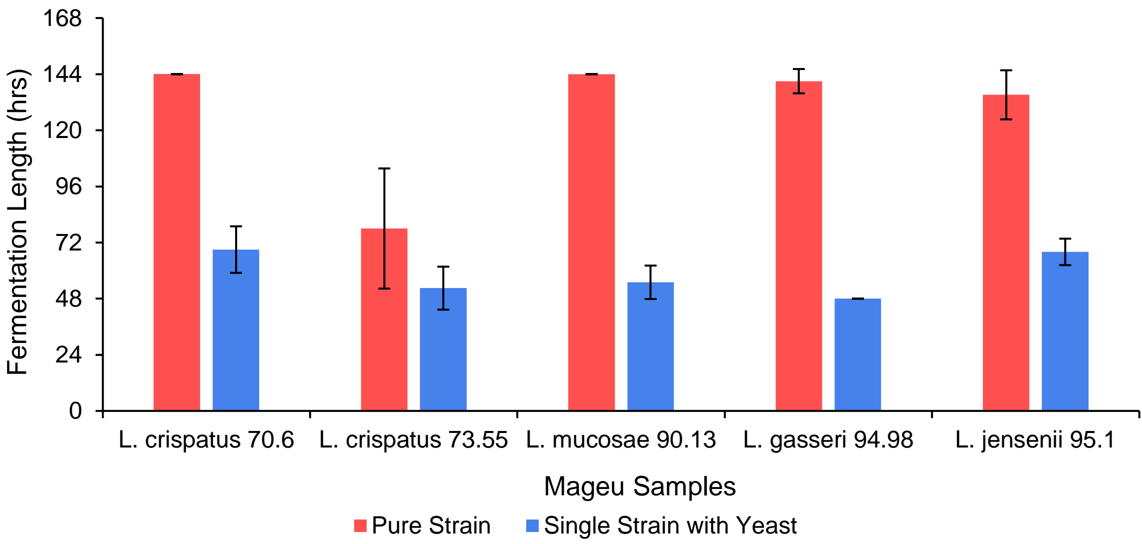

Figure 4-7 Comparison of the average fermentation lengths for the mageu samples produced with pure *Lactobacillaceae* isolates and *Lactobacillaceae* isolates with yeast

4.1.3 Single strain Lactobacillaceae with yeast fermentations

Section 4.1.1 investigated the ability of the individual bacterial strains to ferment maize porridge to mageu. Section 4.1.2 then compared the fermentation profiles of the mageu produced with single strain *Lactobacillaceae* isolates to the mageu produced using mixed cultures. This section compares the pH fermentation profiles of the various mageu samples produced with a single *Lactobacillaceae* isolate and Anchor Yeast to one another. The pH reduction profiles for the fermentation experiments with the mixed cultures are presented in Figure 4.8 and final pH values for the strains are presented in Table 4.2.

As for Figure 4-1, Figure 4-8 represents data gathered from at least two independent runs of the fermentations with each pure strain with yeast, for which each run was performed in duplicate. Compared to the single strain mageu results, the fermentations by the single strains with yeast had a greater spread of pH values over fermentation time. At the beginning of the fermentations, an instantaneous drop in pH was recorded once the inoculum was added. Before inoculation, the pH was between 5.96 and 6.03. Immediately after inoculation, the pH dropped to between 5.46 and 5.50. Since this drop in pH was not observed in the pure strain mageu experiments nor the controls, it can be assumed that it was caused by the addition of the yeast. Specifically for the mixed culture sample with *L. jensenii* 95.1 and yeast, this initial sharp drop in pH meant that the pH of the mixed culture sample was consistently lower than the pH of the pure *L. jensenii* 95.1 mageu sample.

The pH after inoculation was consistent across the various bacterial strains. Between hours 12 and 24, all plots including the negative and positive controls had very similar gradients; however, *L. jensenii* 95.1 mageu and *L. crispatus* 70.6 mageu had the lowest pH values, closely followed by *L. crispatus* 73.55 mageu and the positive control. After hour 24, a steep pH reduction occurred for *L. crispatus*

73.55 mageu and *L. gasseri* 94.98 mageu, reaching the lowest pH of all the mixed cultured mageu samples.

The mageu produced with the bacterial strains and yeast reached their pH end point earlier than the pure strain mageu samples. This is because the pH reduction rate remained high throughout fermentation. After hour 48, following the rapid drop in pH, the pH of *L. crispatus* 70.6 mageu, *L. crispatus* 73.55 mageu, and *L. mucosae* 90.13 mageu only changed gradually. The mageu produced by *L. crispatus* 73.55 with yeast showed a dip in pH between hours 40 and 60 before increasing again. This is because one of the samples had a shorter fermentation time of 42 hours compared to the other runs. A slower rate of change was not observed for the *L. gasseri* 94.98 and *L. jensenii* 95.1 mageu samples. Most notably, the sample *L. gasseri* 94.98 with yeast had overall the shortest fermentation time compared to the other strains with yeast. However, all *Lactobacillaceae* strains with yeast had shorter fermentation times than the positive and negative controls, which was different to that observed with the pure strain mageu. This pattern is different from that observed by Idowu et al. (2016), who achieved the lowest pH at 3.30 after 36 hours with the positive control produced with wheat flour. Their mixed culture of *L. brevis* and yeast (*S. cerevisiae*) achieved the next lowest pH of 3.54 after 36 hours. Again, this shorter fermentation time could be attributed to the larger inoculum size than the one used in this study.

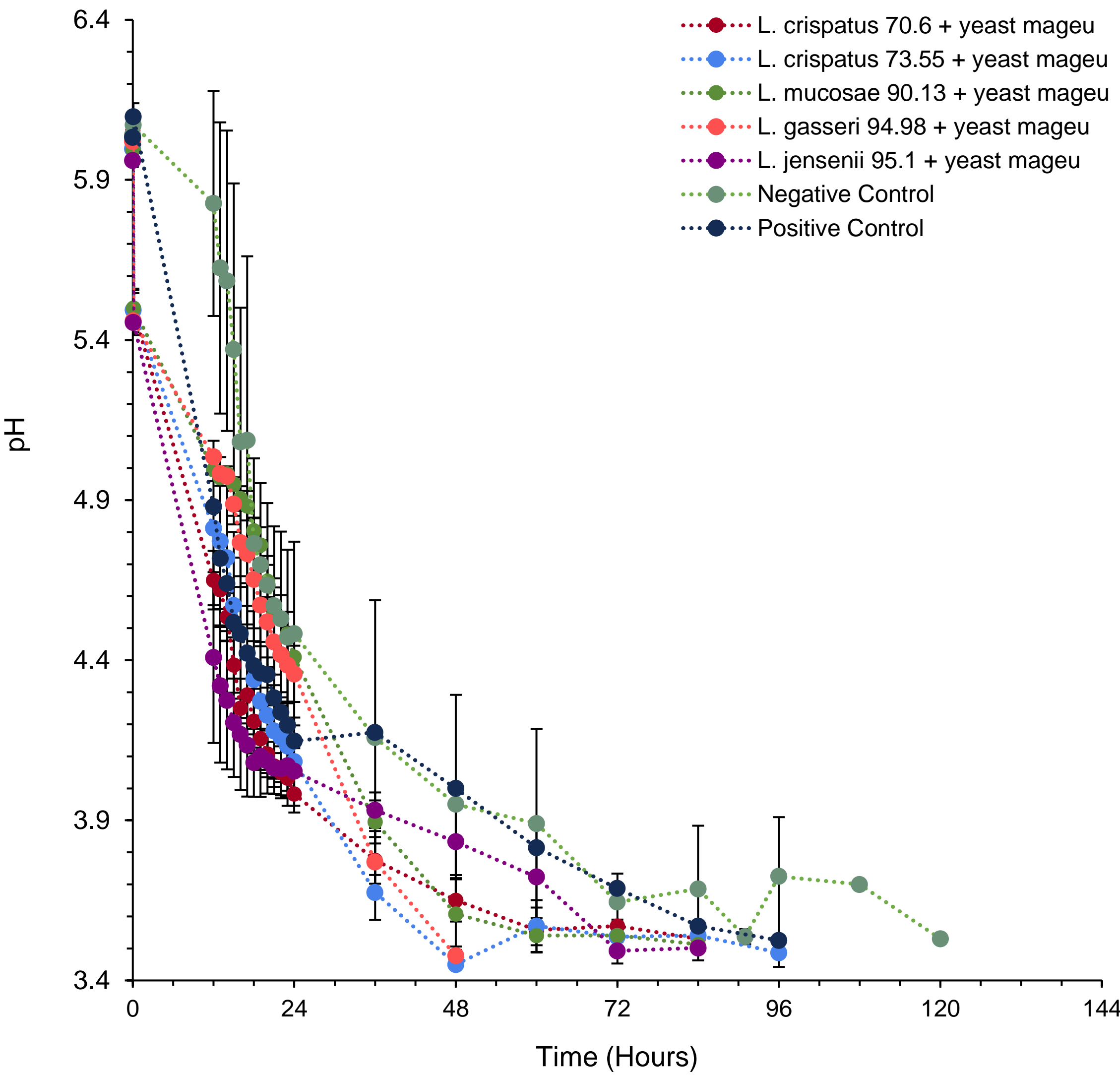

Figure 4-8 Plot of the decrease in pH over mageu fermentation time for the five *Lactobacillaceae* strains with yeast and the controls

While Figure 4-8 shows the pH reduction over time, Table 4-2 presents the final fermentation times and pH end points for the mageu samples produced using the bacterial strains and yeast. There was a larger variance in the data for the mixed culture-mageu compared to the pure strain mageu samples. Matching Figure 4-8, the strain with the smallest variation in fermentation time across all runs was *L. gasseri* 94.98, which consistently had a fermentation period of 48 hours. By contrast, *L. crispatus* 70.6 mageu and the positive control had the next highest variation after the negative control. The positive and negative controls were expected to have a large variance (as expressed by standard deviation) due to the spontaneous fermentation caused by the naturally occurring microorganisms in the maize and wheat flour.

Notably, all fermentations using the combination of the pure strains with yeast had significantly lower ($p<0.05$) fermentation times than the negative control. Therefore, the decrease in fermentation time can be attributed to the fermentation action of the single strain and the yeast (*S. cerevisiae*) together. Of interest, Idowu et al. (2016) measured the pH of mageu produced with only *S. cerevisiae*. Of the samples produced – *L. brevis* alone, *L. brevis* and *S. cerevisiae*, *S. cerevisiae* alone, and the control with wheat flour – *S. cerevisiae* alone had a noticeably higher final pH of 4.26, compared to the other samples, which ranged from 3.30 to 3.57. From that result, *L. brevis* and *S. cerevisiae's* combined action had a more significant effect on fermentation than *S. cerevisiae* alone.

Table 4-2 Average fermentation time for the mageu produced with single strain *Lactobacillaceae* and yeast and the controls

Sample	Average Fermentation Time (hour)	Final pH
L. crispatus 70.6 + Yeast	69 ± 10*	3.52 ± 0.03
L. crispatus 73.55 + Yeast	53 ± 9*	3.49 ± 0.04
L. mucosae 90.13 + Yeast	55 ± 7*	3.51 ± 0.02
L. gasseri 94.98 + Yeast	48 ± 0*	3.48 ± 0.03
L. jensenii 95.1 + Yeast	68 ± 6*	3.50 ± 0.04
Negative Control	94 ± 14	3.54 ± 0.02
Positive Control	87 ± 10	3.52 ± 0.02

*Fermentation times that were significantly different ($p<0.05$) from the negative control.
Fermentation times are displayed as means ± standard deviations from duplicate or triplicate independent experiments performed in duplicates.

4.2 Titratable acidity

Following fermentation, the mageu products were analysed for their acid content, measured as percentage lactic acid. The titratable acidity (TA) of the mageu samples produced using the bacterial strains, both as pure cultures and mixed cultures with yeast, are compared to one another. The influence of yeast addition on TA is also investigated.

Tables 4-3 and 4-4 present the TA values for the mageu fermentations produced using single *Lactobacillaceae* isolates and *Lactobacillaceae* isolates with yeast, respectively. As mentioned, each mageu sample was run in at least quadruplicate. Run 2.1 of *L. crispatus* 70.6 produced a TA outlier (determined statistically using the method in Section 3.9) (highlighted in blue in Table 4-3). Therefore, it was not included in the averages and comparisons amongst samples.

Of the mageu samples produced using the pure bacterial strains, *L. crispatus* 70.6 mageu had the highest TA, and *L. mucosae* 90.13 mageu had the lowest. As mixed cultures, the *L. mucosae* 90.13 with yeast mageu had the highest TA and *L. jensenii* 95.1 with yeast had the lowest. Interestingly, the variance (expressed as standard deviation) was similar for both the pure cultured mageu samples and mixed culture mageu samples.

Table 4-3 Titratable acidity (% (m/m)) for all runs of mageu produced using pure *Lactobacillaceae* isolates

	***L. crispatus* 70.6**	***L. crispatus* 73.55**	***L. mucosae* 90.13**	***L. gasseri* 94.98**	***L. jensenii* 95.1**
Run 2.1	0.625*	0.156	0.137	0.134	0.142
Run 2.2	0.237	0.156	0.119	0.148	0.144
Run 3.1	0.218	0.193	0.144	0.133	0.145
Run 3.2	0.146	0.176	0.124	0.144	0.152
Run 4.1		0.141			0.131
Run 4.2		0.134			0.125
Average	0.200 ± 0.039[a,c]	0.159 ± 0.020[b]	0.131 ± 0.012[a,b]	0.140 ±0.007	0.140 ± 0.009[c]

Means ± Standard Deviation. Average values with matching letters are significantly different from one another (p<0.05). * Denotes the statistical outlier

Table 4-4 Titratable acidity (% (m/m)) for all runs of mageu produced using single *Lactobacillaceae* isolates with yeast

	***L. crispatus* 70.6 + Yeast**	***L. crispatus* 73.55 + Yeast**	***L. mucosae* 90.13 + Yeast**	***L. gasseri* 94.98 + Yeast**	***L. jensenii* 95.1 + Yeast**
Run 2.1	0.254	0.247	0.308		0.240
Run 2.2	0.255	0.237	0.288		0.220
Run 3.1	0.273	0.234		0.279	0.233
Run 3.2	0.272	0.258		0.262	0.243
Run 4.1		0.268	0.338	0.317	0.226
Run 4.2		0.288	0.309	0.301	0.242
Average	0.263 ± 0.009[a,b]	0.255 ± 0.019[c,d,e]	0.311 ± 0.018[a,c,f]	0.290 ± 0.021[d,g]	0.234 ± 0.008[b,e,f,g]

Means ± Standard Deviation. Values with matching letters are significantly different from one another (p<0.05)

Figure 4-9 visually represents the impact yeast addition had on the TA of the probiotic mageu samples. All mageu samples produced using the bacterial strains are also compared to the negative and positive controls.

The pure strain mageu samples had lower TAs across all isolates than the mageu samples produced with the addition of yeast. T-tests confirmed that the increase in TA with the addition of yeast was significant ($p<0.05$) for all *Lactobacillaceae* strains. The addition of yeast had the most significant impact on the TA of the *L. mucosae* 90.13 mageu, which increased by 158%. The lowest increase in TA with the addition of yeast was observed for the *L. crispatus* 70.6 mageu, with an increase of 30%.

The probiotic mageu sample closest to the suggested TA of 0.4% was the *L. mucosae* 90.13 with yeast sample. All pure strain mageu samples had TAs lower than that of the negative control. Holzapfel and Taljaard (2004) suggest that the TA at the end of mageu fermentation should be between 0.4% and 0.5% lactic acid. Nyanzi et al. (2010) achieved very similar TA values for their mageu samples, ranging from 0.39% to 0.45%. Mashau et al. (2020) recorded titratable acidity values of 0.2%, 0.5%, and 0.6% for the control, home-made and laboratory-produced samples respectively.

Most notably, the highest TA was recorded for the positive control at 0.59%. No further reason with the data available could be extrapolated to explain the high TA. However, this result was most similar to the TA achieved by the laboratory-produced sample by Mashau et al. (2020). The method Mashau et al. (2020) used for the laboratory sample is the same method used in this study to produce the positive control, except for the addition of aloe vera powder, which Mashau et al. (2020) added to enhance the shelf-life of the mageu samples.

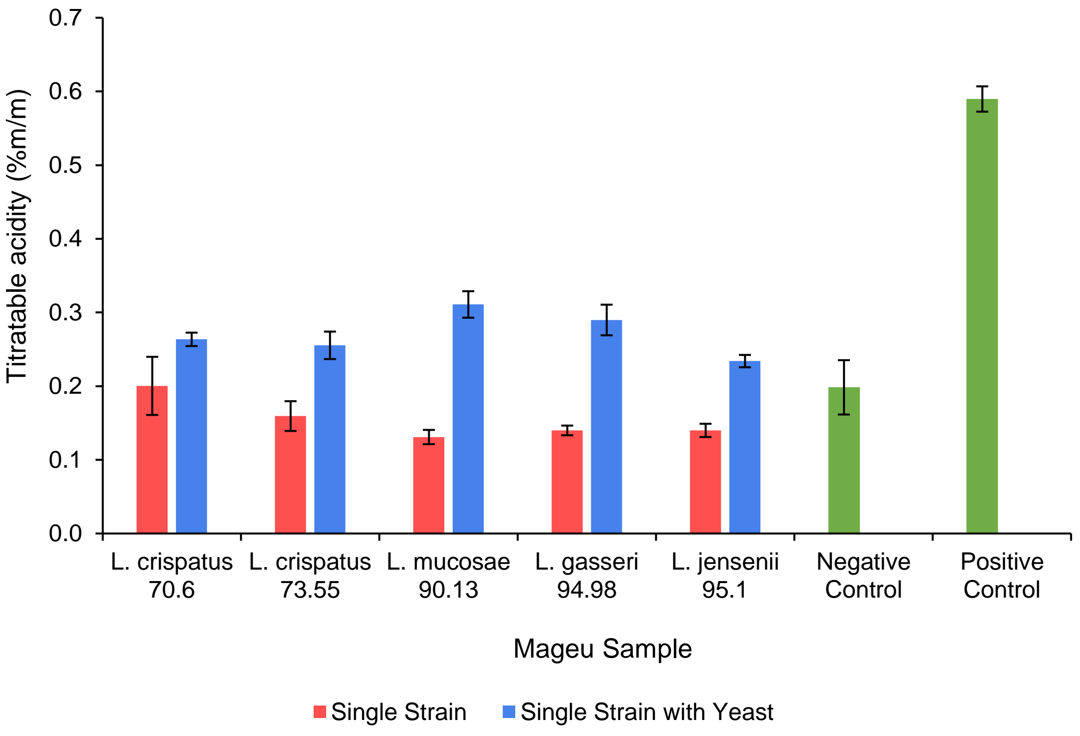

Figure 4-9 Titratable acidity percentages for mageu produced using pure *Lactobacillaceae* strains and *Lactobacillaceae* strains with yeast

Of the five *Lactobacillaceae* strains, all are homofermentative except for *L. mucosae* 90.13 which is heterofermentative (Happel, 2018). This classifier refers to how the bacteria metabolise glucose. While the homofermentative bacteria primarily produce lactate, heterofermentative bacteria produce lactate and other compounds such as ethanol and carbon dioxide (Eiteman and Ramalingam, 2015). In theory, homofermenters produce two moles of lactic acid for every one mole of glucose consumed. Heterofermenters, on the other hand, produce one mole of lactic acid for every one mole of glucose consumed (Eiteman and Ramalingam, 2015). Since heterofermenters produce less lactic acid for the same mass of glucose consumed, this is possibly why the pure *L. mucosae* 90.13 mageu had the lowest TA of the bacterial strains.

