University of Nottingham
UK | CHINA | MALAYSIA

The Growth of Pathogens and Beer Spoilage Organisms in No and Low Alcohol Beers and Strategies to Prevent Their Growth

Supervisors: Dr Stephen Lawrence and Dr Chris Powell

Connor Christopher Bartlett

ID: 20402412

Thesis submitted to the University of Nottingham

For the degree of Master of Research, October 2022

TABLE OF CONTENTS

ABBREVIATIONS ix

ABSTRACT xiii

ACKNOWLEDGMENTS xv

CHAPTER 1: Literature Review

1.1 Introduction 1

1.2 NABLAB Definitions 3

1.3 The NABLAB Market 3

1.4 The Brewing Process 5

1.4.1 Mashing 6

1.4.2 Wort Separation 6

1.4.3 Boiling 7

1.4.4 Fermentation 8

1.4.5 Maturation 9

1.4.6 Filtration and Packaging 10

1.5 NABLAB Production Methods 12

1.5.1 Restricted Fermentation 12

1.5.2 Alcohol Removal 14

1.5.2.1 Vacuum Distillation 15

1.5.2.2 Reverse Osmosis 16

1.6 Microbiological Hurdles 19

1.6.1 Ethanol 19

1.6.2 pH 19

1.6.3 Hops 20

1.6.4 Gases (CO_2 and O_2) 22

1.6.5 Nutrients 24

1.7 Preservatives 23

1.7.1 Sulphur Dioxide 23

1.7.2 Potassium Sorbate 25

1.7.3 Sodium Benzoate 27

1.7.4 Cinnamaldehyde 29

1.8 Microbiology of Beer 30

1.8.1 Lactic Acid Bacteria (LAB) 30

1.8.2 Acetic Acid Bacteria (AAB) 31

1.8.3 Enterobacteriaceae 31

1.8.4 Zymomonas 31

1.8.5 Strict Anaerobes 32

1.8.6 Beer Spoilage Yeasts 33

1.8.7 Pathogens 34

1.9 Microbiology of Draught Beer 37

1.10 NABLAB Microbiology 40

1.10.1 NABLAB 40

1.10.2 Fruit Juice 42

1.11 Research Aims and Objectives 45

CHAPTER 2: Materials and Methods

2.1 Microorganisms 46

2.2 Pathogen Preparation 46

2.3 Culture Medium (Wort Broth) 47

2.4 Inoculum preparation 47

2.5 Microbial Maintenance and Storage 47

2.6 Dosing (Calibration Curve) 48

2.7 Yeast Cell Enumeration 51

2.8 Yeast Cell Viability 51

2.9 Solution Preparations 52

2.10 Present Gravity and ABV 53

2.11 pH 53

2.12 IBU 54

2.13 Carbohydrate Analysis 54

2.14 Elemental Analysis 55

2.15 Challenge Testing 56

2.16 Preservative Dosing for spoilage trials 56

2.17 Spoilage Trials 56

2.18 Pathogen Trials 57

2.18.1 Initial Survival Trials 57

2.18.2 Growth Trials 57

2.18.3 Preservative Sensitivity Trial 58

2.19 Preservative dosing and pH adjustments for OmniLog® trials 58

2.20 OmniLog® trials 61

2.21 Statistical Analysis 61

CHAPTER 3: Results

3.1 Beer Sample Analysis Results

3.1.1 Introduction 62

3.1.2 Results 62

3.2 The Growth of Beer Spoilage Microorganisms in NABLABs Compared to a Full Alcohol Counterpart

3.2.1 Introduction 66

3.2.2 Growth Curves 68

3.2.3 Day Seven Results 70

3.2.4 Discussion 73

3.3 The Effect of Preservatives on the Growth of Beer Spoilage Microorganisms

3.3.1 Sodium Benzoate Introduction 80

3.3.2 Day Seven Results 81

3.3.3 Potassium Sorbate Introduction 84

3.3.4 Day Seven Results 85

3.3.5 Sulphur Dioxide Introduction 88

3.3.6 Day Seven Results 89

3.3.7 Preservative Comparisons Introduction 92

3.3.8 Day Seven results 93

3.3.9 Discussion 96

3.4 Does the Addition of Preservatives to a NABLAB Reduce Growth of Beer Spoilage Microorganisms to a Similar Level to that of its Full Alcohol Counterpart?

3.4.1 Introduction 102

3.4.2 Day Seven results 103

3.4.3 Discussion 106

3.5 Survival of Food-borne Pathogens *E. coli* 0157:H7 and *S.* Typhimurium in NABLABs and a Full Alcohol Counterpart

3.5.1 Introduction 109

3.5.2 Results 110

3.5.3 Discussion 115

3.6 Effectiveness of Preservatives at Inhibiting Food-borne Pathogen Growth in a Modified NABLAB

3.6.1 Introduction 118

3.6.2 Results 120

3.6.3 Discussion 122

Chapter 4: Results of OmniLog® Trials to Assess the Effect of pH and Preservative Concentration on *P. membranifaciens* and *L. brevis*

4.1 OmniLog® Spoilage Trials at Three pH Levels

4.1.1 Introduction 124

4.1.2 Results 125

4.2 Omnilog® Spoilage Trials to Assess Preservative Effectiveness in Reducing Brand 1 0.5% Spoilage to a Level Comparable to Brand 1 4.5%

4.2.1 Introduction 127

4.2.2 Results 128

4.3 Omnilog® Spoilage Trials to Assess Preservative Dose Response at Three pH Levels

4.3.1 Introduction 132

4.3.2 Results 133

4.4 Omnilog® Spoilage Trials to Assess Preservative Combinations at Three pH Levels

4.4.1 Introduction 144

4.4.2 Results 145

4.5 Discussion of Omnilog® Results 156

Chapter 5: Conclusions and Future Work

5.1 Conclusions 160

5.2 Future Work 167

5.3 Key Conclusions 170

CHAPTER 6: References and Appendix

6.1 References 171

6.2 Appendix 183

ABBREVIATIONS

°C	Degrees Celsius
A_{660}	Absorbance at 660 nanometers
AAB	Acetic acid bacteria
ABV	Alcohol by volume
ADI	Acceptable daily intake
ANOVA	Analysis of Variance
ATNC	Apparent Total N-nitroso Compounds
ATP	Adenosine triphosphate
BBPA	British Beer and Pubs Association
CFU/ml	Colony Forming Units per millilitre
CO_2	Carbon Dioxide
DMS	Dimethylsulphide
EBC	European Brewery Convention
FAN	Free Amino Nitrogen
g	Gram
GI	Gastrointestinal
GRAS	Generally recognised as safe
H_2S	Hydrogen sulphide
HPAEC	High Performance Anion-Exchange Chromatography

HSO_3^-	Bi-sulphite
HUS	Haemolytic-uremic syndrome
IBU	International bitterness units
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IPA	India pale ale
iso-α acids	Isomerised alpha acids
ITS	Internal Transcribed Spacer
L	Litre
LAB	Lactic acid bacteria
LPS	Lipopolysaccharide
m	Metre
MB1 0.5%	Modified Brand 1 0.5% ABV
ME	Malt Extract
mg	Milligram
ml	Millilitre
MRS	De Man, Rogosa and Sharpe agar
NA	Nutrient Agar
NABLAB	Non-alcoholic beer and low-alcohol beers
NaCl	Sodium Chloride
NADH	Nicotinamide adenine dinucleotide

NaOH	Sodium Hydroxide
NCTC	National Collection of Type Cultures
NIR	Near-Infrared analysis
Non-STEC	Non-shiga toxin producing *E. coli*
O_2	Oxygen
OD	Optical Density
OG	Original gravity
PEF	Pulsed Electric Fields
PG	Present gravity
pH	Potential of hydrogen
pKa	Negative log of the acid dissociation constant
PMF	Proton Motive Force
PP	Polypropylene
ppb	Parts per billion
ppm	Parts per million
PSI	Pounds per square inch
PU	Pasteurisation unit
rDNA	Ribosomal Deoxy Nucleic Acid
RO	Reverse Osmosis
RT-PCR	Real time polymerase chain reaction

SG	Specific gravity
SMM	s-methymethionine
SO_2	Sulphur Dioxide
SO_3^{2-}	Sulphite
µg	Microgram
µm	Micrometre
v/v	Volume concentration
VDK	Vicinal diketones
WHO	World Health Organisation
WLN	Wallerstein Laboratory Nutrient agar
YMA	Yeast and Mould agar
ΔA_{660nm}	Delta change in absorbance at 660 nanometres

ABSTRACT

There has been an increase in demand for no and low alcohol alternatives to beer, including their serving on-trade through draught dispense systems. Ethanol aids in regular beers microbiological stability, reducing this could lead to product spoilage. Additionally, the methods used to produce no and low alcohol beers can significantly affect other beer parameters, which may be important for susceptibility to microbial growth. This new environmental niche of no and low alcohol beers has already been shown to be more susceptible to microbial spoilage and pathogenic growth than their full alcohol counterparts.

This study assessed the growth of *Pichia membranifaciens, Levilactobacillus brevis, Rahnella spp., Escherichia coli* 0157:H7 and *Salmonella* Typhimurium in three low alcohol beers and one full alcohol counterpart. Along with the antimicrobial effects of commonly used food and beverage preservatives, sodium benzoate, potassium sorbate and sulphur dioxide were studied on these microorganisms. Using an Omnilog® the effects of pH, varying doses and combinations of preservatives was tested on the growth of *P. membranifaciens* and *L. brevis* on one of the low alcohol beers. The differences in spoilage and pathogen growth between the beers was discussed in relation to the pH, sugar composition, elemental composition, and production method.

The growth of *P. membranifaciens* was greater in every low alcohol beer tested compared to the full alcohol beer in spoilage and Omnilog® trials. This appeared to be caused by the reduction in ethanol content. The growth of *L. brevis* was highest in Brand 1 0.5% this appeared to be related to the higher glucose content. *Rahnella spp.* Only grew in Brand 1 0.5% and Brand 2 0.5%. *E. coli* 0157:H7 (Non-STEC) didn't grow in any of the beers tested. *S.* Typhimurium was only able to grow slowly in Brand 2 0.5%. It was observed to grow rapidly in a modified Brand 1 0.5% but was inhibited by all the preservatives tested. Sodium benzoate and potassium sorbate were effective at reducing the growth of all the spoilage organisms, with *P. membranifaciens* and *L. brevis* being reduced to growth levels comparable with a full-alcohol beer. Sulphur dioxide was able to completely inhibit growth of *Rahnella spp.* in Brand 1 0.5%. The differences in growth of spoilers and pathogens appears to be related to the combination of pH, ABV, glucose, fructose, and maltose content of the beer. There were no synergistic effects observed when using combinations of the preservatives. Reducing the pH to 3.8 or raising it to 4.45 did not appear to influence the effectiveness of any of the preservatives tested against *P. membranifaciens* or *L. brevis*. The use of preservatives should be considered when producing NABLABs destined to be served on draught dispense to reduce their susceptibility to spoilage and prevent the growth of *S.* Typhimurium.