The yeast investigated in this project, *S. cerevisiae*, produces ethanol and carbon dioxide from glucose. Ethanol is primarily produced under anaerobic conditions (Walker and Stewart, 2016). Eiteman and Ramalingam (2015) suggest that *S. cerevisiae* could produce lactic acid from glucose if ethanol production were stunted. However, this has only been achieved previously by supressing enzymatic activity involved in ethanol production or producing acetic acid if the yeast have been metabolically manipulated through heterologous expressions of particular genes (Porro et al., 1995; Tokuhiro et al., 2009). Ethanol is an amphoteric compound and is able to act as either a base or acid. However, because ethanol is a very weak acid, it does not significantly lower the pH. Since the TA levels increased for the mageu samples produced with both bacteria and yeast, this indicates that addition of yeast may have increased the bacteria's lactic acid production. Alternatively, the yeast may have had a positive effect on the bacteria by increasing the bacterial growth rate. With higher bacterial cell counts, more lactic acid would have been produced, thus leading to an increased TA. However, the final pH for both the pure strain and bacteria and yeast mageu samples were not significantly different, despite the TAs being significantly different ($p<0.05$). Mashau et al. (2020) had similar results. For one of their samples, at a pH of 3.9, the TA was 0.2%. However, for another sample with a pH of 4.0, the TA was 0.5%. Also, the TA for two other samples were both 1.3%, despite having different pH values of 2.7 and 3.4. Therefore, while it would be expected that a decrease in pH would result in an increase in TA, the data do not show a consistent relationship between pH and TA. Happel (2018) also reported the discrepancy

between culture pH and concentration of lactate produced by the bacterial strains, noting that lactate production is not solely responsible for pH reduction.

Titrating the mageu samples with sodium hydroxide gives a representation of the total acid content. However, the equation used for TA (Equation 3-5) includes the lactic acid factor only. An alternative approach to finding the TA is to use the relationship between lactic acid concentration expressed as percentage acidity and pH. The alternative TA derivation is determined as follows.

Lactic acid dissociates into lactate by donating its proton to water (Equation 4-1). Sodium hydroxide dissociates into its sodium and hydroxide ions (Equation 4-2). Equation 4-4 represents the charge equation. The total concentration of sodium hydroxide (F_{NaOH}) is equal to the concentration of sodium ions (Equation 4-5). Similarly, the total concentration of lactic acid is the sum of the concentration of undissociated lactic acid and the concentration of its conjugate base (Equation 4-6). K_{LA} and K_W are the ionisation constant for lactic acid and ion-product constant for water, respectively (Gupta and Demirbas, 2010; Sin and Tueen, 2019).

$$H_2O + C_3H_6O_3 \leftrightarrow H_3O^+ + C_3H_5O_3^- \quad \text{Equation 4-1}$$

$$NaOH \leftrightarrow Na^+ + OH^- \quad \text{Equation 4-2}$$

$$pH = -\log_{10}[H_3O^+] \quad \text{Equation 4-3}$$

$$[H_3O^+] + [Na^+] = [OH^-] + [C_3H_5O_3^-] \quad \text{Equation 4-4}$$

$$F_{NaOH} = [Na^+] \quad \text{Equation 4-5}$$

$$F_{LA} = [C_3H_6O_3] + [C_3H_5O_3^-] \quad \text{Equation 4-6}$$

$$K_{LA} = \frac{[H_3O^+][C_3H_5O_3^-]}{[C_3H_6O_3]} = 1.38 \times 10^{-4} \quad \text{Equation 4-7}$$

$$K_W = [H_3O^+][OH^-] = 1.00 \times 10^{-14} \quad \text{Equation 4-8}$$

Using Equations 4-6 and 4-7, $[C_3H_5O_3^-]$ is solved for and Equation 4-9 is formed.

$$[C_3H_5O_3^-] = \frac{K_{LA}F_{LA}}{[H_3O^+]+K_{LA}} \quad \text{Equation 4-9}$$

Equation 4-8 is rearranged to solve for $[OH^-]$ and then substituted into Equation 4-4, along with Equations 4-5 and 4-9. The resulting equation is:

$$[H_3O^+] + F_{NaOH} - \frac{K_W}{[H_3O^+]} = F_{LA} \times \frac{K_{LA}}{[H_3O^+]+K_{LA}} \quad \text{Equation 4-10}$$

Equation 4-10 is rearranged to solve for the lactic acid concentration, F_{LA}:

$$F_{LA} = \frac{\left[[H_3O^+]+K_{LA}\right]\left[[H_3O^+]+F_{NaOH}-\frac{K_W}{[H_3O^+]}\right]}{K_{LA}} \quad \text{Equation 4-11}$$

Since the pH at which phenolphthalein turns pink is between 8.3 and 8.5, using Equations 4-2, 4-3, and F_{LA}, the lactic acid concentration in the mageu sample can be determined (Bouteille et al., 2013; Nout et al., 1989). From Equation 4-3, the concentration of hydronium ions can be determined by taking an average pH of 8.4 for the phenolphthalein indicator change.

$$10^{-8.4} = [H_3O^+] = 3.98 \times 10^{-9} M \quad \text{Equation 4-12}$$

For each mageu sample, F_{NaOH} could be determined by multiplying the molarity of the NaOH solution (0.1 M) by the volume of the NaOH solution titre, and dividing by a volume of 15 mL. The 15 mL was made up of 10 mL of mageu sample diluted with 5 mL of deionised water. The 15 mL was a rough volume as the viscosity of the mageu samples played a role in how easily the pipette was able to measure out 5 mL accurately.

As an example, this equation is demonstrated using the mageu sample with *L. jensenii* 95.1 with yeast Run 2.1, which had an NaOH titre of 2.2 mL. This sample had a thin consistency and therefore the volume was likely to be closer to 15 mL than for the thicker samples. The calculation of F_{NaOH} (equal to the concentration of sodium hydroxide) using the experimentally determined titre volume was:

$$F_{NaOH} = 0.1\ \mathrm{M} \times 2.2\ \mathrm{mL} \div 15\ \mathrm{mL} = 0.0147\ \mathrm{M}$$ Equation 4-13

Considering that we are only looking at the neutralisation of lactic acid in these equations, the F_{NaOH} value could be converted to grams of lactic acid per grams of mageu sample:

$$TA\ \%\ \left(\frac{m}{m}\right) = 0.0147 \frac{mol}{L} \times 90 \frac{g}{mol} \times 15\ mL \times \frac{1\ L}{1000\ mL} \times \frac{1}{13.2} g \times 100 = 0.150$$ Equation 4-14

where 90 g/mol is the molecular weight of lactic acid, 15 mL is the volume of the mageu-water sample, and 13.2 g is the weight of the mageu-water mixture.

This value is lower than the TA reported in Table 4-4 of 0.240%. Therefore, the sodium hydroxide reacts with acids other than lactic acid present in the mageu samples, and therefore produces a higher TA than for lactic acid alone. As mentioned, this discrepancy could also be partly caused by the inaccuracy of the volume of the mageu sample due to the high viscosity. The bacteria and yeasts may have been producing other metabolites which contributed to the lowering of the pH and reacted with the sodium hydroxide during titrations to give higher TA results. Pswarayi and Gänzle (2019) noted that while lactic acid was the major fermentation metabolite, noticeable levels of acetic acid were also present in their mageu fermentations. Additionally, LAB have been shown to produce various levels of other acids such as formic, propionic and butyric acid during food production (Ozcelik et al., 2016). However, the acids produced and their concentrations were dependent on the bacterial strain and growth media used. While ethanol and carbon dioxide are the primary metabolites of *S. cerevisiae* fermentation, the yeast can also produce other acids as secondary metabolites such as succinic acid and acetic acid (Walker and Stewart, 2016).

Since TA is a measure of lactic acid equivalence, further investigations were done using a more accurate method for determining lactic acid concentration using high-performance liquid chromatography (HPLC) (presented in Section 4.3).

4.3 Lactic acid and ethanol concentrations

This section investigates how the lactic acid and ethanol concentrations, measured using HPLC, differ for the mageu samples produced using the various bacterial isolates, and whether yeast addition had an impact on the levels. The lactic acid and ethanol concentrations for the probiotic mageu samples will also be compared to the negative control, positive control, and the levels present in maize porridge before inoculation. Lactic acid concentration will be investigated first, followed by ethanol concentration.

4.3.1 Lactic acid concentration

Lactic acid is the primary metabolite produced by LAB during food fermentations. Therefore, it is of interest to accurately determine if the lactic acid levels differ for the various *Lactobacillaceae* isolates and how the addition of yeast impacts them. Lactic acid concentrations are measured using HPLC analysis, which compares peaks of lactate in the mageu samples to a standard of known lactate concentration. Lactate is the conjugate base of lactic acid. While lactic acid is the acid of interest, HPLC measures the analyte lactate. Therefore, the two terms will be used interchangeably in this discussion.

The lactate concentration was measured at the end of fermentation for all mageu samples. These concentrations, presented in Figure 4-10, were all significantly higher than the lactate acid concentration at the beginning of fermentation without an added inoculum (0.045 ± 0.028 g/L). This result confirms the lactic acid fermentation. The positive control had the lowest overall lactate concentration of 0.122 ± 0.087 g/L. However, it had the highest titratable acidity of all samples. Therefore, another acidic compound was produced during this spontaneous fermentation, which produced high titratable acidity levels and was responsible for lowering the pH. The negative control, by contrast, had the third-lowest lactate concentration of 0.823 ± 0.086 g/L, after the pure *L. crispatus* 70.6 fermentation of 0.602 ± 0.280 g/L.

The lactate levels in the mageu were generally higher with the added yeast than with the pure strains. This trend corresponds to that of the titratable acidity (Section 4.2). However, the effect of yeast addition on TA was greater than for lactate concentration. The exception to this was the *L. jensenii* 95.1 mageu, which had a lactate concentration of 1.14 ± 0.15 g/L for the pure strain-produced mageu and 0.890 ± 0.318 g/L with the addition of yeast.

The greatest difference observed with the addition of yeast was for the fermentations with *L. crispatus* 70.6. In this case, the mageu produced with yeast had a concentration of 1.05 ± 0.22 g/L, compared to 0.602 ± 0.280 g/L for the single strain sample. However, the pure *L. crispatus* 70.6 mageu also had the highest variation (expressed as standard deviation) of all the pure strain samples (Figure 4-15). The runs for pure *L. crispatus* 70.6 mageu which were thicker, had the higher lactate concentrations. As mentioned in Section 3.6.2, mixing only occurred at the beginning of fermentation as suggested by literature. Therefore, in the thicker samples the lactic acid concentration may not have distributed evenly but instead formed pockets of higher concentrations. This may have skewed the pH values of the samples which were not shaken before being taken. However, before taking a sample for HPLC analysis, the fermentation bottles were shaken thoroughly to give a representative sample. It is therefore possible, that the thicker samples had an overall lower pH (which corresponds to the higher lactate concentration), than was recorded.

For the samples produced with both single strains and yeast, a larger variation in standard deviation was observed. This was especially prominent for the *L. gasseri* 94.98 mageus. Runs 3.1 and 3.2 using *L. gasseri* 94.98 with yeast produced a very thin consistency mageu compared to Runs 4.1 and 4.2 (analysed further in Section 4.6). At this lower viscosity, the lactic acid may have diffused more evenly throughout the fermentation bottles and thus presented higher lactate concentrations.

The lactic acid concentration values determined by HPLC gave more similar results to the TA values calculated using the alternative equation (Equation 4-14) proposed in 4.2. For example, converting the lactic acid concentration in g/L for *L. jensenii* 95.1 with yeast Run 2.1 to a TA gave 0.156% (m/m). This is very similar to the TA calculated (0.150%) in Equation 4-14. However, when selecting a very thick sample (*L. gasseri 94.98* Run 3.1), the results differed. The theoretical TA values calculated using the alternative equations proposed (Equation 4-14) gave a value of 0.0827%. By contrast, converting the lactic acid concentration obtained by HPLC to an acidity percentage gave 0.132%, which was most similar to the value obtained via titration at 0.133%.

The lactic acid concentrations for all samples were lower than those found for the naturally fermented samples produced in Zimbabwe which had concentrations ranging from 2.59 g/L to 4.37 g/L (28.7 mM - 48.5 mM) (Pswarayi and Gänzle, 2019). However, those fermentations used millet malt as the inoculum source instead of wheat flour and had lower final pH values between 2.96 and 3.38. The preparation of the millet malt was also performed by the households and not purchased. Therefore, the millet malt may have had a larger consortium of microorganisms in a higher concentration than the store-bought wheat flour used in this study.

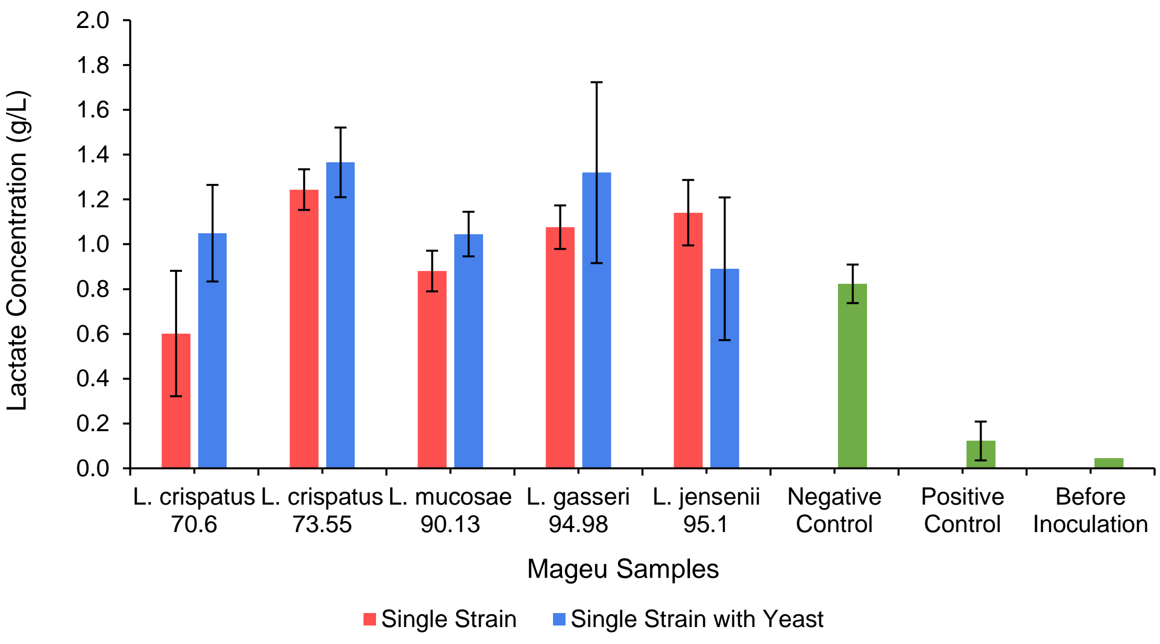

Figure 4-10 HPLC determined lactate concentrations of all mageu samples

4.3.2 Ethanol concentration

The mageu products' ethanol concentrations were monitored to ensure the alcohol levels did not exceed the regulations imposed for mageu production. The ethanol concentration results of all mageu samples are displayed in Figure 4-11, with comparison to the negative control, positive control and to the ethanol levels present in maize porridge before inoculation.

The ethanol concentrations before inoculation of the maize-water mixture were negligible, measured as 0% and 0.00419 g/L in the two samples tested (run in duplicate). Therefore, the ethanol concentrations reported for all mageu samples were as a result of the fermentation action of the microorganisms, both naturally-occurring and deliberately added.

The variance in the ethanol concentrations (expressed as standard deviation) of the positive and negative controls was high relative to the measured concentration values. For example, Run 2.2 of the negative control reported an ethanol concentration of 0.803 g/L, which was double the concentration of Run 2.1, and quadruple the concentrations of Runs 3.1 and 3.2. The positive control yielded similarly variable results, with Run 4.2 having a concentration of 0.659 g/L, 18 times greater than the smallest concentration of 0.0356 g/L for Run 4.1. Since the negative and positive controls were produced by spontaneous fermentation, there would have been different microbial populations in each sample, which produced ethanol and caused the variance.

All mageu samples produced using pure strains had lower ethanol concentrations than both the negative and positive controls. Of all the pure strain samples, the *L. crispatus* 70.6 mageu had the lowest ethanol concentration of 0.0768 ± 0.0111 g/L, followed next by the *L. crispatus* 73.55 mageu at 0.0815 ± 0.0882 g/L. Since isolates 73.55 and 70.6 are of the same species, it is expected that they would have displayed similar results as they are homofermentative and do not produce ethanol. Despite *L. mucosae* 90.13 being the only heterofermentive isolate, the mageu it produced did not have higher ethanol concentrations in comparison to the other pure strains; suggesting that metabolism primarily involved the production of lactic and acetic acid rather than ethanol (Ganzle, 2015).

The pure strain mageu samples' ethanol concentrations were significantly lower ($p<0.05$) than the negative control. This indicates that the added strains may have outcompeted the naturally occurring microorganisms in the maize meal, and so did not give them a chance to proliferate and produce ethanol to the levels seen in the negative control.

All mageu samples produced with yeast had higher ethanol concentrations than their pure strain counterparts. This was expected as *S. cerevisiae* produces ethanol and carbon dioxide from glucose. Although the ethanol concentration levels for pure *L. mucosae* 90.13 mageu were not noticeably higher than the other pure strain mageus, the mageu sample produced with *L. mucosae* 90.13 and yeast had the highest ethanol concentration of all the mageu samples. However, it is not known whether the increase in ethanol concentration from the pure *L. mucosae* 90.13 mageu sample is due to the yeast's metabolism or a mutualistic relationship between the two microorganisms. All mageu samples produced with bacteria and yeast had a larger variance in their ethanol concentrations than their pure strain counterparts. Since the Anchor Yeast used in this study was store-bought, it contained food grade rehydrating agent and ascorbic acid in addition to the baker's yeast. While theoretically the same mass of Anchor Yeast should contain similar yeast cell numbers, there may have been slight differences between each packet. This may have resulted in the variance in the ethanol concentration if the added yeast cell concentration was not consistent. A recommendation for future research is that the dried yeast should be diluted in YPD media and left to incubate before inoculating the maize-water mixture based on the measured optical density to correct this.

In comparison to these results, spontaneously fermented mageu produced at the household level has been reported to have ethanol concentration values of between 6.8 g/L and 15.5 g/L (148 mM – 336 mM) (Pswarayi and Gänzle, 2019). These values are much higher than the maximum allowable limit specified by the South African National Standard for mageu production and those obtained in this study. Since the household fermentations were not controlled, there may have been more microorganisms (both in diversity and concentrations) able to produce ethanol than those used in this study.

Importantly, the South African National Standard for mageu production has a maximum alcohol requirement of 2.5 g/L (0.25 %(m/m)) (South African Bureau of Standards, 2011). The average ethanol concentration of all samples fell below this requirement. However, the average ethanol concentration for the *L. mucosae* 90.13 with yeast sample came closest to not meeting this requirement at an average concentration of 2.43 ± 1.43 g/L. Of the four runs performed for *L. mucosae* 90.13 with yeast, three were below the threshold of 2.5 g/L, but Run 2.1 had a concentration of 4.79 g/L. While this concentration was not statistically an outlier, it was more than double the concentration of the next highest run.

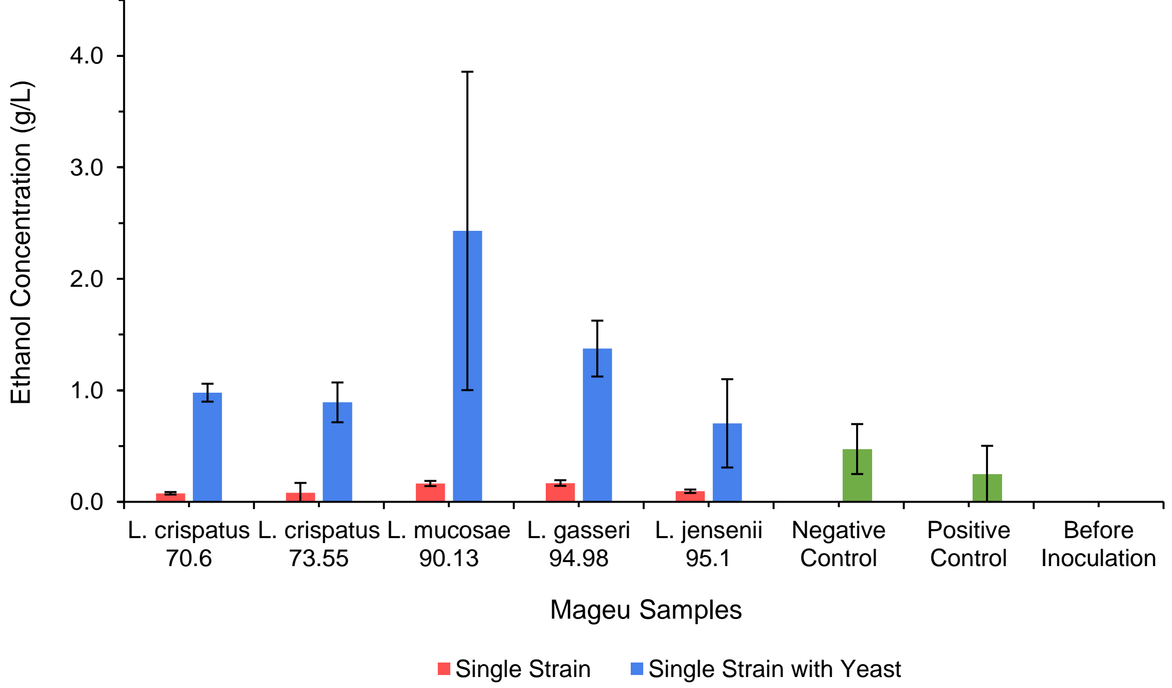

Figure 4-11 HPLC determined ethanol concentration of all mageu samples

4.4 Total solids content

This section investigates the total solids content for all the mageu samples produced and whether there were any differences between samples. Evaporation was used to determine total solids content. The South African National Standard for mageu production sets the total solids content at a minimum of 8% (m/m). To ensure this requirement was met, 12% (m/v) of maize meal was added to water in the mageu preparation. The results for all mageu samples are presented in Figure 4-12.

As expected, there was little difference between the total solids content of the mageu produced using the different strains and between the mageu produced with pure strains and those produced with single strains and yeast. In addition, the samples produced with the isolates had a total solids content comparable to the controls. None of the samples were statistically different from the negative control ($p>0.05$). The pure strain samples compared to the single strain with yeast were not statistically different in total solids content ($p>0.05$), except for the *L. mucosae* 90.13 mageus. Overall, the *L. mucosae* 90.13 with yeast sample had the lowest solids content. These mageu runs were noticeably thinner than the pure strain mageu samples. Therefore, when pipetting a sample to measure the mass, it is possible that despite the bottle being mixed, more water per volume sample was extracted from the mageu than representative of the entire product. However, overall, the consistency of the samples, which differed considerably, did not impact the solid content of the product.

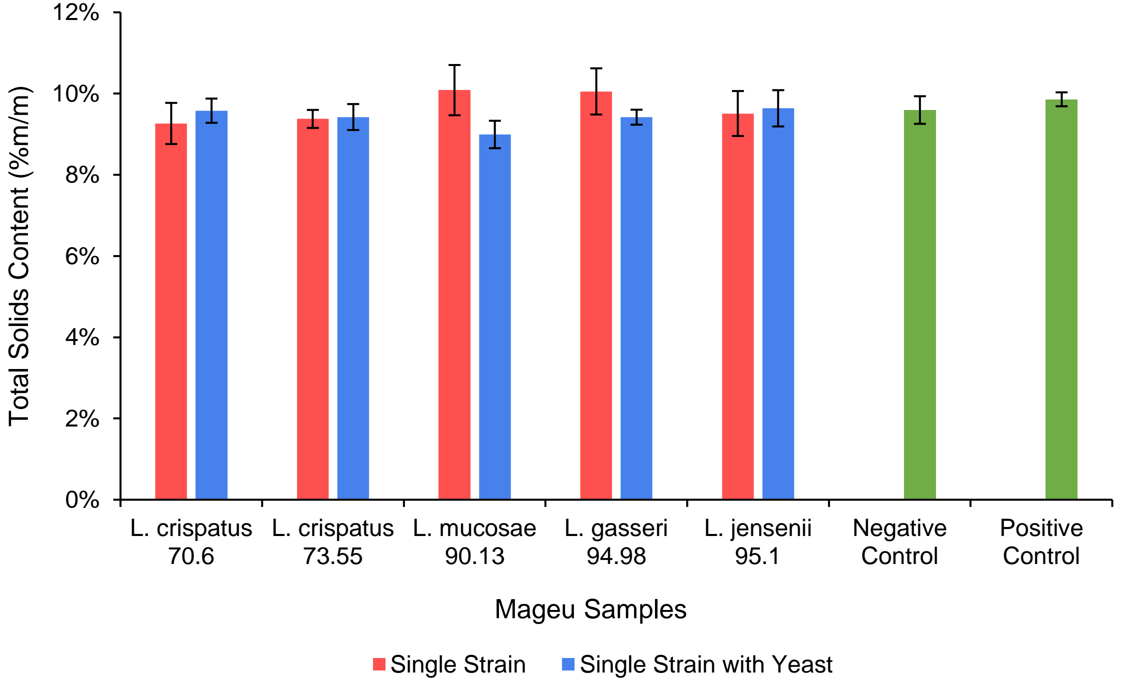

Figure 4-12 Total solids content for all mageu samples

4.5 Cell counts

Before fermentation, the five *Lactobacillaceae* strains were plated from serum bottles onto MRS agar. At the end of fermentation, the mageu samples were diluted and plated on MRS and YPD agar. MRS media is the favoured media for the *Lactobacillaceae* isolates and YPD media is preferred for *S. cerevisiae* growth. These cell counts give an indication of the viable cells at the beginning and end timepoints. Cell counts were performed to determine whether the cell concentrations of the various bacteria increased during fermentation, and whether yeast addition influenced the bacterial growth.

Since no inoculum was added to the negative control, cell counts were only performed on the product. Similarly, for the positive control, the inoculum was a spontaneous fermentation and the cell counts were therefore only measured at the end of fermentation.

Different colony morphologies were observed on the MRS plates following fermentation. Typically, two morphologies could be seen: whiter spherical colonies and brown/purple spherical colonies, both of similar size. On the YPD plates, there were also commonly two morphologies observed: larger white colonies and much smaller translucent colonies. Photographs of the MRS and YPD plates showing the different colony morphologies are presented in Figure 4-13. In Figure 4-13a, the green circle highlights a whiter colony, while the blue circle shows a brown/purple colony. In Figure 4-13b, the blue circle shows a larger whiter colony while the purple circle highlights the translucent colonies. Included in MRS are yeast, peptone, and glucose – the three compounds which make up YPD media. Yeasts can also grow anaerobically, and while the colony morphologies differed on YPD and MRS agar, the white colonies grown on both plates may have been yeast. However, either a gram stain (to identify the larger yeast cells) or form of gene sequencing, for example, sequencing of the 26S rRNA gene, would need to be performed to confirm this (Winters et al., 2018). The brown/purple morphologies seen on the MRS plates did not mirror the characteristic morphologies of the isolates grown on agar plates when inoculating. The colony morphologies may have altered because of the different growth nutrients available in the mageu. Only gene sequencing, for example, 16S rRNA, would be able to confirm the individual bacteria. For this study, only the total colony counts present before and after fermentation were quantified.

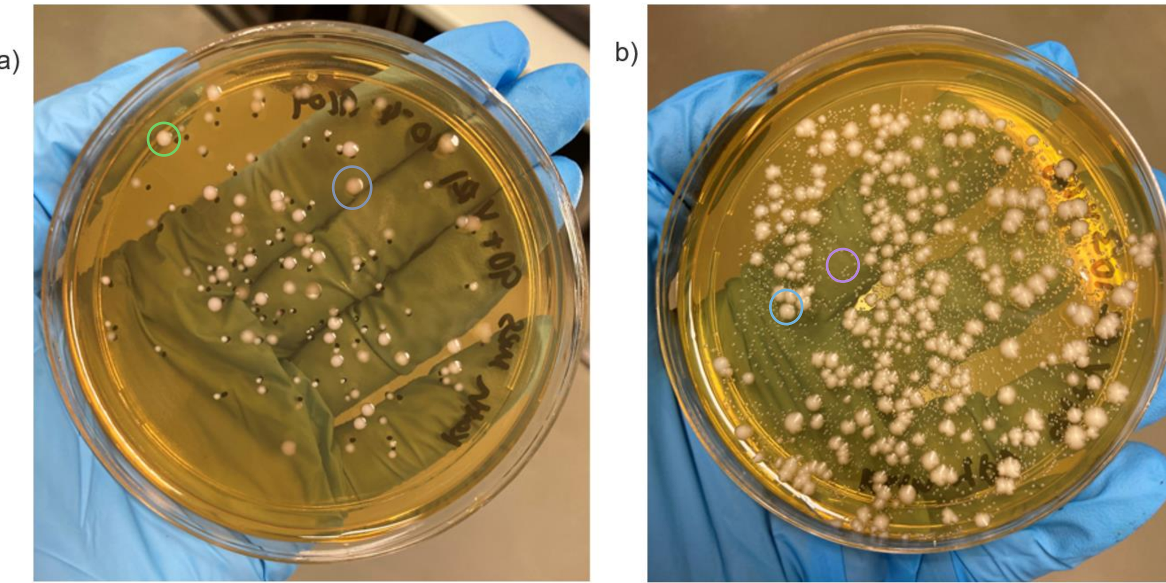

Figure 4-13 Different colony morphologies for mageu microorganisms grown on a) MRS and b) YPD

In a), the green circle shows the white colonies and blue circle shows the brown/purple colonies.
In b), the blue circle shows the whiter colonies and the purple circle shows the translucent colonies.

First, the cell concentrations of the *Lactobacillaceae* isolates inoculated into the maize porridge, and the growth of cells on MRS plates after mageu fermentation are compared. Then the cell concentrations of the *Lactobacillaceae* isolates inoculated into the maize porridge with yeast and the growth of cells on MRS following the mixed culture mageu fermentations are compared. The next comparison looks at the cell concentrations on YPD plates following the mixed culture mageu fermentations. Lastly, the overall change in cell concentrations before and after mageu fermentation for all mageu samples is investigated.

The cell counts of the pure strain mageu microorganisms plated on MRS before and after fermentation are presented in Table 4-5. For the pure strain fermentations, all strains exhibited marked increases in

the cell concentrations by the end of fermentation, with each having increased by at least one factor of 10 as suggested by MRS plate counts. However, *L. jensenii* 95.1 Runs 4.1 and 4.2 had lower cell concentrations than Runs 3.1 and 3.2 by the end of fermentation. When analysis (serial dilutions and cell counts) could not be done immediately after fermentation, samples were stored in the fridge at 4°C until the appropriate setups were ready. This was the case with *L. jensenii* 95.1 Runs 4.1 and 4.2, and so this may have affected this result. Capela et al. (2006) investigated cell concentrations of lactobacilli in yoghurt during storage at 4°C for four weeks following production. From week zero to two, the cell counts had dropped from roughly 8.93 $\log_{10}$ cfu/mL to roughly 8.52 $\log_{10}$ cfu/mL and 7.82 $\log_{10}$ cfu/mL by week 3. Therefore, it is reasonable to assume that the cell counts performed two weeks after fermentation ended will have lower counts than the bottles counted immediately after. Therefore, these two runs can be assumed to not accurately represent cell growth during mageu fermentation.

Despite being inoculated at the same optical density, the initial cell concentrations varied between Runs 3 and 4 for strains *L. crispatus* 73.55 and *L. jensenii* 95.1 (Table 4-5). The difference in cell concentration may be due to cultivation time before inoculation or the natural variances that occurred during the growth of the microorganisms.

The cell concentrations of the mageu produced using the bacterial isolates and yeast were plated on MRS and YPD, and are displayed in Table 4-6 and Table 4-7, respectively. The bacterial cells used for inoculation of the fermentation bottles grown with yeast were cultivated for 24 hours longer than the bacterial cells used for the pure strain fermentations. The initial cell concentrations plated on MRS for the bacterial strains used to produce the mixed culture mageu samples were lower than the initial cell concentrations of the bacterial isolates used to produce the pure strain mageu samples. This trend was observed for all bacterial strains except *L. crispatus* 70.6, which increased very slightly from 6.43 $\log_{10}$ cfu/mL to 6.49 $\log_{10}$ cfu/mL (Table 4-6). The extra 24 hours of growth may have meant that the bacteria were no longer in their exponential phase and had even entered their death phase for some strains, reducing viable cell concentration.

The cell concentrations of the cells grown on YPD after the mixed culture mageu fermentations are displayed in Table 4-7. Since the YPD plates were grown aerobically, it is assumed that the colonies grown after mageu fermentation were yeasts. On average, the highest yeast counts after fermentation were reported for co-fermentation with *L. gasseri* 94.98, followed closely by *L. crispatus* 70.6 and then *L. crispatus* 73.55. Overall, *L. mucosae* 90.13 had the lowest yeast counts even compared to the negative and positive control. The ethanol concentrations were the highest for the co-fermentation of *L. mucosae* 90.13 with yeast (Section 4.3.2). However, this sample had the lowest bacteria and yeast cell counts of all the strains. Therefore, the growth nutrients were possibly used to produce ethanol rather than prioritizing biomass growth. With reference to the results of the positive control, for Run 4.4 many translucent colonies and bigger white colonies were observed on the YPD plates. These translucent colonies were very difficult to count and therefore a total colony count could not be performed.

For all the mageu products with single strains and yeast, the yeast and bacteria counts (Tables 4-6 and 4-7) at the end of the fermentations were in the same order of magnitude (10^7 cfu/mL). The only exception was for *L. mucosae* 90.13, which had lower yeast cell counts than bacteria cell counts.

Table 4-5 Cell counts of pure strain mageu plated on MRS before and after fermentation

	Cell counts plated on MRS agar (Log_{10} cfu/mL)											
	***L. crispatus* 70.6**		***L. crispatus* 73.55**		***L. mucosae* 90.13**		***L. gasseri* 94.98**		***L. jensenii* 95.1**		**Neg. Control**	**Pos. Control**
	Before	After	Before	After	Before	After	Before	After	Before	After	After	After
Run 3.1	6.43	7.64	4.25	7.75	6.78	7.76	6.83	7.36	6.08	7.46	7.93	
Run 3.2	6.43	7.66	4.25		6.78	7.57	6.83	7.29	6.08	7.61	7.89	
Run 4.1			5.42	6.58					6.33	6.78	7.23	5.70
Run 4.2			5.42	6.90					6.33	5.60	6.95	5.48
Run 4.3												6.00
Run 4.4												Contam-inated

Table 4-6 Cell counts of single strain with yeast mageu plated on MRS before and after fermentation

	Cell counts plated on MRS agar (Log_{10} cfu/mL)											
	***L. crispatus* 70.6**		***L. crispatus* 73.55**		***L. mucosae* 90.13**		***L. gasseri* 94.98**		***L. jensenii* 95.1**		**Neg. Control**	**Pos. Control**
	Before	After	Before	After	Before	After	Before	After	Before	After	After	After
Run 3.1	6.49	7.92	4.10	7.72			5.74	7.95	5.97	8.01	7.93	
Run 3.2	6.49	8.02	4.10	8.26			5.74	7.96	5.97	8.13	7.89	
Run 4.1			5.08	7.77	6.08	7.09	5.55	7.75	6.33	7.20	7.23	5.70
Run 4.2			5.08	7.84	6.08	7.19	5.55	7.73	6.33	7.23	6.95	5.48
Run 4.3												6.00
Run 4.4												Contam-inated

Table 4-7 Cell counts for mageu samples produced with *Lactobacillaceae* isolates and yeast plated on YPD agar after fermentation

	Cell counts plated on YPD (Log_{10} cfu/mL)						
	***L. crispatus* 70.6**	***L. crispatus* 73.55**	***L. mucosae* 90.13**	***L. gasseri* 94.98**	***L. jensenii* 95.1**	**Neg. Control**	**Pos. Control**
Run 3.1	7.49	7.67		7.56	7.60		
Run 3.2	7.67	7.83		7.51	7.00		
Run 4.1		7.23	6.28	7.76	7.05	7.15	6.32
Run 4.2		7.41	6.68	7.61	7.14	6.30	7.85
Run 4.3							6.67
Run 4.4							Uncountable

The positive control had lower cell counts on the MRS plates at the end of fermentation than the mageu samples inoculated with the *Lactobacillaceae* strains. In addition, Run 4.4 of the positive control showed cloudy growth on the plates, which grew around bacteria colonies with similar morphologies to those seen for the other runs of the positive control. This was assumed to be contamination as it was only observed in one bottle, but it may also have been associated with the spontaneous fermentation of the wheat flour. A photograph of the cells from the positive control Run 4.4 grown on MRS is presented in Figure 4-14.