ACKNOWLEDGEMENTS

Firstly, I would like to thank Dr Stephen Lawrence and Dr Chris Powell for their supervision, guidance, and support throughout this project. Additional thanks go to Dr Joshua Reid for kindly performing the carbohydrate and elemental analysis, Dr Adriano Gigante for advising on, and running the Omnilog® trials and to the American Society of Brewing Chemists for funding them.

I would also like to thank Adnams PLC (especially the Quality and Brewing teams) for encouraging me to start this project and for supporting me throughout. By funding the majority of the work, investing in new premises, equipment and allowing me to remain a full-time employee while completing this work. I am grateful to work for a company that sees the value in scientific research, cares for the quality of their products, and the safety of their and other no and low alcohol beer producers' customers.

Finally, I would like to thank my family for always supporting and encouraging me to do what I believe in, and Bex for her encouragement, support, and motivation to keep moving forward.

Chapter 1: Literature Review

1.1 Introduction

Beer has been one of the most popular beverages for thousands of years. One reason it has persisted is due to its inherent microbiological stability as a standard beer made from malted barley, water, yeast, and hops has many hurdles which prevent microorganisms from growing. These hurdles include a high ethanol content (3.5-5.5%), low pH (<4.5), isomerised-α acids, low sugar content, low oxygen, and high CO_2 concentration (Vriesekoop *et al.*, 2012; Suzuki, 2011; Menz *et al.,* 2011).

These hurdles allowed beer to travel long distances in the 1700s when beer was regularly shipped to India to support troops of the British empire. The beers that survived the trip unspoiled had a higher hopping rate, greater ABV and were fully attenuated, this led to the origination of the India pale ale (IPA)(Steele, 2012).

Microbiological hurdles inhibit the growth of spoilage microorganisms by employing a combination of different preservative factors (Vriesekoop *et al.,* 2012). This makes an inhospitable environment for many microorganisms while maintaining good organoleptic properties. The few microorganisms that can survive and grow in beer have adapted to do so by becoming resistant to some of these hurdles and are known as beer spoilers (Sakamoto and Konings, 2003). Removing any of these hurdles potentially increases the severity by which beer spoilers can affect the beer and also increases the chances of other non-beer spoilage microorganisms being able to spoil beer.

The more potential culprits for contamination, the harder it is to manage the microbiological stability of a product. Previously, there have been very few reports of pathogenic microorganisms being found in beer with all research showing strong inhibition (Menz *et al.,* 2008**).** However, removing any of these microbiological hurdles will increase the risk that pathogens could survive for extended periods of time or even grow (L'

Anthoën and Ingledew, 1996; Menz *et al.,* 2011) . This is especially concerning in the industrial fermentation environment of breweries, who historically have not had to manage the risks of pathogens in their products, both in their production and out in trade (Bunker, 1955; Sheth *et al.,* 1988; Menz *et al.,* 2011; Friedman *et al.,* 1997).

No-alcohol (<0.05%) and Low alcohol beers (<1.2%) (NABLABs, defined in Section 1.2) have been increasing in popularity in recent years. Consumer demands have shifted, with many being more health conscious and so looking for lower alcohol alternatives (IWSR, 2022). Many brewers have reacted to this and started to produce more no and low alcohol products with a variety of different methodologies. NABLABs have a reduced ethanol content, often a much higher sugar content, and can also have an elevated pH due to the lack of acidification from reduced fermentation, or as an effect of the alcohol removal process (Muller *et al.,* 2020*;* Branyik, 2012). This reduction in some of the hurdles that were protecting the beer from spoilage and food-borne pathogen growth, could cause more frequent and more severe spoilage incidents, which in turn could lead to considerable economic losses.

Recently there has been an increased interest in serving NABLABs through draught dispense systems. The environment of a draught dispense system is not a sterile one. Long dispense lines, warm temperatures, un-sanitary nozzles and poor cleaning regimes, all contribute to beneficial conditions for survival and growth of beer spoilage microorganisms (Quain, 2016). The serving of NABLABs through these means may well exacerbate these spoilage issues, leading to poor customer perception of NABLABs from both landlords and patrons. However, the risks of these issues occurring for NABLABs in trade has been under researched and so these risks still need to be directly investigated.

1.2 NABLAB Definitions

In the UK there are four different descriptors for reduced alcohol beverages; low alcohol, Non-alcoholic, alcohol free and de-alcoholised. Low alcohol is any beverage at or below 1.2% ABV (Department of Health and Social Care, 2018). Non-alcoholic is a term that cannot be used in conjunction with a name already associated with an alcoholic drink i.e. beer and is reserved solely for soft drinks, apart from the exception of non-alcoholic communion wine. Alcohol free can only apply to drinks which have had their alcohol extracted to a level of no more than 0.05% ABV. De-alcoholised is the term that can be applied to drinks which have had their alcohol removed and contain no more than 0.5% ABV (Department of Health and Social Care, 2018). In most EU countries any beer under 0.5% can be called alcohol free and anything below 1.2% can be labelled low-alcohol (EU Regulation 1169, 2011). In the Netherlands an alcohol-free beer must be below 0.1% ABV and a low alcohol beer must be between 0.1%-0.5% ABV (De Nederlandse Reclame Code, 2022). In the US any malt or cereal based beverage under 0.5% can be called non-alcoholic, under 0.05% alcohol free and low-alcohol or reduced alcohol up to 2.5% ABV. However, these cannot be named beer and must be labelled as either malt beverage, cereal beverage or near beer (Code of Federal Regulations, 2021).

For the purposes of this dissertation NABLABs will refer to any beer-like beverage produced with an ABV of 0.0%-1.2% ABV unless otherwise stated.

1.3 The NABLAB Market

The NABLAB market has grown considerably over the past 10 years, while the growth of the alcoholic beer market has slowed. The general drinking population have an increased concern about health and wellbeing with 22% of global consumers looking to actively reduce their alcohol intake (Anderson *et al.,* 2021). With some in Germany using alcohol

free beer as a rehydrating isotonic beverage after physical activity (Anderson *et al.,* 2021). This combined with other factors such as religion, stricter drink driving laws and the banning of alcohol sales in certain retail stores has led to the growth and rise in interest in low alcohol alternatives to beer, wine, and spirits (Bellut and Arendt, 2019).

Although there are other low alcohol products on the market, NABLABs and ciders make up 75% of the market share in the 10 core markets (IWSR, 2022). So now, many breweries are looking for alternative ways to pursue growth from new lower alcohol products. Although most of this growth has been in bottle and can packaging formats, there is an increased interest in having NABLABs in pubs and restaurants on draught dispense systems (Drinks International, 2021; IWSR, 2022). Heineken were providing pubs with Heineken 0.0% in 8L kegs that fit with the Blade dispense system but are now looking to serve it on a more traditional dispense system (Heineken, 2021). Diageo have developed a bespoke font dispenser which uses pint sized cans of Guinness 0.0% (Independent, 2021). There are also some craft breweries producing NABLABs that are being served through standard keg lines from either steel keg or Key Kegs.

According to a report by the International Wine and Spirits Record (IWSR) although in the UK NABLABs only account for 2% of the total beer market, it is the fastest growing segment in the UK and estimated to grow 6% per annum on average by 2023, with the regular beer market stagnating. In another IWSR report new data shows that consumption of no and low products will increase by a third worldwide by 2026 (IWSR, 2022). It is also noted that the real potential for growth in the market is in serving NABLABs on-trade, which is particularly attractive to brewers due to the better profit margins of keg beer.

1.4 The Brewing Process

The brewing of beer is a complex multistep process when compared to the production of other alcoholic beverages. Beers are mostly produced from four main ingredients: Water, malted barley, hops and yeast. These ingredients are processed through mashing, wort separation, boiling, cooling, fermentation, maturation, filtration, and packaging with some products also undergoing pasteurisation (Briggs *et al.,* 2004) (Figure 1).