Figure 4-14 Contamination observed on plate of dilution 10^4 for positive control Run 4.4

A comparison of the change in viable cells between the pure strain mageu and strains with yeast is displayed in Figure 4-15. For the mixed culture fermentations, all bacterial strains saw increases in the MRS plate cell counts after fermentation. These increases were more pronounced than the pure strain samples. The largest change was observed for *L. gasseri* 94.98, which saw a 0.49 log_{10} cfu/mL increase in cell counts as a pure strain, but had a 2.20 log_{10} cfu/mL increase when inoculated with yeast. Furthermore, all strains except *L. mucosae* 90.13 had overall higher cell counts at the end of fermentation when inoculated with yeast, despite having lower or on par initial cell concentrations.

Overall, the largest increase in cell concentration during fermentation was observed for *L. crispatus* 73.55, despite having the lowest initial cell concentration at inoculation. As mentioned previously, despite the inoculation for all strains being at the same initial optical density, the cell concentrations were not the same due to differing morphologies. Lower initial cell concentrations may have been beneficial to *L. crispatus* 73.55 as there was a greater availability of growth nutrients per cell than the other strains.

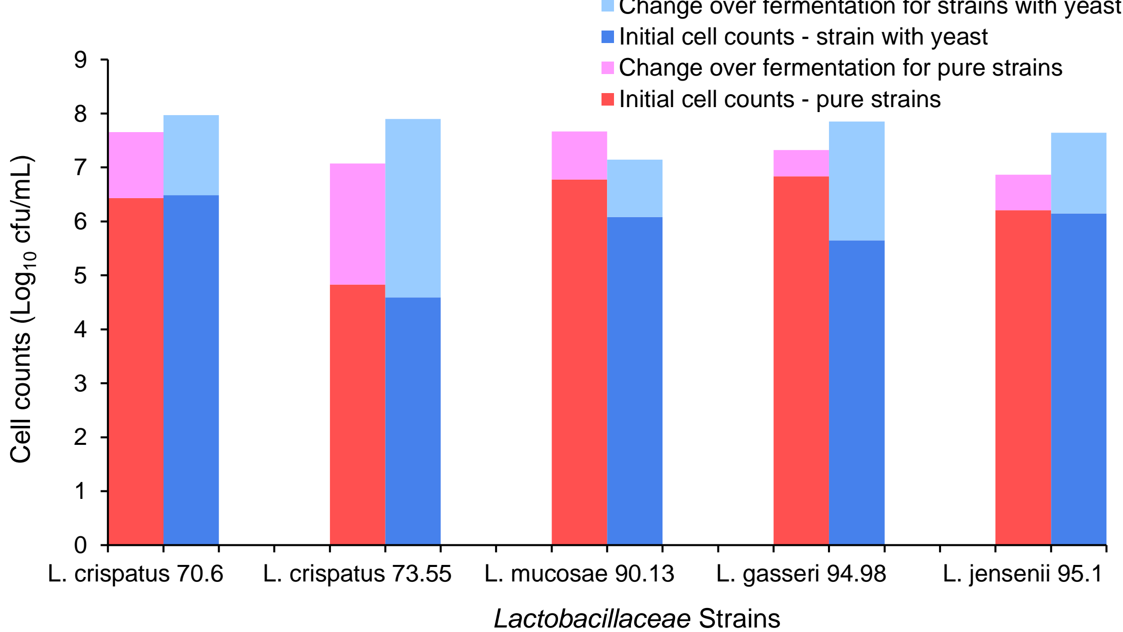

Figure 4-15 Change in MRS plate cell counts over fermentation for pure strain mageu and single strains with yeast mageu

4.6 Qualitative analysis of mageu

The qualitative analysis of the mageu samples investigated smell, consistency and any other noticeable characteristics without tasting the samples. The homogenisation of the samples was also investigated. The smell of the samples was measured on a sourness scale, and any prominent smells were described.

The scale used to rank the samples on sourness ranged from neutral, level 1 (least sour), to level 5 (extremely sour). The smell of yeast, characteristic of a brewery, was also noted across samples where it was present. The results of this analysis are presented in Table 4-8.

The different strains produced varying degrees of sourness compared to one another. On average, *L. crispatus* 70.6 produced the sourest mageu samples as both a pure strain and when combined with yeast. However, *L. jensenii* 95.1 produced two samples with sour scales of 4, though these were distinctly different to the neutral smell of the other four runs. While a yeast smell was commonly associated with yeast samples, pure *L. mucosae* 90.13 mageu also had a noticeable yeast-like smell. This smell may have been caused by small amounts of ethanol production in some of the runs since *L. mucosae* 90.13 is a heterofermenter. Overall, the addition of yeast increased the sourness of the samples. However, there was some variance in the smell of the samples produced across runs with the same strains. The differences in the samples produced using the same bacterial strain may have been caused by the naturally-occurring microorganisms in the maize meal, which were not inactivated during the heat treatment prior to inoculation. This was observed as the negative control still fermented despite no active inoculum being added (Section 4.1), and the samples had varying degrees of sourness.

During the earlier hours of fermentation, the positive control produced a sweet smell. Then, the samples were placed in the fridge until analysis, which was typically performed within 24 hours after fermentation. By the time of analysis, the positive control had continued to ferment and became very sour. These results are also consistent with the shelf-life results, which are examined further in 4.7.

In a review article, Wang et al. (2021) report that lactic acid bacteria can aid in the flavour of fermented foods; however, research on the specific metabolic pathways which produce flavour-enhancing compounds needs to be conducted. This research would give insight into which metabolic pathways could be targeted to enhance the flavours of fermented foods. While limited research has been performed on mageu, similar fermentations using lactic acid bacteria and yeasts are used with other cereals. Winters et al. (2018) investigated the impact that lactic acid bacteria (lactobacilli) and yeasts had on the flavour of sourdough. This study found that different combinations of *Lactobacillus* strains with yeasts produced different volatile compounds. The aroma profile of the sourdough samples was analysed using headspace solid phase micro-extraction gas chromatography mass spectrometry. Therefore, this method could be used to examine the aroma profiles of the mageu samples produced in this project in a follow-up study.

Table 4-8 Analysis of smell of all mageu samples

Smell									
	Sample	**Run 2.1**	**Run 2.2**	**Run 3.1**	**Run 3.2**	**Run 4.1**	**Run 4.2**	**Run 4.3**	**Run 4.4**
L. crispatus 70.6	*Pure*	Sour level 3	Sour level 3	Sour level 3	Sour level 3				
	With Yeast	Sour level 3	Sour level 3	Sour level 3 + Yeast smell	Sour level 3 + Yeast smell				
L. crispatus 73.55	*Pure*	Neutral	Neutral	Sour level 3	Sour level 3	Sour level 1	Sour level 1		
	With Yeast	Sour level 1	Sour level 1	Sour level 1	Yeast smell	Sour level 2	Sour level 2		
L. mucosae 90.13	*Pure*	Neutral + Yeast smell	Neutral + Yeast smell	Sour level 3	Sour level 3				
	With Yeast	Sour level 3	Sour level 2 + Yeast smell			Sour level 2 + slightly tangy	Sour level 2 + slightly tangy		
L. gasseri 94.98	*Pure*	Sour Level 1	Neutral	Sour level 1	Sour level 1				
	With Yeast			Yeast smell + slightly sweet	Sour Level 1	Sour level 3	Sour level 3		
L. jensenii 95.1	*Pure*	Neutral	Neutral	Sour level 4	Sour level 4	Neutral	Neutral		
	With Yeast	Sour level 2	Sour level 2	Sour level 3	Sour level 2	Sour level 2	Sour level 2		
Negative Control		Sour level 2	Sour level 2	Sour level 1	Sour level 1	Sour level 2	Neutral		
Positive Control						Sour level 3	Sour level 3	Sour level 3	Sour level 2

The consistency of the mageu samples was ranked on a scale from very thin, thin, slightly thick, thick, very thick, and extremely thick. The results are presented in Table 4-9.

Variation in consistency was observed among runs of the same samples. Overall, the samples produced with yeast were thinner than the pure strain samples. However, of all the samples, the positive control was the thinnest. The positive control bottles showed two distinct layers during fermentation: a watery layer at the top and a solids layer at the bottom. This was not observed in any of the other samples. *L. mucosae* 90.13 and *L. jensenii* 95.1 produced the thickest mageu samples as pure strains. The differences in thickness could be attributed to the different cell morphologies of the bacterial strains (mentioned in 4.1.1), or differences in metabolites produced and their concentrations. Furthermore, the bacterial strains may also produce different extracellular matrices and degradative enzymes that, when acting on the available substrates in the mageu matrix, may also influence the mageu's consistency. The consistency results reflect the titratable acidity results for the mageu samples: the positive control had the highest titratable acidity and the thinnest consistency. Similarly, the mageu samples produced with the bacterial strains and yeast had higher titratable acidities and thinner consistencies than the

pure bacterial strain mageu samples. As mentioned in Section 4.2, Ozcelik et al. (2016) noted that secondary metabolites and their concentrations were dependent on the LAB strain.

Table 4-9 Consistency analysis of all mageu samples

Consistency									
	Sample	**Run 2.1**	**Run 2.2**	**Run 3.1**	**Run 3.2**	**Run 4.1**	**Run 4.2**	**Run 4.3**	**Run 4.4**
L. crispatus 70.6	*Pure*	Thin with maize clumps	Thin with maize clumps	Slightly thick	Thick				
	With Yeast	Thin	Thin	Thin	Thin				
L. crispatus 73.55	*Pure*	Thick	Thick	Thin	Thick	Thick	Thick		
	With Yeast	Thick	Very thick	Thick	Thin	Slightly thick	Slightly thick		
L. mucosae 90.13	*Pure*	Very thick	Very thick	Very thick	Very thick				
	With Yeast	Thin	Thin			Thin	Thin		
L. gasseri 94.98	*Pure*	Very thick	Very thick	Very thick	Very thick				
	With Yeast			Thin	Thin	Thin	Thin		
L. jensenii 95.1	*Pure*	Very thick	Very thick	Thick	Thick	Extremely thick	Very thick		
	With Yeast	Thin	Thin	Very thin	Very thin	Thick	Very thick		
Negative Control		Thin	Thin	Slightly thick	Slightly thick	Thin	Thin		
Positive Control						Very thin	Very thin	Very thin	Very thin

The final qualitative analysis reported any other noticeable characteristics of the mageu samples, captured in Table 4-10. The only other distinguishing factor between mageu bottles was that some produced gas, which was noticed primarily at the end of fermentation during analysis. Since this was a qualitative analysis, the test for gas production was whether a gas-releasing sound was made upon opening the bottles. In Table 4-11, the samples marked with an 'x' show that gas was produced and the blocks marked out by a diagonal line show that no sample was produced for that run.

Again, the results varied for samples produced using the same bacterial strains. However, out of the 16 bottles which produced gas, 13 of them were produced with yeast. Since yeast can produce carbon dioxide from carbohydrates, this is an expected result. However, it is also surprising that not all samples containing yeast produced gas. For runs 4.1, 4.2, 4.3, and 4.4, the fermentation bottle lids were left slightly loose as required by the CeBER protocol for fermentation bottles. This would have let some gas escape during fermentation, to not over-pressurise the fermentation vessel.

The other instances where gas production was noted was for the pure strain fermentation using *L. crispatus* 70.6 (Runs 2.1 and 2.2) and Run 4.2 of the negative control. This indicates that there may have been microorganisms naturally occurring in the maize meal that produced carbon dioxide and were not removed during heat treatment at the beginning of the mageu production process.

Table 4-10 Table of mageu samples which released gas when opening the fermentation bottles for analysis

Gas Produced when opening bottle									
	Sample	Run 2.1	Run 2.2	Run 3.1	Run 3.2	Run 4.1	Run 4.2	Run 4.3	Run 4.4
L. crispatus 70.6	*Pure*	x	x						
	With Yeast			x	x				
L. crispatus 73.55	*Pure*								
	With Yeast					x	x		
L. mucosae 90.13	*Pure*								
	With Yeast	x	x				x		
L. gasseri 94.98	*Pure*								
	With Yeast			x	x	x	x		
L. jensenii 95.1	*Pure*								
	With Yeast			x	x				
Negative Control							x		
Positive Control									

4.7 Shelf-life of mageu samples

The shelf-life of the mageu samples was analysed by measuring pH for both refrigerated and room temperature samples after 7 days. Due to time constraints, shelf-life was only measured for Runs 2 and 3. Therefore, the results presented below are averages of the Runs 2.1, 2.2, 3.1 and 3.2. The pH of the sensory analysis mageu samples was also measured at the end of fermentation and before the taste testing. These results are also included in this section. The shelf-life of the mageu samples produced using the pure bacterial strains will be compared first, followed by a comparison of the shelf-life of the mixed culture mageu samples. The pH values at the end of fermentation (day 0), and of the refrigerated and room temperature samples after 7 days for the pure strain mageu samples are displayed in Figure 4-16.

For all the mageu samples produced using the single bacterial strains, the pH had dropped when placed at room temperature for 7 days, except for the *L. jensenii* 95.1 mageu sample which saw no change in pH. The negative control similarly experienced a pH reduction for the sample placed at room temperature. The greatest drop occurred for the *L. crispatus* 73.55 mageu which saw a change in pH of 0.13 over the 7 days. The *L. jensenii* 95.1 sample may have reached its pH tolerance level and therefore, did not continue growing and acidifying the mageu.

All mageu samples reported in Figure 4-16 saw only slight decreases or even increases in the pH values for the refrigerated shelf-life bottles. As mentioned in Section 4.3.1, mixing only occurred at the beginning of fermentation and so the lactic acid may not have distributed evenly through the fermentation bottle when the final pH was taken. The fermentation bottle was shaken before being

distributed into the two shelf-life bottles. Therefore, the true final pH at the end of fermentation may have been different to the values recorded. Despite this, the results for the shelf-life show higher pH values were obtained with the samples kept refrigerated than at room temperature over 7 days.

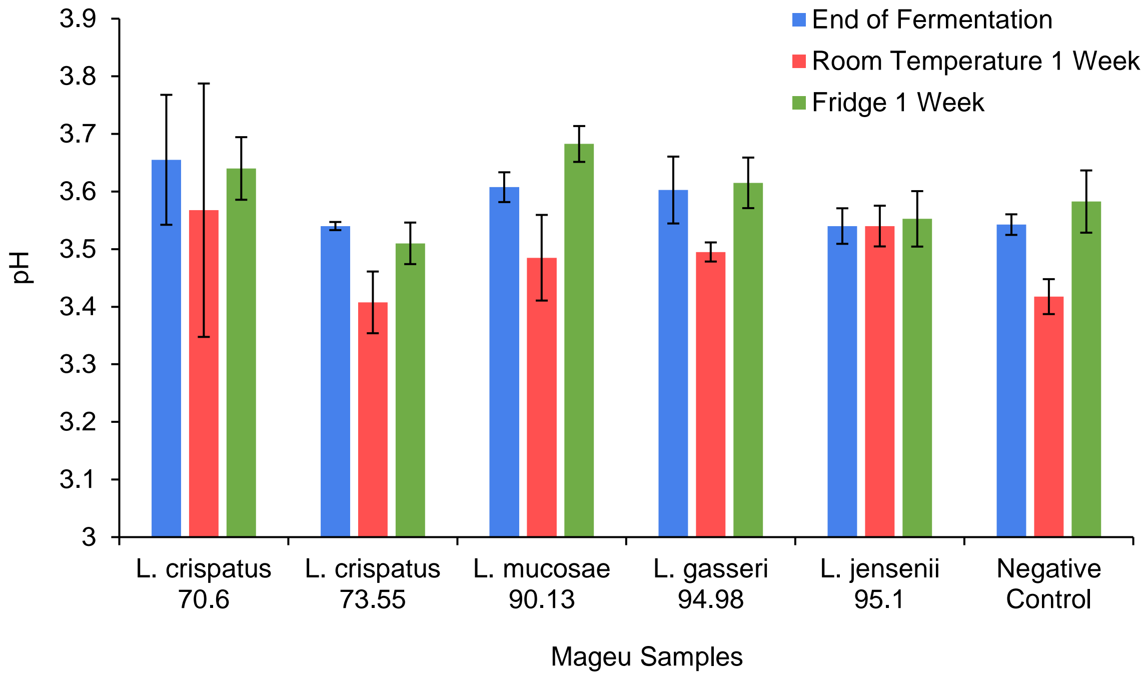

Figure 4-16 The pH values of mageu samples produced using single *Lactobacillaceae* isolates kept at room temperature and refrigerated for 1 week

The shelf-life of the mageu samples produced using the bacterial strains and yeast is investigated next. The results are presented in Figure 4-17. The drop in pH for the samples kept at room temperature was more noticeable than for the pure *Lactobacillaceae* strain mageu samples. The lowest pH value reached was 3.06 for the *L. mucosae* 90.13 with yeast sample and all mixed mageu samples saw a pH drop of at least 0.25 for the sample kept at room temperature. For the refrigerated samples all samples, except *L. jensenii* 95.1 with yeast, also saw a drop in pH over the 7 days; which was also larger than the pH drops for the refrigerated single culture mageu samples. But the pH did not reduce as dramatically as for the room temperature samples. The *L. jensenii* 95.1 with yeast sample had a pH increase after being refrigerated for a week. Since the pH reduced more for the mixed culture mageu samples than the single culture mageu samples, the mixed culture continued to ferment the maize meal even after reaching the pH end point. This may have been due to the increased inoculum size of both the bacteria and yeast or because of an increase in microbial activity caused by the favourable co-fermentation.