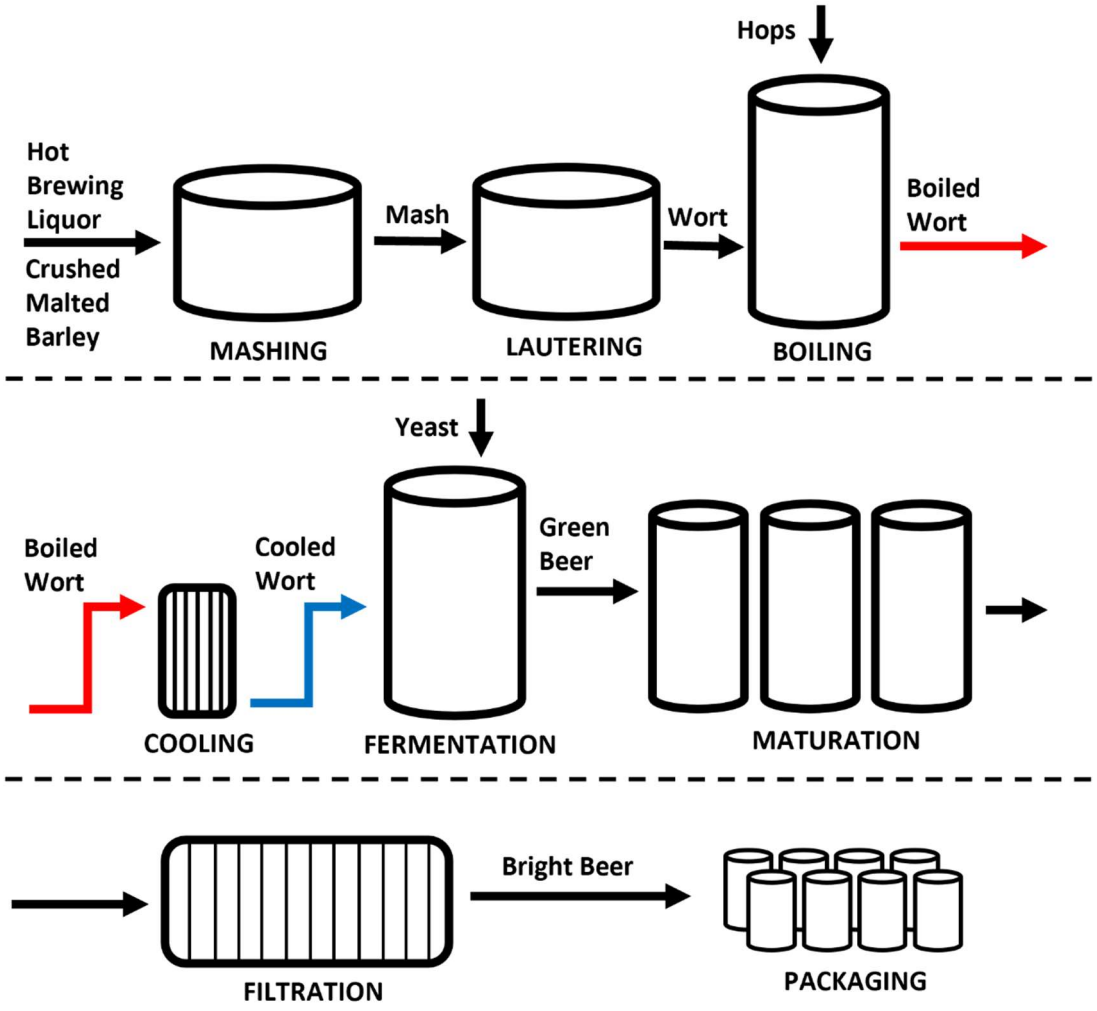

Figure 1: Overview of the brewing process

1.4.1 Mashing

Mashing consists of the introduction of the brewing liquor (heated and treated water) to the crushed malted barley. Once the desired amount of barley and water have been introduced into the mashing vessel, the mash (water and malted barley) is allowed to stand for a time, usually around an hour. This allows the enzymes in the malt to break down the starch found in the grain into sugars (glucose, fructose, sucrose, maltose and maltotriose), the resulting sugary liquid is called wort. The two most important enzymes in the mash are α-amylase and β-amylase. The α-amylase breaks down starch molecules into shorter oligosaccharides which have non-reducing ends, its optimum mash temperature range is 65-75°C. The β-amylase produces maltose from the non-reducing ends of these oligosaccharides, with an optimum temperature range of 60-65°C. It cannot reduce larger starch molecules effectively and so the synergistic activity of both enzymes is essential for wort fermentability (Eßlinger, 2009).

1.4.2 Wort Separation

Wort separation from the mash is achieved through a process known as lautering. The mash is transferred to the lauter tun which has a false bottom full of gaps to let the liquid wort through (Briggs *et al.,* 2004). The insoluble husks of the barley also act as a natural filter bed when on top of the false floor. The first wort collected is hazy and contains many particles, so it is pumped back into the lauter tun. The wort collection begins when it is running clear. The wort is allowed to runoff until the grain bed in the lauter tun becomes visible, at which time 75°C treated water is sprayed over the grain bed in a process called sparging, which is used to wash the mash of any remaining sugars (Willaert, 2006).

1.4.3 Boiling

The freshly sparged wort is then moved directly to the kettle to begin boiling. Boiling wort is simple in a practical sense, but it causes many complex chemical and biochemical reactions to occur. One of these reactions is isomerisation of α-acids (humulone, adhumulone, cohumulone, prehumulone and posthumulone). Hops, specifically the lupulin oil produced in the hop cone, contain these compounds. When boiled, the α-acids are converted into cis- and trans- isomer α-acids (Jaskula *et al.,* 2008). These acids confer a bitter taste to beer and are also an important preservative, as they inhibit the growth of Gram-positive bacteria which could spoil the beer (Suzuki *et al.,* 2006). Boiling the wort also causes what is known as the hot break, this is where proteins and polyphenols derived from the malt coagulate to form large clumps which can be removed from the wort at the end of the boil. If these proteins weren't removed, they could cause clarification issues, and influence the colloidal stability of the final product. The boil also serves as an important microbe reducing step, killing almost all microbes that could have been introduced earlier in the process from the malt, hops, water, or other adjuncts. As well as killing microbes the boil also denatures any enzymes left from the mashing process, this fixes the amount of fermentable sugar available in the wort. The colour of the wort will change throughout the boil through Maillard reactions which forms dark coloured melanoidins. The boil also helps to remove unwanted volatiles such as s-methylmethionine (SMM) which is a pre-cursor molecule to dimethyl sulphide (DMS) which gives a vegetal, corn-like aroma in finished beer (Willaert, 2006; Briggs *et al.,* 2004).

After the boil, the resulting spent hops and hot break, known as trub, are separated from the freshly boiled wort. The wort is then cooled as quickly as possible, most often with the aid of a heat exchanger, and transferred into a fermentation vessel. The wort is cooled to 10-12°C for a lager or 20°C for ale. Although many steps in the brewing process are

designed to specifically avoid oxygen ingress, the freshly cooled wort is aerated in-line on the way to the fermenter. The presence of 8-20ppm oxygen is essential for yeast to synthesise sterols and fatty acids that are required for membrane synthesis which is critically important in cell division (Hornsey, 2013; Eßlinger, 2009).

1.4.4 Fermentation

The yeast is immediately added so fermentation can begin. Fermentation can take as little as three days for some ales and 2-3 weeks for some lagers. The cleanliness of the fermentation vessel and the yeast is of utmost importance at this stage, as boiled cooled wort is a perfect growth medium for a whole host of microbes. For this reason, it is also important that the yeast starts fermenting as soon as possible. Once introduced to the sugary wort, yeast begins fermentation despite the presence of oxygen due to the Crabtree effect. In simple terms, yeast will forgo aerobic respiration in favour of fermentation when there is sufficient sugar in a solution. The yeast converts the fermentable sugars into ethanol via the glycolytic pathway (Hornsey, 2013). The yeast also acidifies the wort by excreting organic acids such as lactic, malic, citric and acetic acids which will quickly prevent most other microbial growth (Coote and Kirsop, 1975; Vriesekoop *et al.,* 2012).

Brewers have a choice of using dry or wet yeast for fermentation. Dry yeast is lyophilised and stored in a sealed packet to retain sterility, it is mostly used for a single brew and the yeast remaining at the end of fermentation is sent to waste. Alternatively, wet yeast is reused from brew to brew, every re-use is known as a generation with many breweries re-using yeast up-to five times. However, some breweries reach hundreds of generations before refreshing their yeast culture. To refresh the culture, a pure yeast that is stored at -80°C under liquid nitrogen is propagated up in wort or other nutrient medium in vessels of increasing size, until the number of desired cells to pitch a single fermentation is reached. The advantage of this method is that it is much more cost effective, however with every

generation there is an increased chance of the yeast possibly becoming contaminated, or in the case of some yeast strains, genetic variants being produced, possibly moving the yeast away from its desired brewing characteristics (Powell and Diacetis, 2007).

Once the fermentation begins to reach its end, the yeast begins to flocculate. The process of flocculation involves specific proteins on the cell surface membrane called lectins. These lectins selectively bind to mannose residues found on other yeasts cell walls, this causes the yeast cells to clump together (Verstrepen, 2003). When clumped together they gradually settle out to the bottom of the vessel where the yeast can be separated from the beer.

1.4.5 Maturation

The main purposes of maturation are:

- Removal of compounds such as vicinal diketones (VDK), sulphur compounds and acetaldehyde
- Improvement of colloidal stability
- Sedimentation of yeast cells
- Carbonation

The sedimented yeast is removed from the bottom of the vessel and the beer is left warm (14-16°C) before transferring. This is to allow for the yeast to continue fermenting slightly, and performing a process known as a diacetyl rest, which allows reduction of any VDK present into other non-flavour active compounds (Boulton and Quain, 2001). This can also help any remaining acetaldehyde to be reduced into ethanol by the yeast. Once the diacetyl rest has finished, the 'green' beer is transferred to another vessel where it undergoes the remainder of its maturation. This secondary fermentation also carbonates the beer as the vessel that the beer was transferred into is sealed, this reduces the need for

carbonation later in the process. The beer can then be cooled to as low as -1°C which aids in the improvement of colloidal stability and sedimentation of yeast which can be removed from the bottom of the vessel (Briggs *et al.,* 2004; Willaert, 2006).

1.4.6 Filtration and Packaging

If the beer is to be kegged, bottled, or canned there are usually a few more processes the beer must go through. The matured beer must be moved to another vessel called a bright or conditioning tank, this can be just a direct transfer, but in most cases a centrifuge is used which helps to remove any remaining yeast that was still suspended in the beer. Then the beer is moved onto filtration which removes the remainder of the yeast, particulate matter and even some bacteria, depending on the type and pore size of filter (Briggs *et al.,* 2004). At the same time the beer is carbonated and, if required, has deaerated liquor (low dissolved oxygen dilution water) added. Before packaging into kegs there may be another filter often tasked with removing any bacteria from the beer. In the brewing environment an absolute 0.45µm filter is known as a sterile filter (a medical sterile filter is 0.2µm). Depending on the brewers goal, beer destined for bottle or can are pasteurised, sterile filtered, or not filtered at all.

If choosing to pasteurise, bottles are filled, capped, and then tunnel pasteurised. This involves the bottles riding a conveyor at a specific speed through a tunnel heated to a set temperature usually around 60-65°C. The temperature and time equation results in a number of PU's (Pasteurisation units), the higher the number of PU's the less likely any bacteria or yeast will have survived the process (Rachon *et al.,* 2018)**.** The EBC recommend that lagers and pilsners have a minimum PU of 15 whereas the recommendation for Non-alcoholic beer is 80 PUs (EBC, 1995). Cans go through a mostly similar process apart from the fact that the beer is pasteurised in line (known as flash pasteurisation) and then aseptically filled into the can instead. This method is also commonly used for kegged beer.

If destined for cask beer the final step would be to take the beer directly from the fermentation vessel or a secondary tank which it was transferred to, known as a racking tank. It is then put into cleaned casks with some isinglass finings. The carbonation of cask beer is achieved by keeping some yeast and fermentable sugar in the beer, allowing for secondary fermentation (Hornsey, 2013).

1.5 Non-Alcoholic Beer Low-Alcoholic Beer (NABLAB) Production methods

There are many ways to produce NABLABs, and with it being a new area of production there is no agreed best method. Currently, low and no alcohol beer production is split up into two categories, restricted fermentation (biological) and alcohol removal (mechanical) (Table 1) (Salanță *et al.,* 2020).

Table 1: Summary of advantages and disadvantages of common NABLAB production methods

	Advantages	*Disadvantages*
Biological		
Specialist yeast	Low cost, no specialist equipment required	Yeast can produce phenolic off-flavours
Adjusted Mashing	Low cost, can use regular yeast strain	Wort-like flavour from higher concentration of aldehydes
Mechanical		
Reverse Osmosis	Low temperature	High running costs
Vacuum Distillation	Can easily achieve 0.05% ABV	High energy requirements, loss of volatile compounds

1.5.1 Restricted Fermentation

To produce a low alcohol beer without having to remove alcohol in another process, there must be a reduction in the fermentation of wort sugars by yeast. This can be achieved by reducing the amount of available sugar in the wort, changing the sugar composition of the wort, cold fermentation, low yeast pitching rates or by using specialist yeast strains. The yeast can also be removed from the wort when the desired ABV has been reached.

The amount of sugar in the wort is decided by the brewer when they create their recipe, simply put the more malted barley and other grains used in the mash the greater the

potential sugar content of the wort produced, depending on the mashing regime. A higher fermentable sugar content in the wort increases the alcohol content in the final beer, so the opposite logic can be applied if we wish to produce a low alcohol beer. In terms of specific gravity (SG) a standard beer may have an original gravity (OG) of 1.040 and a final/present gravity (PG) of 1.008 which would achieve a beer of around 4.2% ABV, whereas if we reduced the OG to 1.012 and fermented to the same PG of 1.008 the ABV would be around 0.53%. However, preventing the yeast from fermenting lower than 1.008 may require some other methods.

The composition of sugars in wort has a large effect on its fermentability, the three main sugars found in wort are glucose, maltose and maltotriose. Sucrose and fructose are also present at lower concentrations. Glucose, sucrose, and fructose are the first to be fermented by yeast and accounts for 10-20% of the total sugar available. Maltose makes up the majority of sugar composition (50-60%) and comprises two glucose molecules. Maltotriose accounts for around 20% of the wort and comprises three linked glucose molecules (Lewis and Young 2001). Yeast generally ferments these sugars in order of complexity (Glucose -> Maltose -> Maltotriose) with maltotriose only being partly fermented in most cases (Londesborough, 2001). As yeast often struggles to ferment maltotriose, increasing the percentage of the worts maltotriose (and other long chain sugars) can decrease both the rate and amount of fermentation of a beer. There are two basic ways to increase the level of long chain sugars in wort. Increasing the mash temperature which decreases the activity of the β-amylase enzyme and increases activity of the α-amylase enzyme and so increases the amount of long chain sugars (Eßlinger, 2009). The type of malt used can also affect the sugar composition, higher roasted malts have a similar amount of available sugar as pale malt but they have a higher degree of caramelisation from the roasting process which produces longer chain sugars in the mash (Brányik *et al.,* 2012)**.**

As restricted fermentations are so short and overshooting ABV could cause significant processing issues, slowing the rate of fermentation is a viable strategy. This can be achieved by fermenting at 14-16°C rather than a more standard 18-20°C, often in combination with reducing yeast pitching rate from standard 0.5 million cells/ml/ °Plato to 0.25 million cells/ml/ °Plato (Salanta *et al*., 2020; Muller *et al.,* 2020). The cooler temperatures slow down the yeast cells metabolism, slowing division, and increasing the length of the lag phase of growth (Bisson, 2005). This creates a more manageable fermentation rate. To prevent any further fermentation the beer can be centrifuged to remove the majority of yeast from solution (Briggs *et al.* 2004).

Increasing in popularity now is the use of specialist yeast strains specifically developed for the production of NABLABs. These yeasts, sometimes termed 'lazy yeasts', can only ferment glucose, sucrose and fructose and cannot utilise maltose or maltotriose like a traditional brewing yeast strain. This has a significant impact on the potential fermentability of wort, as maltose is the most abundant sugar in wort. Examples of these yeasts include *Saccharomycodes ludwigii*, *Saccharomyces cerevisia*e var. *chevalieri* and *Zygosaccharomyces lentus* (Bellut and Arendt, 2019; Capece *et al.,* 2021; Brányik *et al.,* 2012; Liu *et al.,* 2020; Krogerus *et al.,* 2021). These yeasts won't remove the need for the previously mentioned techniques, but will reduce the level to which they are required making restricted fermentation a simpler process to control.

1.5.2 Alcohol Removal

Processes which produce NABLABs by alcohol removal start with a beer which is brewed and fermented to full or partial strength. It is often treated in a very similar way to brewing a regular full-strength beer, although there may be some changes to mashing parameters (higher mash temperature to increase body) or additions (acid to lower pH) to aid in

producing a dealcoholized beer with favourable organoleptic properties (Muller *et al.,* 2020; Salanță *et al.,* 2020).

1.5.2.1 Vacuum Distillation

One such method for removing alcohol from beer is vacuum distillation. In a closed system a vacuum is pulled on the beer, which lowers the boiling point of ethanol from 78°C to 40°C (Muller *et al.,* 2020). This greatly reduces the amount of heat energy required to remove the alcohol. The beer is first heated under the vacuum removing some volatile compounds and CO_2 from the beer in an aroma stripper tank. These volatile compounds and CO_2 are recovered to a recombination tank. The degassed beer then moves into the vacuum column where alcohol can be stripped from the beer. This alcohol is then condensed, it can also be distilled again to separate the alcohol from any remaining volatile flavour compounds and water which can then added back into the beer (Figure 2). The remaining de-alcoholised beer is pumped to the recombination tank where the previously recovered volatiles are. It is often sprayed in to increase surface area to pick up these volatiles (Salanță *et al.,* 2020). This method can achieve a beer with an ABV as low as 0.05%. The alcohol removed from the beer can be used as a valuable co-product in other industrial applications such as vinegar production.

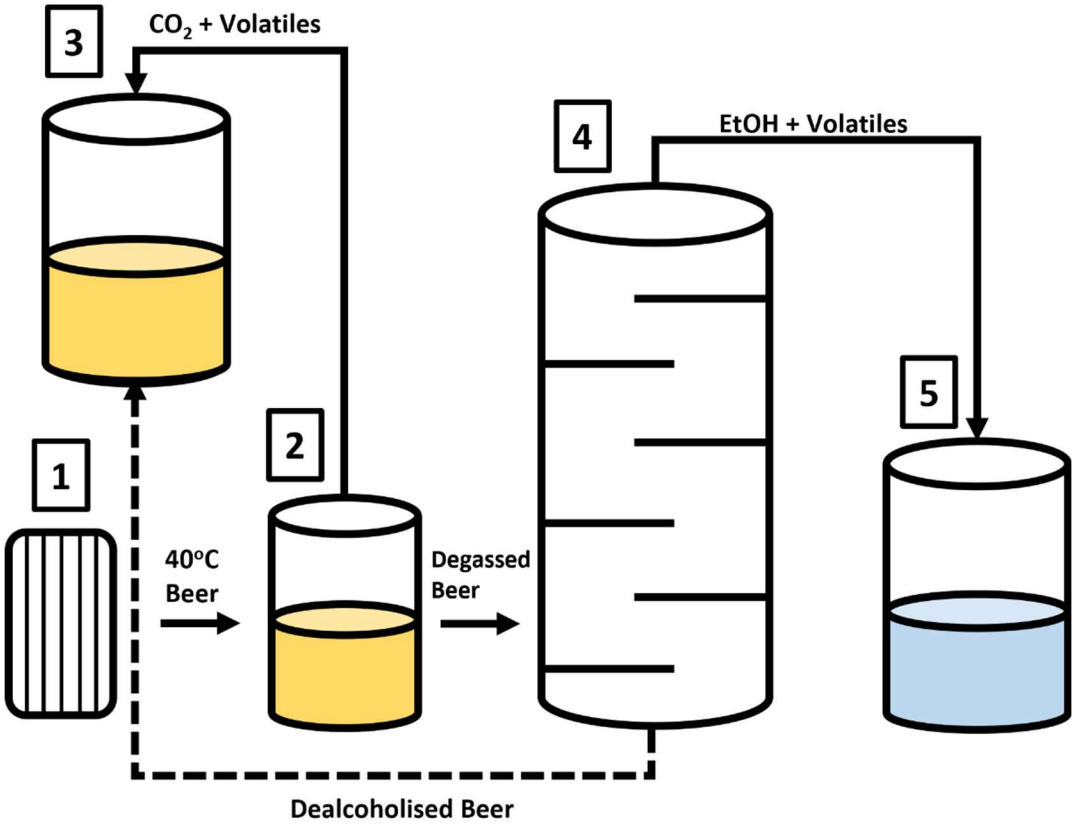

Figure 2: Simplified vacuum distillation process. (1) Plate heat exchanger. (2) Aroma stripper. (3) Recombination tank. (4) Vacuum column. (5) Distillate condenser

1.5.2.2 Reverse Osmosis

Another popular method is reverse osmosis (Figure 3), which can remove alcohol at temperatures as low as 10°C. This is beneficial as beers organoleptic properties are often negatively affected by heating (Bamforth, 2011). Osmosis is the process of a solvent moving through a selectively permeable membrane from low solute concentration to high solute concentration to balance the concentration of the solute on both sides of the membrane (Cath *et al.,* 2006). To achieve the reverse of this process the beer is placed under pressures of 20-80 bar against a semi-permeable membrane. The pressure pushes water and ethanol across the membrane from the retentate side (beer) to the permeate side (water and ethanol) against osmotic pressure (Figure 4). The small pore size of the membrane prevents larger aroma and flavour molecules from crossing the membrane and leaving the beer, although the membranes themselves can significantly adsorb aromatic

compounds (Purwasasmita *et al.,* 2015). The beer is passed tangentially to the semi-permeable membrane, this crossflow method prevents the accumulation of rejected solutes and helps to prevent scaling. The water and ethanol that passes through the membrane to the permeate side can be recovered (Figure 3). The ethanol is often removed via vacuum distillation and the water either added back to the retentate, diluting the beer in a process known as diafiltration, or used as dilution water elsewhere. Using this method, it is common to be able to achieve a beer of around 0.5% ABV, with some new systems being able to get down as low as 0.05% (Salanță *et al.,* 2020).