While there is no set final pH for mageu products, the majority of past studies suggest an ideal pH between 3.5 and 3.9. However, Pswarayi and Gänzle (2019) reported a final pH of 2.96 of traditionally produced mageu and the patented method states that a final pH between 3 and 4.2 is acceptable (Fourcassie et al., 2017). For all mageu samples, the samples placed in the fridge were closer to the ideal final pH than the samples kept at room temperature after 7 days. However, all samples still had acceptable pH values after 7 days, both refrigerated and at room temperature.

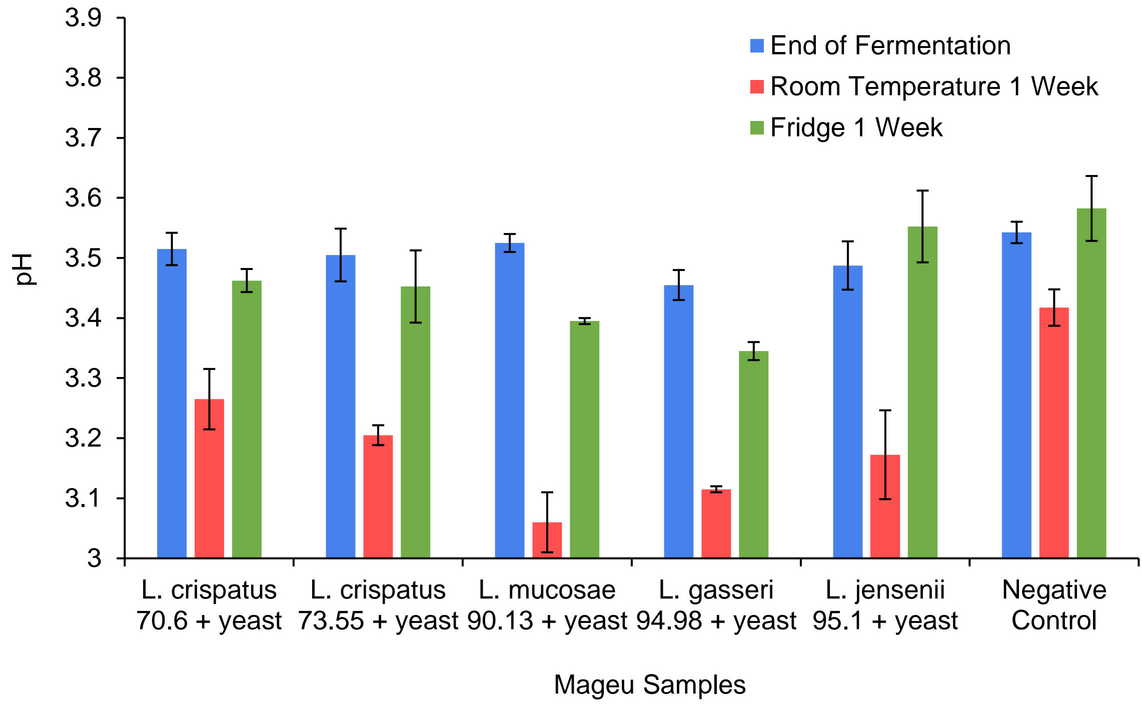

Figure 4-17 The pH values of mageu samples produced using single *Lactobacillaceae* isolates with yeast kept at room temperature and refrigerated for 1 week

Since the positive control was not included in the above results, the pH values taken for the taste testing sample will be used as an approximation for its shelf-life. Two runs were performed for the taste testing samples. The first run had a pH end point of 3.55 and fermentation was terminated at 92 hours. The second run had a final pH of 3.59 and fermentation ended at 116 hours. Due to the positive control undergoing spontaneous fermentation, it is not unexpected that the fermentation times differed between the runs. The bottles were kept in the fridge until the tasting. The pH was next measured before the taste testing. For the first run, the pH was measured 2 days 4 hours after fermentation ended and for the second run pH was measured 1 day and 4 hours after fermentation. Over this time, the first run had a pH drop from 3.55 to 3.45. The second run had a pH drop of 3.59 to 3.50. The pH reduction was most similar to the drop in pH observed for the mixed culture mageu samples placed in the fridge for 7 days. Therefore, the positive control had similar rates of pH reduction over 2 days 4 hours, compared to the probiotic samples over 7 days. So, the positive control continued to ferment at a faster rate while in storage (postacidification) than the other mageu samples. However, it is not known whether the rate of pH reduction would have slowed by day 7 and what the final pH would have been.

4.8 Sensory evaluation of mageu samples

A consumer acceptability test was performed for the sensory analysis of the mageu samples. This type of test requires untrained consumer panellists to rate samples on a liking scale (Lawless and Heymann, 2010). Each sample is ranked on a scale rather than the samples being compared to one another. The following factors were taken into account when selecting the mageu samples for tasting. Firstly, a limit of six samples would be given to each panellist, not to cause sensory overload. Of the six samples, one had to be a commercial sample and a positive control using the traditional South African production method. The other four samples were selected from the mageu samples produced with pure strains and single strains with yeast. Prior to the tasting, the mageu samples were analysed for *E. coli* and *Clostridium* spp. contamination to confirm their safety for consumption. No anaerobic spore forming bacteria, nor *E. coli* were observed in any of the samples.

Since *L. crispatus* strains are currently gaining traction as desirable probiotic candidates for vaginal health applications, one of the *L. crispatus* strains was selected for the tasting. As *L. crispatus* 73.55 showed varying degrees of growth during the runs of mageu production, *L. crispatus* 70.6 was selected as fermentations with this strain were more consistent. Since *L. crispatus* 70.6 is a homofermentative *Lactobacillus* spp., it was expected that a heterofermentative bacteria strain would have the most considerable difference in taste due to the different metabolites produced. Therefore, to compare the heterofermentative bacteria to the homofermentative bacteria, *L. mucosae* 90.13 was selected as the second bacterium. To study the impact of yeast on mageu flavour, the mageu samples produced using these strains with yeast were also investigated. The two bacterial isolates produced varying levels of sourness and consistencies in the mageu product when grown as pure cultures and with yeast (refer to Tables 4-8 and 4-9). Therefore, to summarise, the four mageu samples selected were produced using: pure *L. crispatus* 70.6, pure *L. mucosae* 90.13, *L. crispatus* 70.6 with yeast, and *L. mucosae* 90.13 with yeast.

As expressed in the section above, while the positive control produced a sweet smell during fermentation, it had soured considerably by analysis time. The pH of the tasting positive control had dropped from 3.55 at the end fermentation to 3.45 before tasting within 48 hours. Thus, the positive control had the lowest pH at the tasting, which was evident in its sour smell.

The panellists (n=21) were asked to rate each mageu sample on a degree of liking and likelihood of purchasing the sample. The results are displayed in the box and whisker plots shown in Figure 4-18 and Figure 4-19. In Figure 4-18, the higher scores indicate a larger degree of liking. For all samples, except the commercial mageu, there is a large spread across the degree of liking. This large spread shows that individual preferences played a role in how the panellists scored each sample. Additionally, many panellists may have been most familiar with traditional versions of the product, produced with varying inoculum sources.

The samples which received the lowest scores were pure *L. mucosae* 90.13 and the positive control. Of the laboratory-produced samples, *L. crispatus* 70.6 with yeast had the highest median score of 6.5. Since a score of five indicates neither liking or disliking a sample, scoring above this indicates the sample was, on average, liked more than disliked. Additionally, of the laboratory-produced samples, only the two *L. crispatus* 70.6 samples were graded a score of 9 (like extremely) by one or more panellists. By contrast, the pure *L. mucosae* 90.13 and positive control samples had medians that fell below average (score of 5). Overall, the commercial sample had the highest scores. It did not receive a score below 6 (like slightly). This was expected since it was the only sample with additives such as sugar and flavouring. No sugar was added to any laboratory-produced samples so as not to influence the flavour nuances produced by the *Lactobacillaceae* isolates and *S. cerevisiae*.

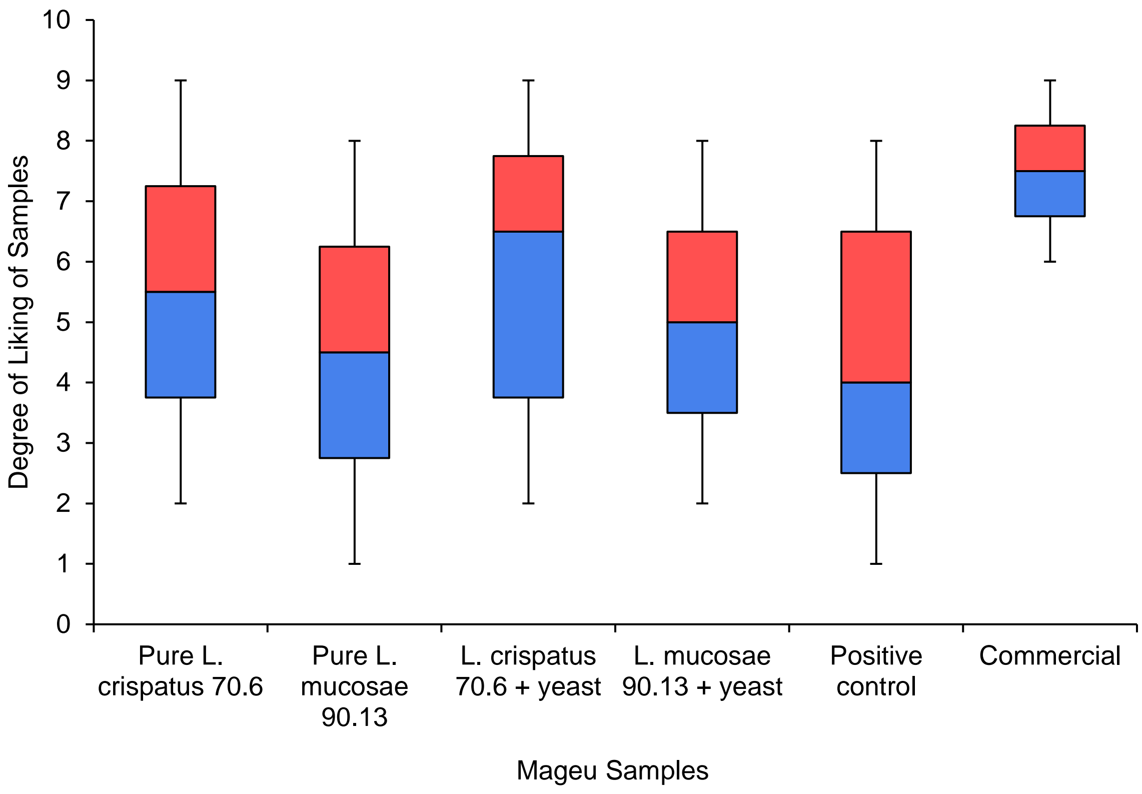

Figure 4-18 Box and whisker plot of degree of liking of the mageu tasting samples

The next box and whisker plot looks at the likelihood to purchase the mageu samples (Figure 4-19). Likelihood to purchase is ranked on a different scale with a score of 5 for ‘definitely would buy’ and a score of 1 representing ‘definitely would not buy’. Therefore, the average score of 3 shows neither preference nor dislike for the sample and is described as ‘maybe/maybe not’.

All mageu samples except pure *L. mucosae* 90.13 received at least one ‘definitely would buy’ response. However, all laboratory-produced samples also received at least one response of ‘definitely would not buy’. Similarly to the degree of liking results, the spread of the likelihood to purchase was high. Reflecting the liking results, the sample that most panellists would purchase was the commercial sample. Furthermore, 16 out of 21 responses said that they definitely would buy the commercial sample. The pure *L. mucosae* 90.13 and positive control samples had medians of score 2.5, which lies between ’probably would not buy’ and indifference about purchasing those samples. However, the three other laboratory-produced samples had medians of score 3, indicating indifference about purchasing those samples.

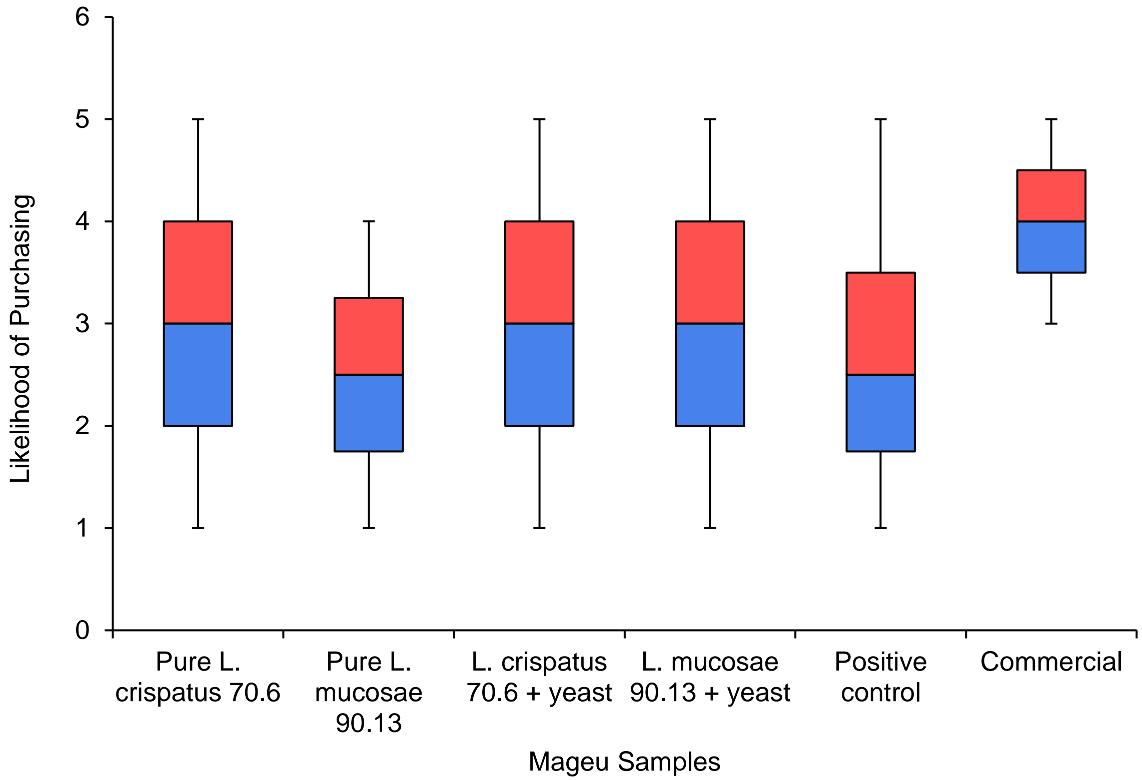

Figure 4-19 Box and whisker plot of likelihood of purchase for the mageu tasting samples

While the box and whisker plots showed the spread of the scores for each sample, they did not show the frequency of the scores received. Therefore, the means and standard deviation of the frequency distribution of scores are displayed in Figure 4-20.

Agreeing with the box and whisker plots, the commercial sample received the highest mean score, with the least variation expressed as standard deviation. By contrast, the laboratory-produced samples had large standard deviations, in line with the box and whisker plot results. On average, the mageu samples produced with the *Lactobacillaceae* isolates and yeasts achieved higher scores than the pure *Lactobacillaceae* strains. Furthermore, both *L. mucosae* 90.13 mageu samples performed better in the degree of liking than their *L. crispatus* 70.6 counterparts. However, pure *L. crispatus* 70.6 mageu received slightly higher scores (2.57 compared to 2.43) for the likelihood of purchasing compared to pure *L. mucosae 90.13* mageu. But both these averages fell in the unlikely to purchase category (scores below 3). Therefore, while the pure *L. mucosae* 90.13 mageu received a higher degree of liking to pure *L. crispatus* 73.55 mageu, neither of them was likely to be purchased.

The large standard deviations and spread of data can be attributed to the panellists' individual preferences. A larger consumer panel (n=100) is typically used to normalise individual preferences (Lawless and Heymann, 2010). However, due to the COVID-19 pandemic, students may have been hesitant to participate in in-person research projects, despite all the protocols put in place. Moreover, not all students were on campus because of most classes being offered remotely. Therefore, only 21 panellists participated in this consumer tasting. However, clinical trials using mageu as a probiotic delivery vehicle will occur subsequent to this study, and these results still provide insight into consumer preferences of the *Lactobacillaceae*-produced mageu.

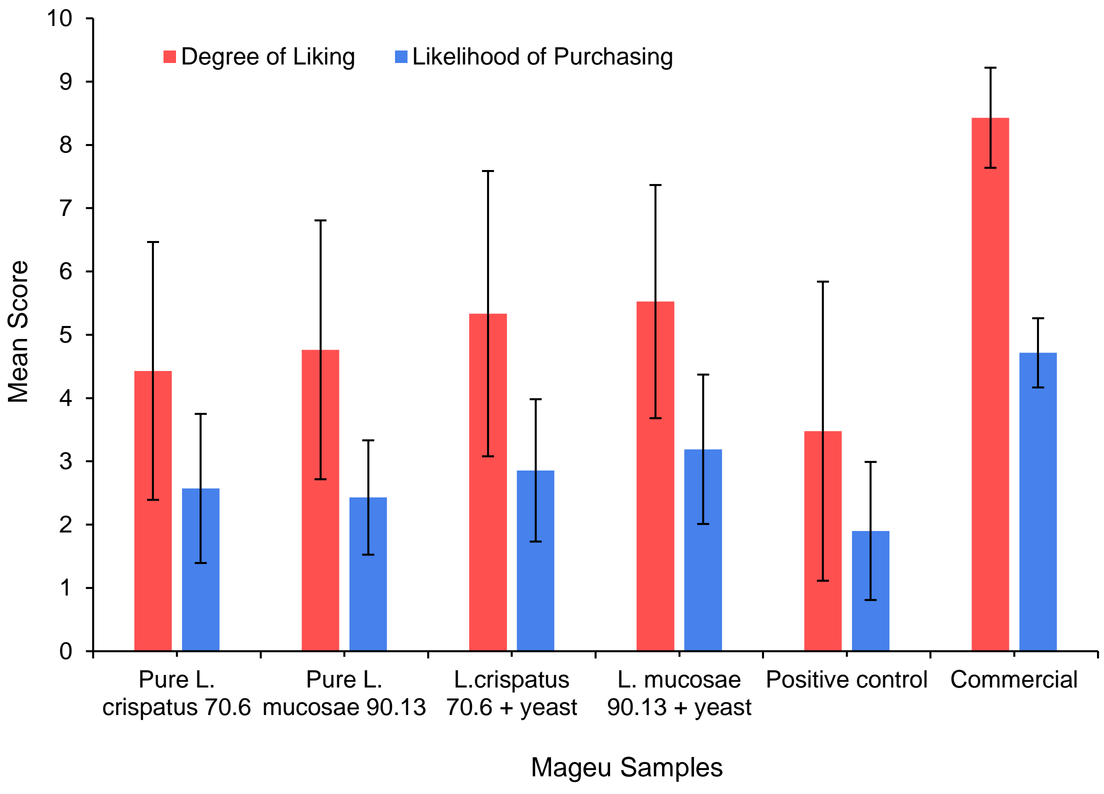

Figure 4-20 Mean scores of degree of liking and likelihood of purchasing for the mageu samples

5 Yoghurt Results and Discussion

This section investigates the ability of the various bacterial strains to ferment milk to yoghurt, both as pure cultures and mixed cultures with *S. thermophilus*. Crafty Cultures' 'Natural Yoghurt' culture containing *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* was used to produce the positive control. No active inoculum was supplied to the negative control. Fermentation was monitored by measuring a reduction in pH. The products were then analysed qualitatively and by measuring titratable acidity.