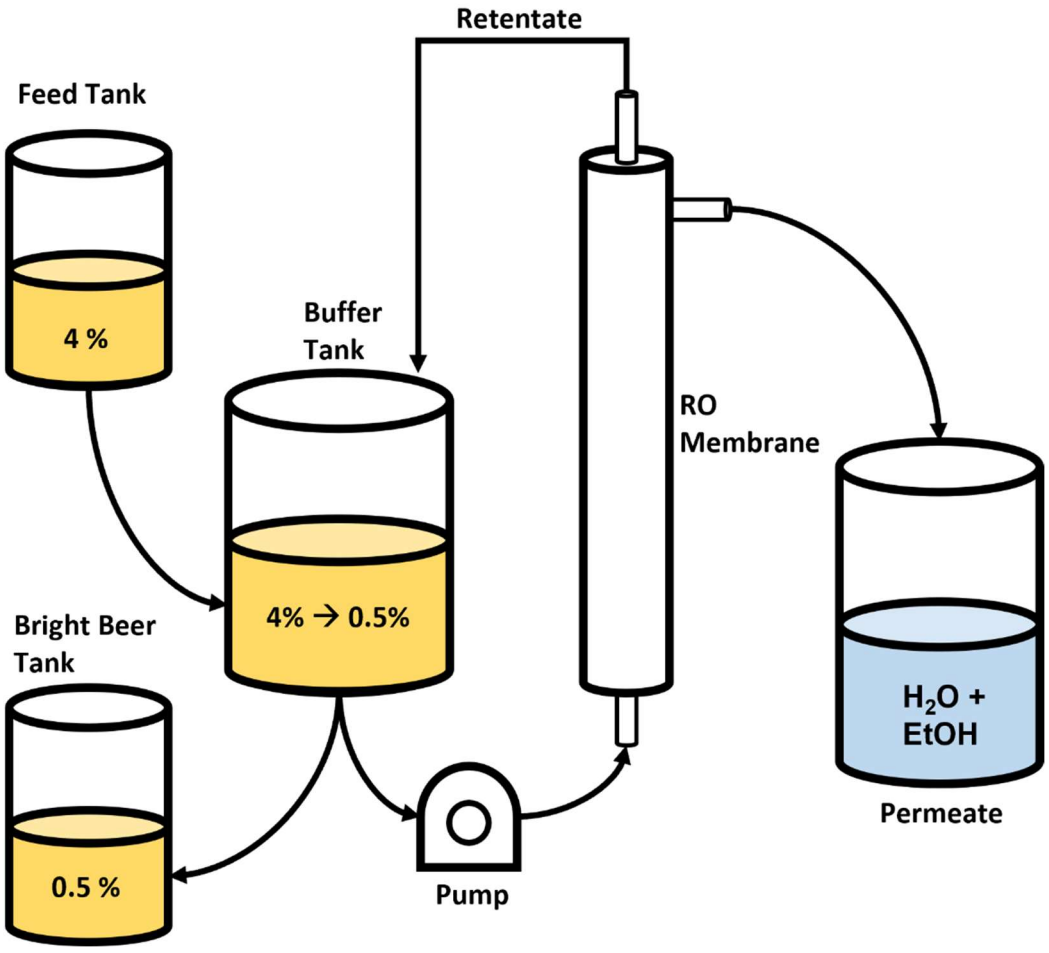

Figure 3: Process diagram of reverse osmosis for production of low alcohol beer

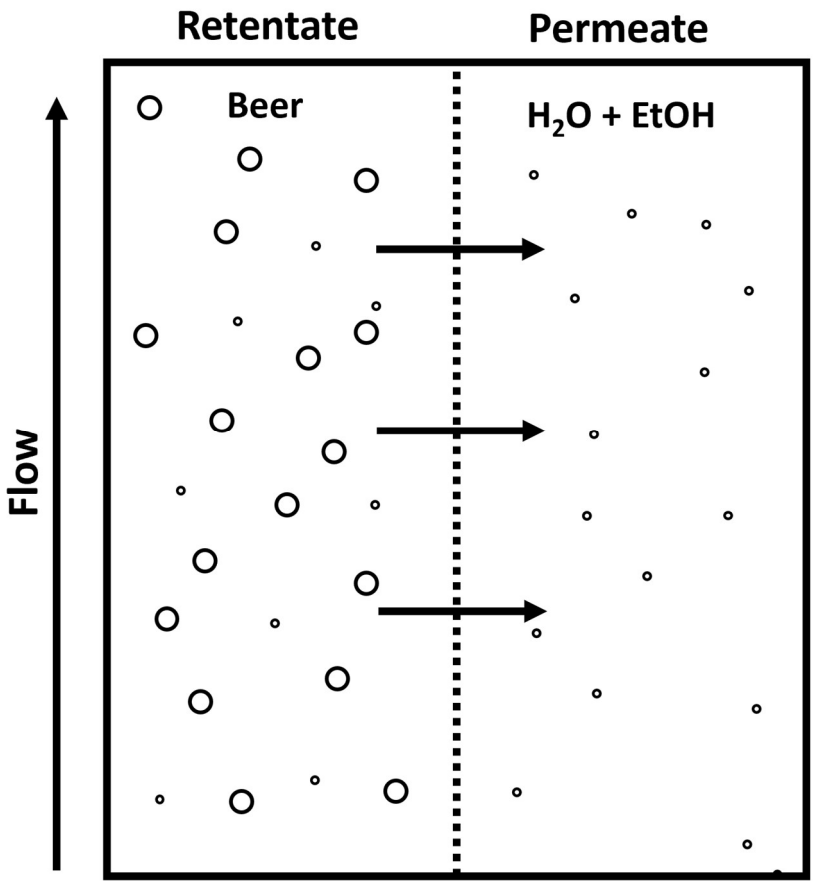

Figure 4: Reverse osmosis membrane function diagram

1.6 Microbiological Hurdles

A beers microbiological hurdles are imperative to its resistance to spoilage, and so removing or changing these hurdles significantly could have severe implications for the microbial stability, both throughout the brewing process and at the point of dispense. Recently a drive to produce alternative beer-like beverages for changing customer demands, has led to a significant change to some of the products breweries produce. NABLABs are one of the main ways in which breweries are adapting. The main microbiological hurdles in beer are: a high ethanol content (3.5-5.5%), low pH (<4.5), isomerised-α acids, low sugar content, low oxygen, and high CO_2 concentration (Vriesekoop *et al.,* 2012).

1.6.1 Ethanol

Ethanol is such a significant anti-microbial agent a 70% solution of it is often used to sterilise laboratory surfaces after they have been cleaned (Graziano *et al.,* 2013). Although a significantly lower concentration of ethanol is found in beer (3.5 – 5.5% typically) it is still thought to be one of the major anti-microbial hurdles. At concentrations found in beer ethanol induces cell membrane leakage, production of uncross-linked peptidoglycan leading to cell lysis and disrupts a range of other cell functions in bacteria (Menz *et al.,* 2008). Cell membrane permeability has been shown to increase in the presence of ethanol, making it difficult for cells to maintain a stable cytoplasmic pH due to increased proton flow (Barker and Park, 2001). Despite these effects there are still many bacteria and yeasts that can withstand and grow in up to 14% ABV beverages (Wang *et al.,* 2022; Pardah, 2015). It had been previously noted that beers with a higher ethanol content are more resistant to spoilage from *Lactobacillus brevis* (Shimwell, 1935). *Lopez et al*. (2020) analyzed 38 craft beers from the Spanish market and found that 100% of beers under 5% were contaminated with wild yeast, over 6% ABV only 41% were contaminated. Quain (2021) dosed ethanol

back into NABLABs to directly assess the effects of ethanol. The growth of spoilage organisms was seen to reduce by 24% in a NABLAB re-alcoholised to 8% ABV. Interestingly, adding ethanol to raise the ABV of a 4.5% ABV lager to 8% ABV reduced growth by 62%, showing that the antimicrobial effect of ethanol may also be dependent on other factors (Quain, 2021). The common knowledge that a regular strength beer cannot allow growth of pathogens holds up under research, as *E. coli* 0157:H7 and *Salmonella* Typhimurium have been shown to be inhibited in un-hopped wort over 4% ABV (pH of 5.5) and in beer over 2.7% ABV (pH of 4.3) (Menz *et al.,* 2010)**.** In 7% ABV back-sweetened wine and cider models, *E. coli* 0157:H7, *Salmonella enterica,* and *Listeria monocytogenes* showed a reduction in viability within 96hrs (Xiong *et al.,* 2021). Similar results were observed in Korean turbid rice wine at 6-7% ABV when stored at 5°C. However, some viable *E.* coli 0157:H7 was still present after 28 days and *Bacillus cereus* was also able to survive unaffected in its sporulated form (Kim *et al.,* 2014). The foodborne pathogen *Staphylococcus aureus* shows no tolerance to wort or beer and *Listeria monocytogenes* growth was prevented in un-hopped wort at >2%ABV (Menz *et al.,* 2010; Menz *et al.,* 2011).

1.6.2 pH

The pH of beer is usually in the range of 3.6-4.6 providing an inhospitable environment for many bacteria. But this provides little inhibition to yeasts. Those bacteria that can survive are often themselves acid producers e.g. *Lactobacillus* (Lactic acid), *Acetobacter* (Acetic acid) and *Pediococcus* (Lactic acid), so they already have an innate resistance to a low pH (Wang *et al.,* 2018). However, the methods used by bacteria to maintain their desired intracellular pH is energy intensive (Sun *et al.,* 2011; Booth, 1985; Suzuki, 2011). The bacteria need to maintain this intracellular pH to prevent the denaturing of important enzymes related to their metabolism, as well as maintaining their proton motive force

(PMF) (Suzuki, 2011). Dissipation of the PMF and denaturing of enzymes interferes with energy production and inevitably leads to cell death (Hutkins and Nannen, 1993). A low pH also plays a role in isomerised-α acids antimicrobial effects. Isomerised-α acids and a low pH work synergistically as both disturb the intracellular pH of the cell (Simpson and Hammond, 1991).