5.1 Fermentation pH profiles and length

In this section, the ability of the pure *Lactobacillaceae* strains to acidify milk and reduce the pH is investigated and compared. Additionally, the impact of *S. thermophilus* addition on the fermentation pH profiles and fermentation length is investigated. The yoghurt fermentations were measured continuously from the start of fermentation until hour 12. The majority of the methods investigated for yoghurt production end fermentation at a set pH (Capela et al., 2006; Coman et al., 2013; Donkor et al., 2007; Marinaki et al., 2016). The instructions for the freeze-dried yoghurt culture from Crafty Cultures suggested fermenting the milk between eight and 12 hours. However, Dave and Shah (1998) investigated fermentation pH for 24 hours, and Hekmat and Reid (2006) only monitored fermentation for six hours. Therefore, fermentation was ended after 24 hours or at a pH of 4.5, whichever occurred first. However, for the samples produced with the pure strains, the pH at hour 24 was still well above 4.5. Therefore, the samples were kept at an incubation temperature of 37°C, and a final reading was taken at hour 36.

5.1.1 Pure Lactobacillaceae isolates fermentation

The fermentation pH profiles for the yoghurt experiments using the pure strains are presented in Figure 5-1. Three runs were performed to investigate whether the pure bacterial strains could produce yoghurt. The first and second runs used fresh milk, while the third run used UHT milk. Two runs were performed for the negative control, the first using fresh milk and the second using UHT milk. A single duplicate run was performed for the positive control using UHT milk. The reason for the change in milk types is described below. The fermentation pH profiles show the averages and standard deviations of the three runs.

The initial pH of milk before inoculum addition averaged between 6.50 and 6.56. Inoculation did not alter this pH. During hours 0 to 12, the pH remained relatively unchanged for all samples with the pure strains and the negative control. At hour 12, the pure samples had only reached a pH between 6.32 and 6.37 and the negative control had an average pH of 6.41. The pH at hour 12 was not significantly different ($p>0.05$) for all pure strain samples from the negative control. By contrast, the positive control had reached a pH of 4.82 by hour 12. The positive control was the only sample to reach a pH of 4.5, recorded at hour 20. Therefore, the positive control was able to ferment milk as expected. The negative control had a defined lag phase until hour 10, after which a steep dip in the fermentation pH occurred. The standard deviations of the last three time points for the negative control were very high. The run using UHT milk saw a significant drop in pH at hour 20 which suggests that there may have been some naturally occurring microorganisms in the milk that fermented the milk when incubated at 37°C.

Figure 5-1 Decrease in pH over fermentation time for fermented milk samples produced with pure *Lactobacillaceae* isolates

For the first yoghurt run, the fresh milk was pasteurised at 85°C before being incubated at 42°C (Capela et al., 2006; Donkor et al., 2007). Based on a qualitative assessment, all the milk samples containing the pure isolates had curdled by hour 24. From Figure 5-1, it was also at hour 24 that the pH values became more variable. Photographs of these curdled samples are shown in Figure 5-2. Thus, the fermented product was no longer representative of milk nor yoghurt. During fermentation, milk can curdle from either a too high fermentation temperature or fermenting the milk for too long (Katz, 2012). Also, there may have been naturally-occurring microorganisms in the milk which were not removed during pasteurisation that acidified the milk and caused curdling. This would explain why the negative control saw a drop in pH despite no added inoculum. Since the pH remained mostly unchanged for all the samples until hour 12, suggesting very little fermentation occurring, it was possible that the milk curdled due to the fermentation temperature. Therefore, the next run was adapted so that the milk was pasteurised (as before) and then the samples were incubated at a lower temperature of 37°C following inoculation. The same curdling effect was observed for these samples. To remove the temperature effect entirely, the pasteurisation step was removed from the process by replacing the fresh milk with UHT milk. The final run used UHT milk and the samples were incubated at 37°C. Despite this change, the final run still curdled, indicating that it was not the pasteurisation that curdled the milk. Similarly to the pure strain-produced samples, the negative control samples also curdled in all cases. While some

bottles curdled more slowly than others, all eventually curdled without reaching the desired pH of 4.5. The curdling of the samples corresponded to an increase in the pH variance between the runs. During fermentation, no mixing was performed. Therefore, the pH readings taken when the samples had begun to curdle may not have been representative of the entire bottle. This is possibly what caused the highly variable results seen from hours 20 onwards in the pure strain samples and negative control.

The preliminary conclusion drawn from these results is that the pure *Lactobacillaceae* strains by themselves could not ferment milk to yoghurt within a 24-hour window and were thus unable to produce yoghurt. Therefore, no further analysis was performed on the samples.

Figure 5-2 Photographs of the milk samples inoculated with pure *Lactobacillaceae* strains

5.1.2 Single strain *Lactobacillaceae* with *S. thermophilus* fermentation

The fermentation pH profiles for the yoghurt samples produced using single *Lactobacillaceae* strains and *S. thermophilus* are presented in Figure 5-3. Due to time constraints, only one run of duplicates was performed for the yoghurt samples produced with *Lactobacillaceae* and *S. thermophilus* and the positive control. This run aimed to investigate whether the probiotic bacterial strains together with *S. thermophilus* could produce yoghurt. The duplicate run used UHT milk for the fermentations. The fermentation pH profiles show the averages and standard deviations of the duplicate run.

Before inoculation, the starting pH of the UHT milk was consistent with the pure strain samples and ranged between 6.47 and 6.52. After inoculation, the pH remained unchanged. All samples, including the controls, had an approximately constant pH for the first four hours of fermentation. After four hours, an exponential decay was observed for all *Lactobacillaceae* samples and the positive control (Figure 5-3). All fermentation samples with the *Lactobacillaceae* and *S. thermophilus* (including the positive control) displayed the same trend of pH decrease, with all samples seemingly running close to parallel

to one another. However, three samples had consistently lower pH values throughout fermentation. These three samples were the ones produced using the two *L. crispatus* strains and *L. gasseri* 94.98. The sample produced with *L. mucosae* 90.13 and *S. thermophilus* had the highest pH at the end of 24 hours of 4.77. While not all yoghurt samples produced with the mixed cultures achieved a pH of 4.5 in 24 hours, the final pH was in the range of 4.59 to 4.77. Marinaki et al. (2016) stopped fermentation at a pH of 4.7. Therefore, the experimental results obtained for the mixed cultures were close to this endpoint. Again, the positive control was the only system to reach a pH of below 4.5, measured at 20 hours. This could be due to a larger inoculum size (the cfu/ml of the commercial culture was not determined prior to inoculation) or due to the protocooperation between the *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*. An increase in the inoculum size of the *Lactobacillaceae* strains and *S. thermophilus* could potentially increase the rate of fermentation, and thereby allow for a pH of 4.5 to be reached by these systems within the 24-hour window.

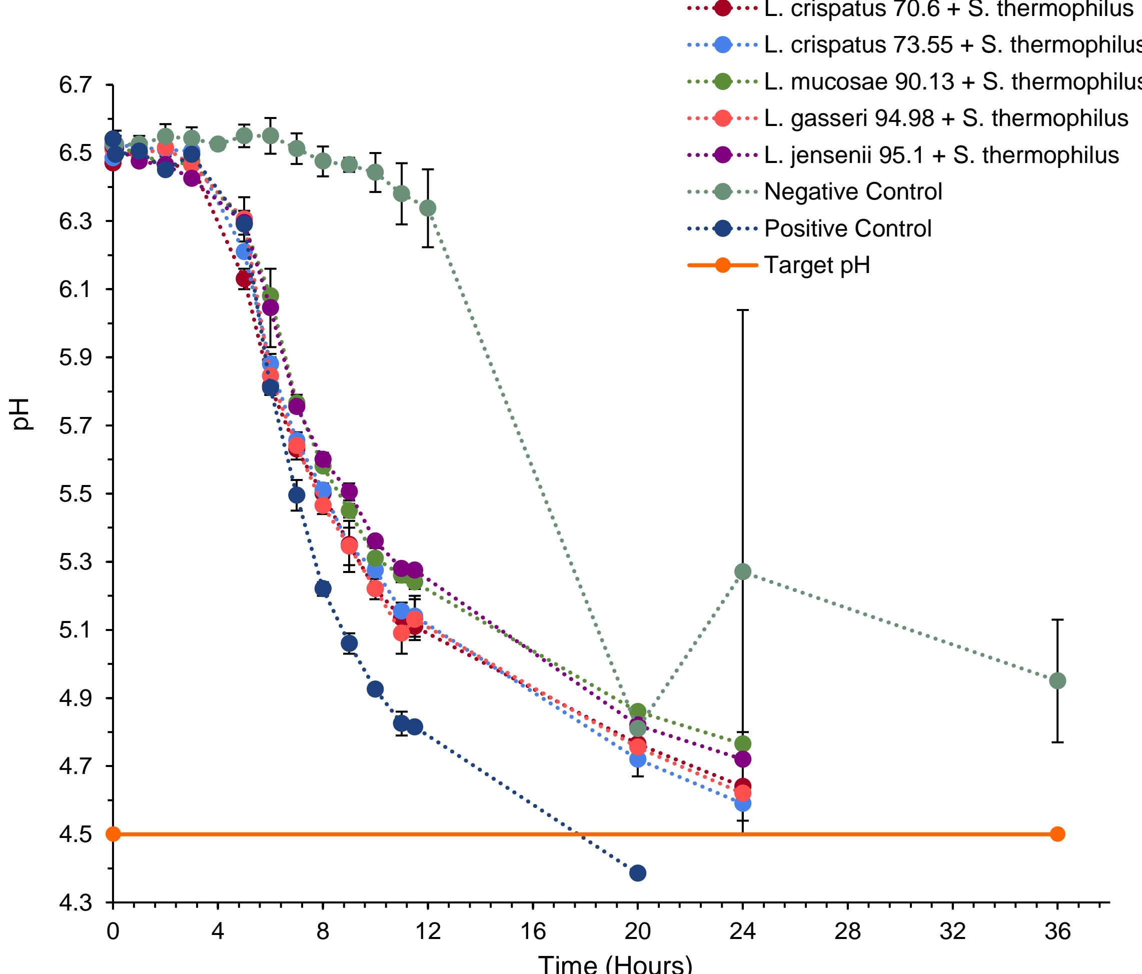

Figure 5-3 Plot of pH over fermentation length for yoghurt produced by single *Lactobacillaceae* strains with *S. thermophilus*

While together the *Lactobacillaceae* strains and *S. thermophilus* were able to ferment milk to a pH consistent with that of yoghurt, it is possible that the fermentation could be attributed to *S. thermophilus* activity alone. Therefore, a follow-up study should include a positive control produced with only *S. thermophilus* to compare the fermentation profiles to the yoghurts produced with the mixed cultures. Additionally, the cultures used for fermentation should be plated to determine the cell concentrations before and after fermentation. The aerobic M17 plates would give *S. thermophilus* counts, and while *S. thermophilus* would grow weakly on MRS anaerobically, the difference in colony morphologies would allow for the *Lactobacillaceae* colonies to be counted.

From a qualitative perspective, all fermentations with *Lactobacillaceae* and *S. thermophilus* produced yoghurt (Figure 5-4). This will be analysed further in Section 5.3, but all samples had thickened and smelled slightly sweet, with no curdling apparent.

Figure 5-4 Photographs of the milk samples inoculated with single *Lactobacillaceae* strains and *S. thermophilus*

5.1.3 Comparison of Lactobacillaceae isolates as single strains and with S. thermophilus

Though the pure strain *Lactobacillaceae* fermentations could not produce yoghurt as observed from qualitative analyses, it was still desired to establish if the *S. thermophilus* addition positively affected the pH reduction rate. The pH at hour 12 was therefore compared for all samples, as the pure strain samples had not yet curdled and were still qualitatively representative of fermenting milk at this hour. The pH values are plotted in Figure 5-5. Since the pure *Lactobacillaceae* isolates did not produce yoghurt, the x-axis represents the fermented milk samples produced by the bacterial strains. In all cases, the pH of pure strain samples showed no significant change from time zero. In contrast, the samples with *Lactobacillaceae* and *S. thermophilus* saw at least a pH change of 1.

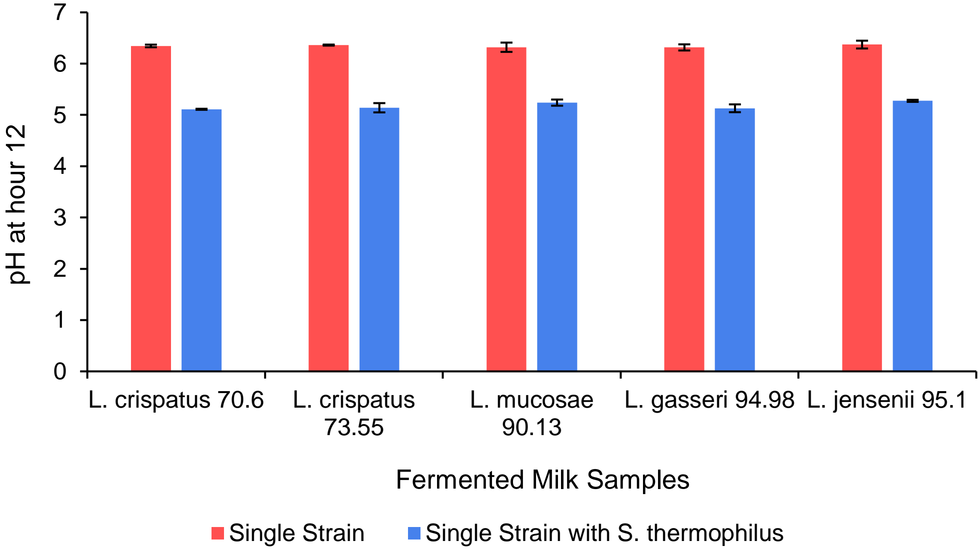

Figure 5-5 Graph of pH at hour 12 for the milk fermented with pure *Lactobacillaceae* isolates and *Lactobacillaceae* isolates with *S. thermophilus*

5.2 Titratable acidity of yoghurt samples

Since the products produced by the pure *Lactobacillaceae* strains and negative control using fresh milk were not yoghurt, no further analyses were performed on those samples. Therefore, TA was only determined for the yoghurt produced with the *Lactobacillaceae* strains and *S. thermophilus*, and both controls. The TA results are presented in Figure 5-6.

The positive control had the highest TA at 0.889 ± 0.003% (m/m), followed next by the negative control at 0.859 ± 0.026% (m/m). As mentioned in 5.1, the positive control was produced using UHT milk. Since the negative control runs produced using fresh full cream milk curdled, these results for the negative control include only those produced using UHT milk. The yoghurt samples produced with the *Lactobacillaceae* strains and *S. thermophilus* had similar TA values of between 0.770 ± 0.003% and 0.782 ± 0.005%, and were not statistically different ($p>0.05$). The TA results of the probiotic yoghurt compared to the negative and positive controls were much closer than the TA results of the mageu samples. The sample produced with *L. gasseri* 94.98 had the lowest TA, and the sample with *L. mucosae* 90.13 had the highest. Adhikari et al. (2002) terminated fermentation when TA reached values between 0.9 and 0.95%; however, no mention was given if this was a mass or volume percentage. Assuming it was a mass percentage, the negative and positive control results are similar to those reported by Adhikari et al. (2002). According to the Codex Alimentarius Standard for Fermented Milks, yoghurt should have a minimum TA of 0.6% (m/m) lactic acid (CODEX Alimentarius, 2018). Therefore, all milk fermentations using single strains with *S. thermophilus* could produce yoghurt as the TAs were all above 0.6% (m/m).

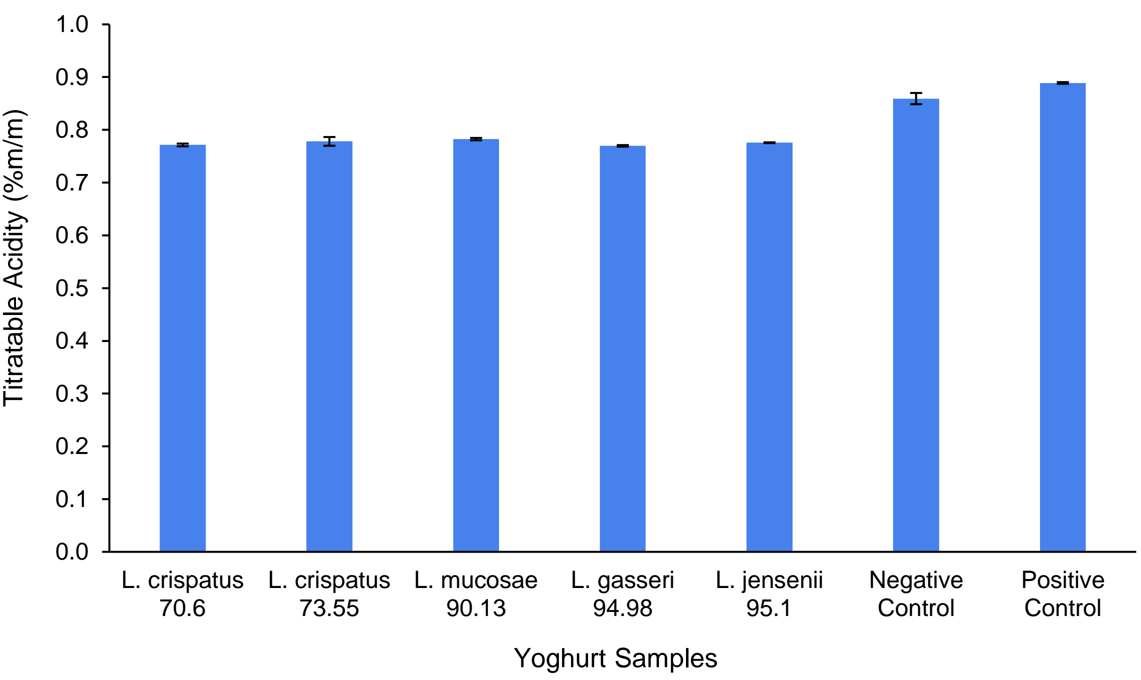

Figure 5-6 Plot of titratable acidity of yoghurt produced using single *Lactobacillaceae* isolates with *S. thermophilus*

5.3 Qualitative analysis of yoghurt

A qualitative analysis of yoghurt was performed by analysing the smell and consistency of the yoghurt and noticing any other additional sensory characteristics. Since all the products produced with pure strains curdled and did not have the consistency of yoghurt, no further sensory analyses were performed for those bottles. Smell was analysed in terms of sourness and sweetness, while consistency described thickness and homogenization of the product.