There are only a few pathogens which can grow in the normal pH range of beer, *Salmonella* Typhimurium and *E. coli* 0157:H7 being two of them. Both being able to grow in wort at a pH of 4.5 and low alcohol beer at a pH of 4.3, while being able to survive in full alcohol beer for just over 20 days at 14°C and possibly even longer if stored at 4°C (Menz *et al*., 2011). The growth of both pathogens in low alcohol beer was prevented at a pH of 4.0, however no data for these pathogens in low alcohol beer exists between 4.3 and 4.0 (Menz *et al.,* 2011; L'Anthoën and Ingledew, 1996).

1.6.3 Hops

The major anti-microbial component of hops are the isomerised-α acids, which also contribute the distinctive bitter taste to beer (Briggs *et al.,* 2004). They inhibit the growth of Gram-positive bacteria but not Gram-negative bacteria (in the concentrations found in beer). However, some Gram-positive bacteria such as *Lactobacillus* and *Pediococcus* have acquired resistance mechanisms involving the genes *horA* and *horC* (Suzuki, 2011). The isomerised-α acids exhibit antimicrobial effects against Gram-positive bacteria by acting as a proton ionophore, this decreases the intracellular pH of the cell and so dissipates the proton gradient, in turn reducing proton motive force causing a decrease in uptake of nutrients and eventual death of the cell (Suzuki, 2011). Once the iso-α acids dissociate with their proton they can pick up valuable divalent cations such as Mn^{2+} and then leave the cell taking the cation with them (Suzuki, 2011).

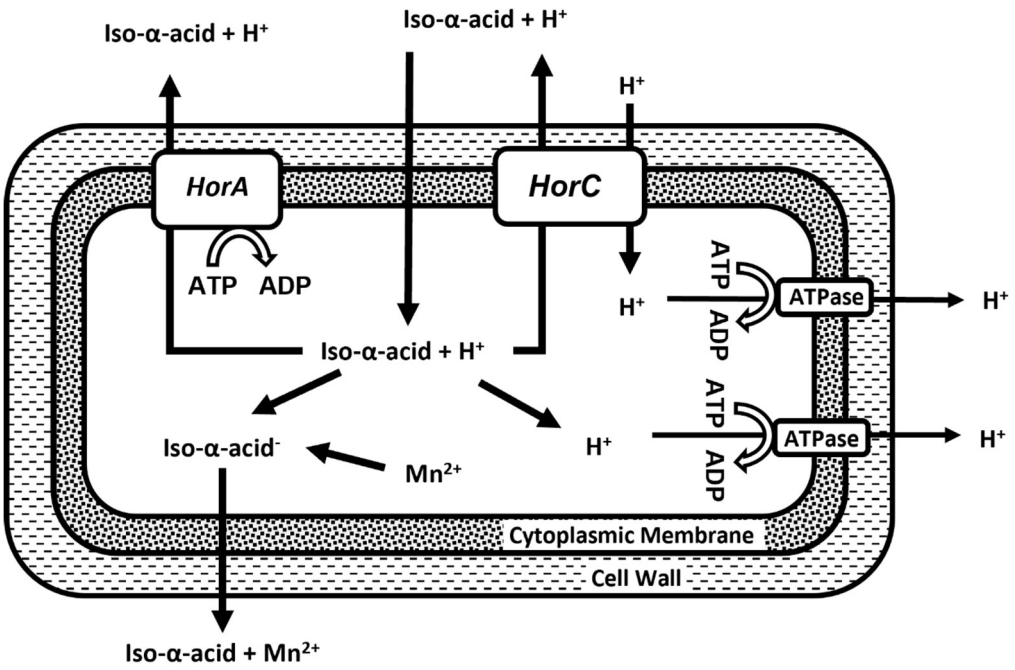

Figure 5: Mechanism of hop resistance by HorA and HorC expression and overexpression of ATPase protein. Adapted from Suzuki et al., 2006

To prevent this from happening, HorA, which is an ABC type multidrug transporter when expressed, expels the hop compounds from the cell before they can release protons and exchange for cations, thus preventing or reducing the acidification of the intracellular fluid (Suzuki, 2015). HorC acts as a proton motive force dependent multidrug transporter, although this doesn't help with the acidification of the intracellular fluid it does prevent Iso-α acids from binding cellularly important cations (Figure 5) (Suzuki *et al.,* 2006). Hop resistant lactic acid bacteria often overexpress proton translocating ATPases which move protons out of the cell at the cost of ATP (Figure 5).

Most Gram-positive pathogens that have been tested in the presence of isomerised-α acids are sensitive to them, and don't appear to possess any hop resistance genes, with the maximum of 5 IBUs being withstood by *Listeria monocytogenes* (Menz *et al.,* 2010). Most beers should be comfortably above this level. However, with certain production methods of alcohol-free beers, very low IBUs may be required to achieve the correct organoleptic properties (Quain, 2021). However, as *E. coli* 0157:H7 and *Salmonella* Typhimurium are Gram-negative they are not affected by isomerised-α acids, at least at the concentrations

found in beer (Menz *et al.,* 2011). There are other antimicrobial compounds in hops such as the beta acids which also show greater antimicrobial activity than the isomerised-α acids (Karabín *et al.,* 2016). However, the beta-acids are much less soluble in water and are found in much lower concentrations, so in beer they are less likely to play a major role in antimicrobial activity (Krofta and Mikyska, 2014). Both *E. coli* 0157:H7 and *Salmonella* Typhimurium have been grown in low alcohol beer, and so any other antimicrobial compounds possibly found in the hops also did not appear to affect them significantly in the beers tested (Menz *et al.,* 2011). This is not to say that they may not be affected by them at higher concentrations should the hop dosing increase or an extract of a specific hop compound be employed.

1.6.4 Gases (CO_2 and O_2)

The concentration of carbon dioxide in finished beer is usually in the region of 5000ppm. In the process of fermentation and carbonation the levels of dissolved oxygen are often reduced below 200ppb which will limit the growth of most aerobic microorganisms. There are a variety of mechanisms by which CO_2 inhibits growth, apart from just creating an anaerobic environment (Vriesekoop *et al.,* 2012). It decreases the pH of the solution by reacting with water to form carbonic acid and can also affect carboxylation and decarboxylation reactions (Daniels *et al.,* 1984). It has been shown that increased concentrations of CO_2 can slow the growth rates of pathogens such as *E. coli, L. monocytogenes* and *Bacillus cereus* in milk at 15°C (Martin *et al*., 2003) but did not prevent growth of any in this study. Menz and colleagues tested the survivability of *E. coli 0157:H7* in full strength beer with normal unopened bottle levels of CO_2, compared to a 100ml sample in a 250ml Erlenmeyer flask to represent the falling CO_2 levels of their experimental conditions, they saw no significant differences in survival (Menz *et al*., 2011).

1.6.5 Nutrients

There is a high concentration of nutritive substances in wort which are highly desirable for effective yeast growth and fermentation. These include; sugars such as glucose, fructose, sucrose, maltose and maltotriose. Nitrogenous compounds including free amino nitrogen, peptides, and proteins. As well as vitamins, ions, and trace elements (Smart, 2002). Many of these nutrients are metabolised by yeast throughout the fermentation, leaving the resulting beer with minimal residual nutrients (Sakamoto and Konings, 2003). The distinct lack of nutrients reduces the risk that spoilage microorganisms will be able to grow, with well-attenuated beers having been shown to be less likely to spoil (Rainbow, 1971). Higher free amino nitrogen and total soluble nitrogen levels have been shown to the increase the growth of a range of beer spoilage LAB (Fernandez and Simpson, 1995). In addition, citrate, pyruvate, malate and arginine specifically, have been shown to be utilised by beer spoilage LAB. This is particularly pertinent to the environment of beer, as these amino acids can positively influence proton motive force generation in low nutrient environments (Suzuki *et.al,* 2005; Suzuki, Iijima, Sakamoto *et.al,* 2006). Residual fermentables (glucose, fructose and maltose) have been shown to be a primary driver of microbial spoilage in NABLABs (Quain, 2021). However, this same study showed no clear relationship between spoilage and FAN. Possibly indicating that the effects of FAN on spoilage may be minimal when considering under-attenuated beers with a large quantity of residual fermentables, such as NABLABs produced by restricted fermentation.

1.7 Preservatives

A preservative is a substance used to aid in maintaining a products quality and safety. In food and drink, they are mainly used to prevent microbial contamination from spoilage and pathogenic microorganisms and reduce oxidation. Both of which would otherwise impair the products organoleptic properties or make it unsafe to consume. Some common preservatives used in the food and drink industry are, sorbic acid, benzoic acid, sulphur dioxide, nitrites, ascorbic acid and phosphoric acid. The use of many preservatives in food and drink is regulated, the concentration allowed differs depending on the food or drink being produced and the country where it is being produced. In soft drinks for example, potassium sorbate can be used with a maximum limit of 300ppm in the EU and UK (EU Regulation 1129, 2011), whereas in the US it is permitted up to 1000ppm (Code of Federal Regulations, 2022).

1.7.1 Sulphur Dioxide

Sulphur dioxide (SO_2) is one of the most common food and drink preservatives, it has a long history of use especially in wines and ciders (Garcia-fuentes *et al.,* 2015). In beer, sulphur dioxide is found at levels from <1 to 50mg/L. It originates from yeast as a metabolic by-product, from processing aids such as isinglass finings, or added directly as a preservative if permitted. The legal limit of sulphite permitted in kegged NABLABs in the UK is 20mg/L, cask ale is permitted up to a limit of 50mg/L due to the addition of isinglass finings into the cask on racking (EU Regulation 1129, 2011). It is often used as an antioxidant as it can react with carbonyl compounds which will cause stale off-flavours (Guido, 2016).

Sulphur dioxide also has antimicrobial properties, most effective against Gram-negative organisms, although some effect is exhibited against Gram-positive bacteria, yeasts and moulds (Illet, 1995). When in solution SO_2 is involved in a complex equilibration reaction of

SO_2~H_2O, HSO_3^- and SO_3^{2-} (Figure 6). Molecular SO_2 (SO_2~H_2O) has the greatest antimicrobial activity as it can diffuse directly into bacterial cells. Bi-sulphite (HSO_3^-) has some antimicrobial activity but is about 5-10 times less effective than molecular SO_2 (Liett, 1995). Sulphite (SO_3^{2-}) has little to no antimicrobial effects, at least at concentrations commonly used in food and drink (Lisanti *et al.,* 2019). The bias of this equilibrium is affected by pH and temperature (Guido, 2016). However, in the standard pH range of beer the majority of SO_2 species are Bisulphite with a very small amount of molecular SO_2 (Figure 6). Additionally, when SO_2 becomes bound to carbonyls or other compounds in beer its antimicrobial effects are reduced further, these reactions are mostly reversible (Guido, 2016).

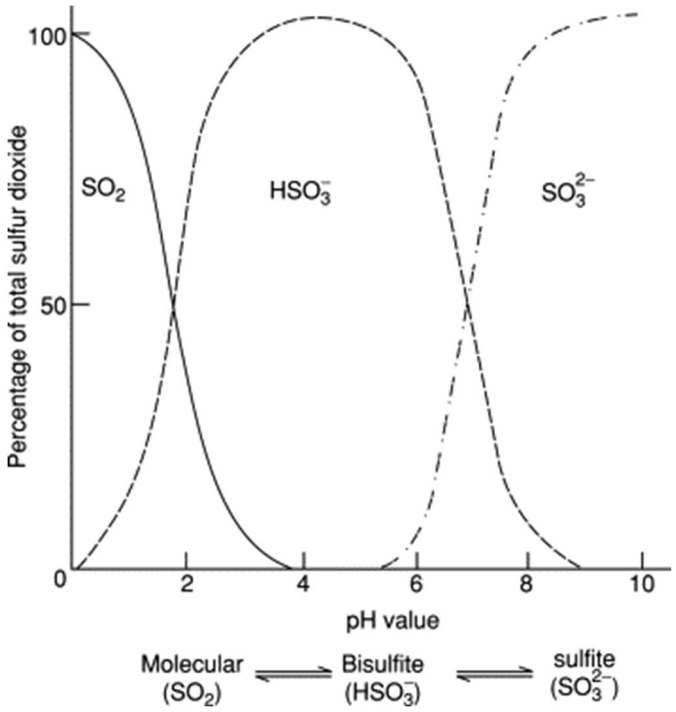

Figure 6: The effect of pH on equilibrium of SO_2 species from (Illet, 1995)

The effect of sulphites in regular strength beer or NABLABs on spoilage has not been extensively studied. Most information comes from food, wine and cider research as sulphites are regularly used in these products and the doses allowed are much higher than that in beer (EU Regulation 1129, 2011). Basaran-Akgul and colleagues researched the effects of varying concentrations of SO_2 on *E. coli* 0157:H7 in apple cider (Juice). Their research showed that even at a dose of 25ppm SO_2 there was antibacterial activity against *E. coli* 0157:H7 in 24 hours at room temperature. This was not seen on the two apple ciders produced with pH ranges closer to beer (Red delicious pH 3.91 and Rome pH 3.76). However, these juices do have fewer antimicrobial hurdles, a high sugar content, and survival was only tested over 24 Hours (Basaran-Akgul *et al.,* 2009).

1.7.2 Potassium Sorbate

The antimicrobial activity of potassium sorbate was first discovered in 1939 and was approved as a food additive in 1953 (Catherine *et al.,* 2014). It is now used in cheese, baked goods, dried meats, wine, apple cider, soft drinks and many others due to its low toxicity even at exceedingly high doses, high organoleptic threshold, good water solubility, large scale manufacturing infrastructure and low use costs (Catherine *et al.,* 2014). Most of the antimicrobial effects of potassium sorbate are related to the undissociated acid molecule (Lück and Jager, 1997). The amount of undissociated acid present is consequently tied to the pH of the substrate, with the upper limit for activity being pH 6.5 (Liewen and Marth, 1985) (Figure 7). This gives a much wider pH range of effective activity than sulphites and sodium benzoate, with a pKa of 4.69. At the higher end of average beer pH potassium sorbate is still around 65% the undissociated acid, which could lead to good antimicrobial activity and the ability to use low doses (Figure 7). The inhibition mechanisms of potassium sorbate are not fully understood. However, it is believed that it can inhibit transport of carbohydrates by inhibiting enolase and lactate dehydrogenase, uncouple oxidative phosphorylation, interfere with other dehydrogenases, and may also have action against

cell walls (Liewen and Marth, 1985). This may help to explain sorbic acids broad spectrum effectiveness. It is not strictly a microbiocidal agent and is more well known as microbiostatic, with the organisms it is most commonly used to control being yeasts and fungi (Sofos and Busta, 1981).

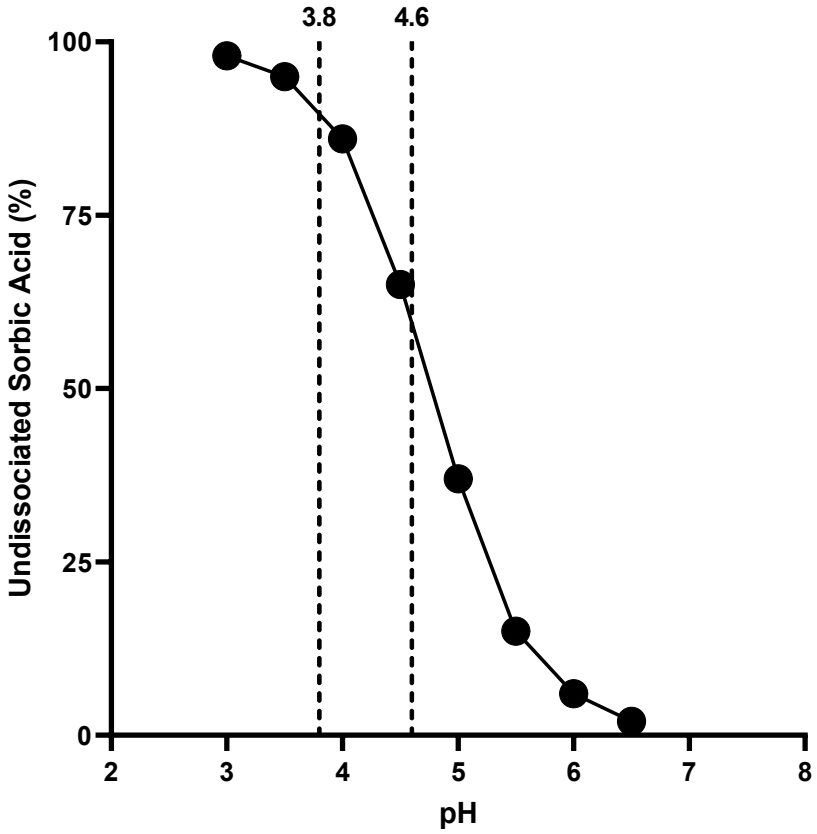

Figure 7: Effect of pH on sorbic acid dissociation in solution with upper (4.6) and lower (3.8) values for typical beer pH (Adapted from Sofos and Busta, 1981)

It is extensively used in wine and cider making at 100-200ppm after primary fermentation has finished to prevent any secondary fermentation, either from the brewing yeast or contaminating yeasts (Catherine *et al.,* 2014). This prevents excess ethanol formation, off flavour development, and carbonation of a usually still and packaged product. It may also have an effect on preventing bacterial contamination, however it has been suggested that it does not provide adequate protection against acetic bacteria or excessive malolactic fermentation (Catherine *et al.,* 2014)**.** In both the cider and wine use cases, potassium sorbate is often used alongside sulphites which could provide additive or even synergistic

microbiostatic affects (Cojocaru and Antoce, 2012). Despite the strong microbiostatic affects of sorbic acid, when there is a high microbial load there is a greater chance of a microorganism being present which can metabolise it (Catherine *et al.,* 2014). This can cause a few problems, firstly being metabolised will reduce the amount of sorbic acid present in the substrate and so reduce its effectiveness at preventing microbial growth. Secondly, the metabolization by-products can be off flavours and aromas (Chisholm and Samuels, 1992). These can include pentadiene (kerosene), ethyl sorbate (honey) and 2-ethoxy-3,5-headiene (geranium) all of which have low enough organoleptic thresholds to be detected even with the relatively low concentration of sorbic acid (Catherine *et al.,* 2014).

1.7.3 Sodium Benzoate

Sodium benzoate is the sodium salt of benzoic acid, it has been used as a preservative in foods since the early 1900s (Lück and Jager, 1997). Its highly effective microbiostatic activity, along with having a high sensory threshold, has ensured it remains a popular preservative choice for acidic foods. Benzoic acid naturally occurs in many foods including cranberries, blackberries, certain varieties of tomatoes and some fermented foods such as yoghurts (Olmo *et al.,* 2017). This is part of the reason why some of these foods resist yeast and mould growth. Although Benzoic acid itself doesn't significantly impact the organoleptic properties of food, it can however stimulate taste cells and cause oral prickling, increased sweetness, and reduced bitter, salt, and sour perception (Otero-losada, 2003). The undissociated molecule of benzoic acid can cross cell membranes, once inside the cell it dissociates due to the higher intracellular pH which releases charged anions and protons (Warth, 1988). This alters the intracellular pH, inhibits enzymes involved in the Krebs cycle, and disrupts the cell membrane (Olmo *et al.,* 2017). Much like sulphites and sorbates the pH of the solution greatly effects the level of undissociated acid present, with

the pKa being pH 4.19 at 25°C (Olmo *et al.,* 2017). There is only 12.9% undissociated acid at a pH of 5.0 (Figure 8).