The results for the bottles produced with single strains and *S. thermophilus* are displayed in Table 5-1. Since the milk was not mixed during fermenting, some lumps had formed by the end of fermentation. However, the bottles were shaken before pouring, which removed all lumps and gave a smooth consistency. The positive control had the sweetest smell, followed by the *Lactobacillaceae* isolates and *S. thermophilus* samples, and then the negative control. The two controls were also thicker than the *Lactobacillaceae* strains and *S. thermophilus* samples. Overall, very little difference was noticed across all categories for the yoghurts produced with the *Lactobacillaceae* strains and *S. thermophilus*.

During fermentation, the added starter culture influences the fermentation of milk to yoghurt by performing three major chemical conversions. Firstly, the bacteria convert the available carbohydrates (lactose) into lactic acid via hydrolysis. Secondly, caseins are converted to peptides and free amino acids during proteolysis. Lastly, free fatty acids are formed from milk fat during lipolysis (Steele et al., 2013). During these processes, compounds that contribute to the flavour of yoghurt are also produced (Smid and Kleerebezem, 2014). The difference in starter culture of yoghurt influences the compounds produced which are partly responsible for flavouring (Tamime and Robinson, 2007). These compounds are lactic acid, the primary acid produced during fermentation, diacetyl, acetaldehyde, and acetoin, which can affect sensory characteristics (Tamime and Robinson, 2007). However, for the *Lactobacillaceae* strains, little change was observed on the aromatic compounds produced compared to the controls, but increasing the inoculation concentration may influence this.

Table 5-1 Qualitative results of yoghurt produced with single *Lactobacillaceae* strains and *S. thermophilus* and the controls

Sample	Smell	Consistency	Other Notes
L. crispatus 70.6 with *S. thermophilus*	Slightly sweet	Smooth and thick	Solid layer of yoghurt had formed on bottom of bottle
L. crispatus 73.55 with *S. thermophilus*	Slightly sweet	Smooth and thick	
L. mucosae 90.13 with *S. thermophilus*	Slightly sweet	Smooth and thick	
L. gasseri 94.98 with *S. thermophilus*	Slightly sweet	Smooth and thick	
L. jensenii 95.1 with *S. thermophilus*	Slightly sweet	Smooth and thick	
Negative Control	Very faintly sweet	Very thick	Before shaking bottle, had a gel-like consistency where it was pulling away from the bottle walls (this was seen at the beginning of curdling)
Positive Control	Sweet	Smooth and very thick	

The differences between the *Lactobacillaceae* isolates runs and strains were less noticeable than the qualitative analysis results for mageu. The fermentation time for mageu is considerably longer than for yoghurt. Therefore, the metabolites produced by the strains during mageu production may be in more significant concentrations and have a more noticeable influence on the product's flavour and other sensory characteristics.

6 Conclusion and Recommendations

6.1 Conclusions

The goal of this research project was to determine whether previously isolated *Lactobacillaceae* strains were able to ferment maize meal to mageu and/or ferment milk to yoghurt to act as potential low-cost probiotic delivery vehicles for the improvement of vaginal health. The bacterial strains' potentials were analysed both as pure strains and supplemented with traditionally used microorganisms. For mageu, the bacterial strains were supplemented with Anchor Yeast for the mixed culture fermentations, while *S. thermophilus* was used with the bacterial strains for mixed culture yoghurt production. Since the aim was to produce probiotic fermented foods, the products also needed to be acceptable for consumption. Therefore, the second part of the research included consumer acceptability tests for four probiotic mageu samples.

To determine whether the bacterial strains were successful in fermenting maize meal to mageu, the fermented products were analysed with respect to their fermentation profiles, lactic acid and ethanol production, total solids content, titratable acidity, viable cell counts, and qualitative characteristics.

Comparing the fermentation profiles of the negative control (uninoculated) to the single strain mageu fermentations showed that the *Lactobacillaceae* strains facilitate mageu fermentation to the same degree as the positive control (traditional flour inoculum), with a steep drop in pH at the beginning of fermentation observed. However, the single strain mageu samples reached a point where the pH reduction rate slowed. This was possibly due to the low pH inhibiting microbial growth since the *Lactobacillaceae* isolates are better suited to the vaginal pH of around 4.5. The fermentation times for the pure strains varied between 78 hours and 144 hours, with *L. crispatus* 73.55 having the shortest fermentation time. From comparison with other studies, increasing the inoculum size has the potential to decrease these fermentation times using the pure strain mageu samples. Despite the long fermentation times, all bacterial isolates were able to ferment maize meal to mageu, with the samples reaching final pH values between 3.52 and 3.66.

To analyse what caused the drop in pH, titratable acidity and lactic acid concentration were measured. While none of the pure strain mageu samples reached the suggested titratable acidity of 0.4%, the results indicated that acid had been produced. This was confirmed with the lactic acid concentration measurement using HPLC. The pure strain mageu samples had lactic acid concentration values between 0.601 g/L and 1.24 g/L.

To determine whether the mageu product requirements were met, ethanol concentration and total solids content were measured. The ethanol concentrations for all pure strain mageu samples were well below the maximum allowable limit of 2.5 g/L. Furthermore, all pure strain mageu samples had total solids contents above the minimum limit of 8 % (m/m).

These results were used to address the mageu part of the first key research question:

- Can pure *Lactobacillaceae* cultures, identified by Happel et al. (2020), ferment milk to yoghurt or ferment maize meal to mageu, as seen by the production of pH-lowering metabolites, and the formation of products meeting the respective product requirements?

To answer this question, the *Lactobacillaceae* isolates were able to produce mageu as single cultures as seen by the production of pH-lowering metabolites to reach a final pH of around 3.5. Furthermore, all mageu samples produced using the pure cultures met the production standards for mageu as set by the South African National Standards.

The second part of the first research question investigated whether the bacterial isolates can ferment milk to yoghurt as pure cultures by producing pH lowering metabolites and meeting the yoghurt production standards. The end-point for yoghurt fermentation is marked by a pH of around 4.5. Based on this, none of the pure *Lactobacillaceae* strains were able to ferment milk to yoghurt as they could

not reach a pH around 4.5. By hour 12, the pure bacterial samples had only reached a pH between 6.32 and 6.37 and leaving the fermentations until 36 hours did not allow the samples to reach the desired pH of 4.5. Instead, the milk curdled and qualitatively, yoghurt was not produced. Therefore, as pure strains, the *Lactobacillaceae* isolates were unable to ferment milk to yoghurt.

The second research question investigated whether the addition of Anchor Yeast (*S. cerevisiae*) positively affected maize meal fermentation:

- Can supplementing isolated *Lactobacillaceae* isolates with *Saccharomyces cerevisiae* for mageu production (maize meal fermentation) positively affect the fermentation process with respect to the fermentation time and the final pH?

The mageu samples produced with the bacterial strains and yeast showed an instantaneous drop in pH following inoculum addition, which aided in the reduced fermentation time for the mixed mageu samples. All mageu samples saw statistically significant decreases in fermentation time, correlated with an increase in hydronium ion activity, with the addition of yeast compared to the pure strain mageu samples. Of all the bacterial strains, the addition of yeast had the greatest impact on decreasing fermentation time for the *L. gasseri* 94.98 mageu.

Since all mixed culture samples had fermentation times shorter than the negative control, the decrease in fermentation time from the pure culture mageu samples was a result of the microbial action of the single strain and yeast together. The final pH values were also closer to a pH of 3.5 for the mixed mageu samples with final pH values ranging from 3.48 to 3.54. These results were echoed with statistically significant increases in titratable acidity with the addition of yeast. However, again none of the mageu samples produced with the bacterial strains and yeast met the suggested titratable acidity of 0.4%. Furthermore, the titratable acidity values did not correspond to the final pH values, and so an alternative equation was formulated using the percentage acidity and pH to determine titratable acidity. However, these results showed that during the titrations the base, sodium hydroxide, reacts with other acids to neutralise the sample, which produces a higher titratable acidity than the concentration of lactic acid alone, as determined by HPLC.

Again, ethanol concentration was investigated to ensure the maize meal fermentations met the maize production standards. There was larger variance in ethanol concentrations for the mixed culture mageu samples than the pure culture mageu samples. This is possibly attributed to varying cell concentrations of yeast used since the Anchor Yeast was store bought and contained additives such as rehydrating agent and ascorbic acid. However, on average all mixed culture mageu samples had ethanol concentrations lower than the maximum allowable limit. Lastly, the mixed culture mageu samples also had total solids contents above 8% (m/m) to meet the mageu production standards.

Therefore, the addition of *S. cerevisiae* to the *Lactobacillaceae* strains positively affected maize meal fermentations by significantly decreasing the fermentation time, allowing the final product pH of around 3.5 to be reached earlier than the pure strain mageu samples. Furthermore, the mixed culture samples met the production requirements for mageu.

To further investigate whether yeast addition positively affected the mageu fermentations, cell counts were performed to determine the increase in cell concentrations before and after fermentation. For all *Lactobacillaceae* strains, the increase in cell colonies on plated MRS during fermentation was greater for the mixed culture mageu samples than the pure culture samples. Therefore, the mixed culture fermentations saw more pronounced increases in the MRS plate cell counts than the pure culture fermentations. However, no 16S rRNA gene sequencing of the microbial cells grown on the MRS plates following fermentation was performed and therefore it could not be confirmed that all of the colonies were the *Lactobacillaceae* isolates.

From a quantitative aspect (measuring pH and fermentation time), the *Lactobacillaceae* strains were able to ferment maize meal to mageu. However, since the aim of the project was to produce fermented foods fit for consumption, qualitative characteristics of the fermentations were also considered. Therefore, a qualitative analysis was performed to determine if the sensory characteristics of the fermented maize meal were consistent with that of mageu. The various bacterial strains produced different aromatic nuances in the final products, with some being sourer than others. Overall, *L.*

crispatus 70.6 produced the sourest mageu samples as both a pure culture and mixed culture. The second qualitative characteristic investigated was consistency. While there was variation in the consistency of the runs for the same samples; overall, the mixed samples with yeast were thinner than their pure sample counterparts. However, the thinnest sample was consistently the positive control. The final characteristic investigated was the production of gas. Of the samples which produced gas, the majority were mixed cultures, indicating that yeast addition was likely the most significant contributor toward gas production.

Lastly, shelf-life of the mageu products was investigated to determine whether postacidification occurred and to what extent. The mageu products were stored at room temperature or in the refrigerator for one week. The refrigerated pure culture mageu samples saw only slight changes in the pH after a week, while the room temperature samples saw a more noticeable decrease in pH. However, the drop in pH for the mixed culture mageu samples at both room temperature and refrigerated were larger than for the pure culture mageu samples. This was possibly caused by the increased inoculum size of the mixed culture (the bacteria and yeast) or due to an increase in microbial activity caused by the favourable co-fermentation. Overall, all probiotic mageu samples had acceptable pH values by the end of one week both at room temperature and refrigerated.

The third research question investigated whether supplementing the *Lactobacillaceae* isolates with *S. thermophilus* could positively affect milk fermentation:

- Can supplementing isolated *Lactobacillaceae* strains with *Streptococcus thermophilus* positively affect yoghurt production (milk fermentation) with respect to the fermentation time and the final pH?

Since the pure bacterial isolates alone could not ferment milk, it was important to determine whether supplementing with *S. thermophilus* would result in successful fermentation to reach the desired end-point pH of 4.5 and qualitatively produce yoghurt. Firstly, an exponential decay in the fermentation pH was observed for the mixed culture fermentation, whereas a more linear trend was observed for the pure strain fermentations. While none of the mixed culture fermentations were able to reach the desired pH of 4.5, the final pH values were not substantially higher at between 4.59 and 4.77. To compare if the addition of *S. thermophilus* positively affected fermentation, the pH at hour 12 was investigated for both pure and mixed culture samples. At hour 12 the mixed culture samples had a pH change of at least 1 lower than their pure culture counterparts. To investigate the role of pH reducing metabolites on pH reduction, the titratable acidity for these samples was measured. All mixed culture samples had titratable acidity values above the minimum requirement of 0.6 % (m/m) set by the Codex Alimentarius Standard for fermented milks. Qualitatively, the mixed culture samples had not curdled by hour 24 but had instead thickened. Little difference in consistency and smell was noticed between the mixed culture yoghurt samples during the qualitative analysis. However, the positive and negative controls had thicker consistencies than the probiotic samples. Therefore, *S. thermophilus* addition positively affected yoghurt production by creating a fermented product resembling yoghurt and achieving a pH close to 4.5 after 24 hours.

The final research question considered the consumer acceptability of the mageu probiotic products:

- What is the consumer acceptability of the mageu probiotic products?

Four probiotic mageu samples were selected for consumer acceptability analysis. Two *Lactobacillaceae* strains were selected for analysis. The mageu samples produced using the pure strains and mixed culture with yeast were included to determine the impact of yeast addition on the taste of the mageu. The two strains selected were *L. crispatus* 70.6 and *L. mucosae* 90.13. *L. crispatus* strains are currently popular candidates for vaginal health applications and of the two *L. crispatus* strains, *L. crispatus* 70.6 performed more consistently during the fermentations. Since *L. crispatus* 70.6 is homofermentative, *L. mucosae* 90.13, a heterofermenter, was selected as the second strain to analyse the differences in taste due to the differences in metabolite production between the two strains.

The results showed a large spread in the degree of liking for the probiotic mageu samples, indicating that individual preference played a large role in the panellists' selection. Of the probiotic samples, *L. crispatus* 70.6 with yeast had the highest median score and on average this sample was liked more than it was disliked. The *L. crispatus* 70.6 samples received better median scores than the *L. mucosae*

90.13 samples. The likelihood of purchasing the samples was also investigated. The *L. crispatus* 70.6 samples again performed better on average than the *L. mucosae* 90.13 samples. However, when investigating the mean scores received for degree of liking, the *L. mucosae* 90.13 results were higher than the *L. crispatus* 70.6 results. But there was substantial variance in the results reported which is again related to individual taste preferences. Overall, the commercial sample performed best of the samples tasted. The commercial sample was sweetened and flavoured whereas the laboratory-produced samples were not.

To contextualise the results while considering the hypotheses for this project, they are stated below. The first hypothesis refers to mageu:

1. Probiotic mageu can be produced by fermenting maize meal using *Saccharomyces cerevisiae* and *Lactobacillaceae* strains that were among the top ten isolates for probiotic development identified by Happel et al. (2020). This is because the isolates are known lactic acid producers and are able to tolerate low pH. Successful fermentation will be characterised by the final product displaying qualitative characteristics of mageu and the strains being able to ferment maize meal to the pH of around 3.5 that is characteristic of mageu. Since lactic acid is the main flavour compound for mageu, the sensory profile of the probiotic mageu will not differ significantly from the control mageu.

The *Lactobacillaceae* strains were indeed able to produce mageu both as pure strains and mixed cultures with *S. cerevisiae* as seen through qualitative analysis and reaching a pH of around 3.5. Despite reaching final pH values of around 3.5, the pure culture samples showed a reduction in fermentation rates as the pH dropped. While the strains can tolerate and lower pH, the characteristic vaginal pH is between 4 and 4.5; therefore, as the pH dropped below this, the growth rate slowed. From the titratable acidity, lactic acid and ethanol results the various *Lactobacillaceae* strains produced varying levels of acidic compounds and ethanol. Therefore, varying degrees of sourness (ranging from neutral to sourness level 4) were recorded for the different bacterial strains. However, since the positive control exhibited sourness levels of 2 and 3, the results were not greatly different compared to the positive control, although slight distinctions in the smell profiles of the various strains was observed. Therefore, the mageu results agree with the first hypothesis.

The second hypothesis refers to yoghurt fermentation:

2. Probiotic yoghurt can be produced by fermenting milk using *Streptococcus thermophilus* and *Lactobacillaceae* strains identified by Happel et al. (2020) as most promising for treatment or prevention of bacterial vaginosis, in replacement of the traditional *L. delbrueckii* subsp. *bulgaricus* yoghurt bacterium. Like *L. delbrueckii* subsp. *bulgaricus*, the *Lactobacillaceae* isolates produce lactic acid and are able to tolerate low pH. This will enable the mixed culture of *S. thermophilus* and the *Lactobacillaceae* strains to ferment milk to the requisite pH of 4.5 for yoghurt.

Different to the mageu results, only when supplemented with the traditionally-used microorganism (*S. thermophilus*), were the *Lactobacillaceae* strains able to produce yoghurt. All the mixed culture fermentation samples were able to reach a final pH of around 4.5. However, it is not known whether the drop in pH was due to the microbial activity of *S. thermophilus* alone or favourable co-fermentation. Despite this, the mixed culture fermentations were able to ferment milk to yoghurt. Therefore, the results from the project support the second hypothesis.

The experimental method developed in this project can be used to test additional probiotic candidates. The results from this study are the first step in developing the fermented foods mageu and yoghurt as low-cost delivery vehicles specifically for the improvement of vaginal health of South African women. This could be tested through observational trials where women are given the fermented foods and their vaginal health monitored.

6.2 Recommendations

The recommendations given below are suggestions for further work and improvements of the methods used to produce the fermented foods, and analysis of the fermentation products in determining the success of the *Lactobacillaceae* strains in producing mageu and yoghurt. The first set of recommendations are specific to the mageu fermentations and sensory evaluation using consumer acceptability tests.

During the mixed culture mageu fermentations dried yeast was weighed and added to the maize-water mixture. However, from the results it is possible that the concentration of yeast added to the mixture varied due to the additives in the store-bought product. It is therefore recommended that the dried yeast be diluted in YPD media and plated on YPD agar to determine the cell concentration at a specific optical density. This known correlation can then be used to determine the desired volume of culture to add to the maize-water mixture. This was the same method used for the *Lactobacillaceae* isolates.

When analysing the fermentation products for cell concentration, it is recommended that the cell counts be performed as soon as possible following the end of fermentation. This would produce more accurate results as the viable cell concentration of the microorganisms in the fermentation products decrease during storage. To identify the colonies grown on MRS and YPD it is suggested that sequencing of the 16S rRNA gene and/or gram staining be performed to classify the microorganisms and determine whether the cell concentration of the *Lactobacillaceae* isolates and yeast increased during fermentation.

For the sensory analysis of the mageu samples, some differences in the flavours produced by the bacterial strains were observed. Past research has investigated the role that lactic acid bacteria have on the flavour of fermented foods. The study performed by Winters et al. (2018) investigated the different metabolites produced by lactic acid bacteria and yeast during sourdough production and how these influenced the aroma profiles of the sourdough. It is suggested for future work that the same method, headspace solid phase micro-extraction gas chromatography mass spectrometry, be used to analyse the aroma profiles of the probiotic mageu samples.

As noted from the consumer acceptability tests for the mageu samples, the commercial sample performed best. It is suspected that this is due to the added sugar and flavouring used to produce the commercially available products. No additives were used to produce the mageu samples, not to mask the flavour differences provided by the various bacterial strains and yeast. However, since taste of health products plays a significant role in whether consumers will purchase products, it is recommended that sugar and/or flavouring be added during production for the mageu samples to be used as probiotic delivery vehicles.

During the consumer acceptability tests, the panellists' individual preferences played a large role in the results for the probiotic mageu samples. These individual preferences lead to a large spread of data points for the degree of liking and likelihood to purchase. Lawless and Heymann (2010) suggest that a large consumer panel (n=100) be used during consumer acceptability tests to normalise individual preferences. As acknowledged, it was difficult to reach this many panellists due to the COVID-19 pandemic. However, for future studies, it is recommended that a larger sample size of panellists be used so that individual preferences do not weigh as heavily in the results.

The next set of recommendations are specific to the yoghurt fermentations. While together the *Lactobacillaceae* strains and *S. thermophilus* were able to ferment milk to a pH consistent with that of yoghurt, it is possible that the fermentation could be attributed to *S. thermophilus* activity alone. Therefore, a follow-up study should include a control produced with only *S. thermophilus* to compare the fermentation profiles to the yoghurts produced with the mixed cultures. Furthermore, the samples should be plated out during fermentation and after to confirm that the isolates are still viable.

For both the mageu and yoghurt fermentations, it is suggested that further studies be conducted to investigate whether an increase in the inoculum cell concentration decreases the fermentation period to meet the final pH values. It would be preferable to determine whether the yoghurt fermentation time

could decrease with an increase in initial cell concentration to match the results of comparable probiotic yoghurt studies.

The final suggestion for further work is that clinical trials be performed to determine whether the mageu and yoghurt samples produced with the *Lactobacillaceae* isolates perform well as probiotic delivery vehicles (confer a health benefit and supply the bacteria in sufficient quantities); and whether the fermented products do improve the vaginal health of South African women when used in conjunction with antibiotics or alone.

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Appendix A: Questionnaire examples

ACCEPTABILITY OF YOGHURT SAMPLES

PLEASE CIRCLE WHICHEVER IS APPLICABLE		
GENDER: Male / Female / Prefer not to say / Other _______________	**AGE**: 18-25 / 26-30 / 31-40 / 41+	**HOW OFTEN DO YOU CONSUME YOGHURT?** Daily / Approx. 2-3 times a week / 2x per month / Approx. 4 times a year / never

INSTRUCTIONS

- Please taste the samples from **left to right**.
- Rinse your mouth with **water** before beginning. Rinse your mouth between the samples. Take a **generous scoop** from each sample.
- Evaluate the samples on the following scales for 1] **degree of liking** & 2] **purchase intent**. In each case, place an X next to the preferred answer.

HOW DO YOU LIKE EACH OF THE FOLOWING SAMPLES? Check one phrase to indicate your overall opinion of the product.

CODE		CODE		CODE		CODE		CODE		CODE	
	Like extremely		Like extremely		Like extremely		Like extremely		Like extremely		Like extremely
	Like very much		Like very much		Like very much		Like very much		Like very much		Like very much
	Like moderately		Like moderately		Like moderately		Like moderately		Like moderately		Like moderately
	Like slightly		Like slightly		Like slightly		Like slightly		Like slightly		Like slightly
	Neither like nor Dislike		Neither like nor Dislike		Neither like nor Dislike		Neither like nor Dislike		Neither like nor Dislike		Neither like nor Dislike
	Dislike slightly		Dislike slightly		Dislike slightly		Dislike slightly		Dislike slightly		Dislike slightly
	Dislike moderately		Dislike moderately		Dislike moderately		Dislike moderately		Dislike moderately		Dislike moderately
	Dislike very much		Dislike very much		Dislike very much		Dislike very much		Dislike very much		Dislike very much
	Dislike extremely		Dislike extremely		Dislike extremely		Dislike extremely		Dislike extremely		Dislike extremely

WOULD YOU PURCHASE THESE SAMPLES??

CODE		CODE		CODE		CODE		CODE		CODE	
	Definitely would buy		Definitely would buy		Definitely would buy		Definitely would buy		Definitely would buy		Definitely would buy
	Probably would buy		Probably would buy		Probably would buy		Probably would buy		Probably would buy		Probably would buy
	Maybe / Maybe not		Maybe / Maybe not		Maybe / Maybe not		Maybe / Maybe not		Maybe / Maybe not		Maybe / Maybe not
	Probably would not buy		Probably would not buy		Probably would not buy		Probably would not buy		Probably would not buy		Probably would not buy
	Definitely would not buy		Definitely would not buy		Definitely would not buy		Definitely would not buy		Definitely would not buy		Definitely would not buy

Thank you very much for your time & assistance – please collect a gift as you leave the room!

ACCEPTABILITY OF MAGEU SAMPLES

PLEASE **CIRCLE** WHICHEVER IS APPLICABLE		
GENDER: Male / Female / Prefer not to say / Other _______________	**AGE**: 18-25 / 26-30 / 31-40 / 41+	**HOW OFTEN DO YOU CONSUME MAGEU?** Daily / Approx. 2-3 times a week / 2x per month / Approx. 4 times a year / never

INSTRUCTIONS

- Please taste the samples from **left to right.**
- Rinse your mouth with **water** before beginning. Rinse your mouth between the samples. Take a **generous scoop** from each sample.
- Evaluate the samples on the following scales for 1] **degree of liking** & 2] **purchase intent**. In each case, place an X next to the preferred answer.

HOW DO YOU LIKE EACH OF THE FOLOWING SAMPLES? Check one phrase to indicate your overall opinion of the product.

CODE		CODE		CODE		CODE		CODE		CODE	
	Like extremely		Like extremely		Like extremely		Like extremely		Like extremely		Like extremely
	Like very much		Like very much		Like very much		Like very much		Like very much		Like very much
	Like moderately		Like moderately		Like moderately		Like moderately		Like moderately		Like moderately
	Like slightly		Like slightly		Like slightly		Like slightly		Like slightly		Like slightly
	Neither like nor Dislike		Neither like nor Dislike		Neither like nor Dislike		Neither like nor Dislike		Neither like nor Dislike		Neither like nor Dislike
	Dislike slightly		Dislike slightly		Dislike slightly		Dislike slightly		Dislike slightly		Dislike slightly
	Dislike moderately		Dislike moderately		Dislike moderately		Dislike moderately		Dislike moderately		Dislike moderately
	Dislike very much		Dislike very much		Dislike very much		Dislike very much		Dislike very much		Dislike very much
	Dislike extremely		Dislike extremely		Dislike extremely		Dislike extremely		Dislike extremely		Dislike extremely

WOULD YOU PURCHASE THESE SAMPLES??

CODE		CODE		CODE		CODE		CODE		CODE	
	Definitely would buy		Definitely would buy		Definitely would buy		Definitely would buy		Definitely would buy		Definitely would buy
	Probably would buy		Probably would buy		Probably would buy		Probably would buy		Probably would buy		Probably would buy
	Maybe / Maybe not		Maybe / Maybe not		Maybe / Maybe not		Maybe / Maybe not		Maybe / Maybe not		Maybe / Maybe not
	Probably would not buy		Probably would not buy		Probably would not buy		Probably would not buy		Probably would not buy		Probably would not buy
	Definitely would not buy		Definitely would not buy		Definitely would not buy		Definitely would not buy		Definitely would not buy		Definitely would not buy

Thank you very much for your time & assistance – please collect a gift as you leave the room!

Appendix B: Ethics application

B.1 EBE assessment of ethics in research form

Application for Approval of Ethics in Research (EiR) Projects
Faculty of Engineering and the Built Environment, University of Cape Town

ETHICS APPLICATION FORM

Please Note:
Any person planning to undertake research in the Faculty of Engineering and the Built Environment (EBE) at the University of Cape Town is required to complete this form **before** collecting or analysing data. The objective of submitting this application *prior* to embarking on research is to ensure that the highest ethical standards in research, conducted under the auspices of the EBE Faculty, are met. Please ensure that you have read, and understood the **EBE Ethics in Research Handbook** (available from the UCT EBE, Research Ethics website) prior to completing this application form: http://www.ebe.uct.ac.za/ebe/research/ethics1

APPLICANT'S DETAILS		
Name of principal researcher, student or external applicant		Katherine Hartzenberg-Aeroe
Department		Department of Chemical Engineering
Preferred email address of applicant:		HRTELL001@myuct.ac.za
If Student	Your Degree: e.g., MSc, PhD, etc.	MSc
	Credit Value of Research: e.g., 60/120/180/360 etc.	180 credits
	Name of Supervisor (if supervised):	Dr Marijke Fagan-Endres and Dr Brian Kullin
If this is a researchcontract, indicate the source of funding/sponsorship		Research costs are funded by NRF CSUR
Project Title		Fermented foods production using isolated probiotic *Lactobacillus* species for the improvement of vaginal health: the case of mageu and yoghurt

I hereby undertake to carry out my research in such a way that:
- there is no apparent legal objection to the nature or the method of research; and
- the research will not compromise staff or students or the other responsibilities of the University;
- the stated objective will be achieved, and the findings will have a high degree of validity;
- limitations and alternative interpretations will be considered;
- the findings could be subject to peer review and publicly available; and
- I will comply with the conventions of copyright and avoid any practice that would constitute plagiarism.

APPLICATION BY	Full name	Signature	Date
Principal Researcher/ Student/External applicant	Katherine Hartzenberg-Aeroe		6 May 2021
SUPPORTED BY	**Full name**	**Signature**	**Date**
Supervisor (where applicable)	Dr Marijke Fagan-Endres Dr Brian Kullin		6 May 2021

APPROVED BY	Full name	Signature	Date
HOD (or delegated nominee) Final authority for all applicants who have answered NO to all questions in Section 1; and for all Undergraduate research (Including Honours).			
Chair: Faculty EIR Committee For applicants other than undergraduate students who have answered YES to any of the questions in Section 1.	Prof H von Blottnitz		16/05/2021

B.2 DSA research access to students application

	RESEARCH ACCESS TO STUDENTS	DSA 100

NOTES

1. This form must be **FULLY** completed by all applicants who want to access UCT students for the purpose of research or surveys.
2. Return the fully completed **(a) DSA 100** application form **by email**, in **the same word format**, together with **your: (b) research proposal inclusive of your survey, (c) copy of your ethics approval letter / proof (d) informed consent letter** to: Nadierah.Pienaar@uct.ac.za. You application will be attended to by the Executive Director, Department of Student Affairs (DSA), UCT.
3. The turnaround time for a reply is **approximately 10 working days.**
4. NB: It is the responsibility of the researcher/s to apply for and to obtain **ethics approval and to comply with amendments that may be requested;** as well as **to obtain** approval to access UCT staff and/or UCT students, from the following, at UCT, respectively: (a) **Ethics:** Chairperson, Faculty Research Ethics Committee' (FREC) for ethics approval, (b) **Staff access:** Executive Director: HR for approval to access UCT staff, and (c) **Student access:** Executive Director: Student Affairs for approval to access UCT students.
5. **Note:** UCT Senate Research Protocols requires compliance to the above, **even if prior approval has been obtained from any other institution/agency. UCT's research protocol requirements applies to *all* persons, institutions and agencies from UCT and external to UCT who want to conduct research on human subjects for academic, marketing or service related reasons at UCT.**
6. **Should approval be granted to access UCT students for this research study, such approval is effective for a period of one year from the date of approval (as stated in Section D of this form), and the approval expires automatically on the last day.**
7. **The approving authority reserves the right to revoke an approval based on reasonable grounds and/or new information.**

SECTION A: RESEARCH APPLICANT/S DETAILS

Position	Staff / Student No	Title and Name	Contact Details (Email / Cell / land line)
A.1 Student Number	HRTELL001	Miss Katherine Hartzenberg-Aeroe	HRTELL001@myuct.ac.za / +27 73 656 9524
A.2 Academic / PASS Staff No.			
A.3 Visitor/ Researcher ID No.			
A.4 University at which a student or employee	UCT	Address if *not* UCT:	
A.5 Faculty/ Department/School	Faculty of Engineering and the Built Environment, Department of Chemical Engineering		
A.6 APPLICANTS DETAILS If different from above	Title and Name	Tel.	Email

SECTION B: RESEARCHER/S SUPERVISOR/S DETAILS

Position	Title and Name	Tel.	Email
B.1 Supervisor	Dr Marijke Fagan-Endres	021 650 1806	Marijke.fagan-endres@uct.ac.za
B.2 Co-Supervisor/s	Dr Brian Kullin	083 663 3769	Brian.kullin@uct.ac.za

SECTION C: APPLICANT'S RESEARCH STUDY FIELD AND APPROVAL STATUS

C.1 Degree – if applicable	MSc (Eng) in Chemical Engineering
C.2 Research Project Title	Fermented foods production using isolated Lactobacillus species for the improvement of vaginal health: the case of mageu and yoghurt
C.3 Research Proposal	Attached: Yes ■ No ☐
C.4 Target population	Students from the EBE faculty
C.5 Lead Researcher details	If different from applicant:
C6. Will use research assistant/s	Yes ☐ No ■ If yes- provide a list of names, contact details :
C.7 Research Methodology and Informed consent	**Research methodology:** In brief, the methodology requires panellists for tasting of fermented foods produced according to the South African food standards. Qualitative tasting and quantitative questionnaire **Informed consent:** Panellists will be informed that the study runs anonymously and will be informed of all ingredients used to produce the fermented foods.
C.8 Ethics clearance status from UCT's Faculty Ethics in Research Committee /Chair (EiRC)	Approved by the UCT EiRC: Yes ■ With amendments: Yes ☐ No ■ (a) Attach copy of your UCT ethics approval. Attached: Yes ■ No ☐ (b) State date / Ref. No / Faculty of your UCT ethics approval: 16/05/2021 Ref. / Faculty: EBE

SECTION D: APPLICANT/S APPROVAL STATUS FOR ACCESS TO STUDENTS FOR RESEARCH PURPOSE

(To be completed by the ED, DSA or NOMINEE)

	Approved / With Terms / Not	* Conditional approval with terms	Applicant/s Ref. No.:
D.1 APPROVAL STATUS	(i) Approved ■ (ii) ~~With terms~~ (iii) ~~Not approved~~	a) Access to students for this research study must only be undertaken after written ethics approval has been obtained. *b)* In event any ethics conditions are attached, these must be complied with before access to students.	HRTELL001 / Miss Katherine Hartzenberg-Aeroe

	Designation	Name	Signature	Date of Approval
D.2 PREPARED BY:	Personal Assistant	*Nadierah Pienaar*		5/08/2021
D.3 APPROVED BY:	Executive Director Department of Student Affairs	*Mr Pura Mgolombane*		*6/08/2021*

Version. DSA 100.2021 Page 1 of 1 DSA 100

Appendix C: Calculations

C.1 Optical density and cell concentration calculations for *Lactobacillaceae* inoculum

L. jensenii 95.1 was used to determine the optical density required for the starting *Lactobacillaceae* isolate inoculum in the 500 mL working volume of either the maize-water mixture or milk before fermentation. *L. jensenii* 95.1 was grown in MRS broth and its optical density measured and serial dilutions performed. The results are listed in Table C-1 below.

Table C-1 Table of optical density and colony counts *for L. jensenii* 95.1

OD_{600nm}	Dilution Factor	Number of colonies observed on MRS plate	Colony counts per mL (cfu/mL)
2.355	10^7	190	1.9×10^9

From Equation 3-7 (Section 3.7.5), the number of viable colonies in colony forming units per mL (cfu/mL) is determined by multiplying the number of colonies observed on the plate by the dilution factor and plating factor of 10. Therefore, an OD_{600nm} of 2.355 was equivalent to a colony count of 1.9×10^9 cfu/mL. However, according to literature, the bacteria should have a cell concentration of 10^7 cfu/mL when inoculating the maize-water mixture or milk. It was assumed that there was a linear correlation between the optical density and cell concentration. To decrease the cell concentration to a factor of 10^7 cfu/m, the initial concentration (1.9×10^9 cfu/mL) and OD_{600nm} (2.355) were divided by a factor of 125. A graphical representation is shown in Figure C-1 (not drawn to scale).

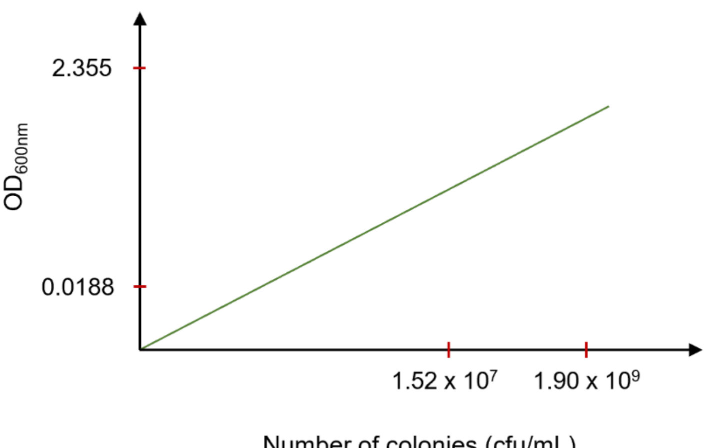

Figure C-1 Graphical representation of relationship between optical density and colony counts per mL

Therefore, the new OD_{600nm} was 0.0188. So, the equation for determining the volume of inoculum to add to the maize-water mixture and milk became Equation 3-2. The starting inoculum for *S. thermophilus* was determined using the same method.

$$V_1[ml] = \frac{C_2V_2}{C_1} = \frac{0.0188 \ \times 500\ mL}{OD_{600nm}\ of\ isolate\ culture\ in\ inoculum} \quad \text{Equation 6-1}$$

Appendix D: Experimental data

The experimental data used for this project can be accessed through the DOI link below:

10.25375/uct.19161173