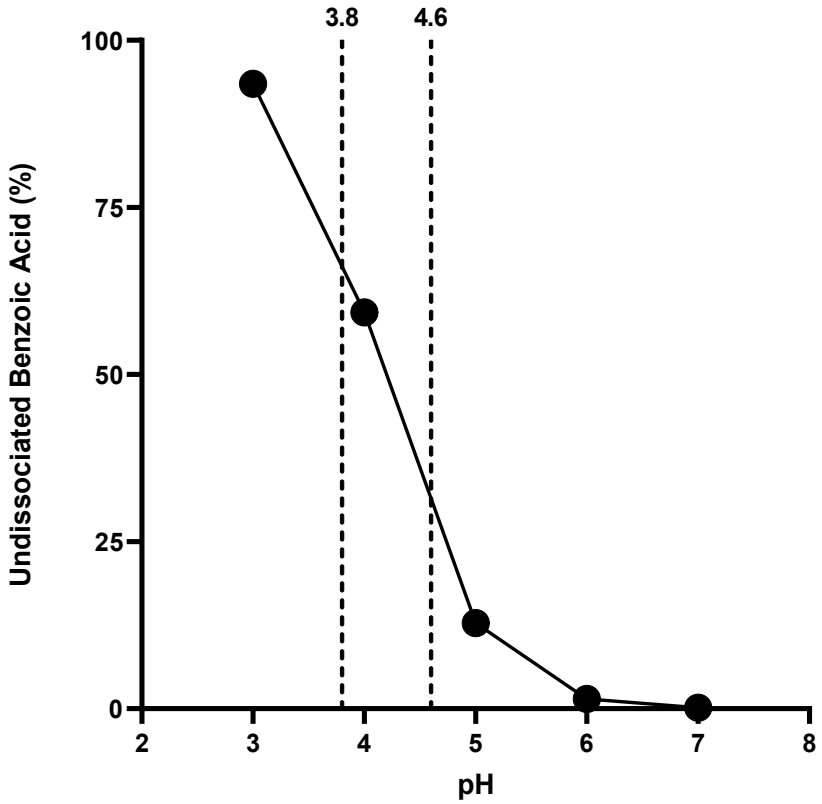

Figure 8: Effect of pH on Benzoic acid dissociation in solution, with upper (4.6) and lower (3.8) values for typical beer pH (adapted from Baird-Parker, 1980)

Although mainly used to prevent the growth of mould and yeast, benzoic acid also exhibits bacteriostatic effects against many spoilage and pathogenic bacteria, notably *E. coli* 0157:H7 (Ceylan *et al.,* 2004). The maximum permitted dose of sodium benzoate for low alcohol beer in keg in the UK is 200ppm (EU Regulation 1129, 2011). However, in the US it is permissible up to 1000ppm (Code of Federal Regulations, 2022). The World Health Organisations (WHO) Acceptable Daily Intake (ADI) for benzoic acid was 5mg/kg body weight/day which equates to 350mg a day for a 70kg person but was recently reviewed and raised to 20mg/kg bodyweight/day (WHO, 2021). There has been some controversy surrounding the safety of benzoic acid as a food preservative, it has previously been shown that benzoic acid can react with ascorbic acid to form the highly carcinogenic compound

benzene (Gardner and Lawrence, 1993). This was a problem as many soft drinks that were using benzoic acid as a preservative also routinely used ascorbic acid (Gardner and Lawrence, 1993). The use of this combination has now been advised against and the majority of producers have reformulated to avoid this. It was shown by Lachenmeier and colleagues that there was no longer a significant correlation between benzoic acid levels and benzene levels, which they attributed to producers reformulating to avoid the mixing with ascorbic acid (Lachenmeier *et al.,* 2008). Due to the low recommended ADI and previous controversy surrounding benzene levels many consumers are wary of products containing benzoic acid, and so producers are looking to reduce its use in their products by lowering the dose or replacing it with a more consumer acceptable preservative.

1.7.4 Cinnamaldehyde

Cinnamon is a spice made from the bark of trees in the genus *Cinnamomum*. The majority (85-90%) of the cinnamon oil found in the bark comprises of cinnamaldehyde with the majority of that being the trans-isomer (Doyle and Stephens, 2019). Trans-Cinnamaldehyde has Generally Recognised as Safe (GRAS) status and is approved for use as a food additive (Doyle and Stephens, 2019). It has been shown to inhibit the growth of Gram-positive and negative bacteria, yeast and moulds as well as inhibiting the formation of biofilms (Firmino *et al.,* 2018; Doyle and Stephens, 2019). However, the sensory threshold is very low (around 5-6ppm) and so if used in food products its dose must be carefully controlled as to not affect the sensory qualities. A study on low alcohol wine preservation with cinnamon essential oils assessed the sensory effects on the products and all those with doses higher than 100ppm were rejected (Mitropoulou *et al.,* 2020). Although this level was below the minimum inhibitory concentration from initial zone of inhibition assays, it did still slow the rate of spoilage by bacteria and yeasts significantly. In challenge tested samples without cinnamaldehyde growth was first detected on day 9 (both bacteria and yeast) of room

temperature incubation whereas the cinnamon essential oil dosed sample spoilage was first detected on day 74 for bacteria and day 81 for yeasts (Mitropoulou *et al.,* 2020).

1.8 Microbiology of Beer

Despite all the microbiological hurdles that bacteria and yeasts need to overcome, beer is still subject to spoilage from a number of microorganisms. There are a range of issues caused by beer spoilage bacteria and yeasts such as: significant production of off-flavours (diacetyl, H_2S, caprylic, butyric etc), increase in turbidity, fermentation of long chain sugars in package, causing over-carbonation and excess alcohol production, and in the case of some *Pediococcus spp.* 'Ropiness' caused by excessive production of exopolysaccharides (Bokulich and Bamforth, 2013).

The main spoilage microorganisms found in beer are Lactic acid bacteria, Acetic acid bacteria, and beer spoilage yeasts.

1.8.1 Lactic Acid Bacteria (LAB)

The Lactic Acid Bacteria (LAB) are a group of Gram-positive, facultative anaerobes including *Lactobacillus brevis*, *L. casei, L. plantarum*, *Pediococcus damnosus* and *P. inopinatus* to name a few. Being Gram-positive they are susceptible to iso-α acids, however some strains have evolved to resist these anti-microbial effects through the genes *horA* and *horC* (Suzuki, 2011) (Figure 5). This has allowed LAB to become responsible for 60-90% of beer spoilage incidents (Suzuki, 2011). Their main spoilage effect is acidification of the beer via production of lactic acid, though some like *Pediococcus damnosus* can produce diacetyl and exopolysaccharides causing 'ropiness'. Interestingly, beer spoilage LAB are often micro-aerotolerant anaerobes and so although they cannot utilise oxygen as an electron acceptor for respiration, they can produce a pseudocatalase, believed to be a Mn^{2+} containing molecule, which can remove toxic peroxides produced by the exposure to oxygen (Priest, 2003). As anaerobes, and often micro-aerotolerant ones, brewery adapted LAB are a

spoilage threat to bottle, can, keg and cask. So therefore, methods for their reduction or elimination from final pack is imperative to prevent major spoilage events.

1.8.2 Acetic Acid Bacteria (AAB)

Acetic acid bacteria (AAB) are a group of Gram-negative, aerobic, rod-shaped bacteria, which have the ability to oxidise ethanol into acetic acid. This includes the Genera *Acetobacter* and *Gluconobacter* (Van Vuuren and Priest, 2003; Lynch *et al.,* 2019). They have a high alcohol tolerance (around 10%), are highly acid tolerant, being able to survive acetic acid concentrations of 6%-10% (v/v) (Lynch *et al.,* 2019). In addition, they are highly resistant to iso-α acids (Paradh, 2015). So, they can grow well in most beers, wines and ciders providing there is sufficient oxygen (De Roos and De Vuyst, 2018). Excess production of acetic acid from AAB infection causes beers to taste vinegary, additionally the intermediary step for oxidation of ethanol to acetic acid is acetaldehyde which can give a characteristic green apple aroma (Gomes *et al.,* 2018). In recent years, there has been a vast improvement to brewery sanitation and dissolved oxygen control, which has reduced the number of spoilage incidences caused by AAB (Paradh, 2015). However, they are still prevalent in packaging areas and in pubs cellars and dispense lines (Jevons and Quain, 2021). Cask beer is particularly sensitive to AAB spoilage as the cask is vented when tapped allowing oxygen and airborne contaminants from the cellar to enter the cask (Hill, 2015). For kegged and bottled products AAB are not of great concern due to the lack of oxygen. However, some AAB are micro-aerophilic and can take part in the formation of biofilms so they should always be considered as a potential spoilage threat (Kubizniaková *et al.,* 2021).

1.8.3 *Enterobacteriaceae*

The *Enterobacteriaceae* are facultatively anaerobic, Gram-negative, rod-shaped bacteria. Some species from this family that have been isolated from breweries include *Obseumbacterium proteus*, *Rahnella spp., Citrobacter freundii* and *Klebsiella spp.* (Van

Vuuren and Priest, 2003). Out of this large family of organisms only *O. proteus* and *Rahnella spp.* have regularly been shown to survive the harsh conditions of fermentation, often surviving in cropped yeast (especially top cropping ale fermentations) and so being able to cause repeat infection in the brewery (Van Vuuren and Priest, 2003). Most *Enterobacteriaceae* are thought to enter the brewery from the water supply. *Obseumbacterium proteus* is tolerant to ethanol of up to 6% ABV, and if found in high enough concentrations can cause beers to have a fruity parsnip like odour from its production of dimethyl sulphide amongst other volatiles (Van Vuuren and Priest, 2003). It also contains the enzyme nitrate reductase which converts nitrates in the wort to nitrites, this increases the levels of the carcinogenic apparent total *N*-nitroso compounds (ATNC) (Paradh, 2015). *Rahnella spp.* has some resistance to ethanol but is not found to survive in high gravity fermentations. It can affect fermentation performance as well as producing large quantities of diacetyl and dimethyl sulphide, depending on the level of infection (Van Vuuren and Priest, 2003).

1.8.4 *Zymomonas*

Zymomonas are a Gram-negative, facultatively anaerobic, short rod-shaped bacteria, with some strains being motile. They readily ferment glucose and fructose by a modified Entner-Doudoroff pathway but cannot utilise maltose (Paradh, 2015). They are highly ethanol tolerant, depending on strain, growth can be observed at 8-10% ABV. Their main spoilage niche is primed cask beer, as the priming sugar provides sufficient glucose for rapid growth. This growth leads to production of acetaldehyde and hydrogen sulphide giving the beer a rotten apple and rotten egg aroma (Priest, 2003). The route of contamination is believed to be from soil, which could easily get onto the outside of casks and then into the cask on racking. They have not been implicated in any incidences of spoilage in lager (Paradh, 2015). New products being developed such as NABLABs produced by restricted

fermentation may have residual glucose or fructose, which could increase the likelihood for *Zymomonas* infection, especially if NABLABs are to be served on draught dispense systems in close proximity to primed cask beers.

1.8.5 Strict Anaerobes

Pectinatus are strictly anaerobic, Gram-negative, curved helical rods that can resist iso-α acids, alcohol (<5.2%) and low pH, with growth above 4.0 pH and survival down to 3.5 pH. Their spoilage effects are strong, producing H_2S (rotten egg), propionic acid (body odour) and turbidity (Juvonen, 2015). But due to their strictly anaerobic nature they haven't been the most prevalent spoilage microorganisms (Paradh *et al.,* 2011). However, as breweries dissolved oxygen control continues to improve their prevalence is likely to increase, especially as some strains of *Pectinatus* have been shown to grow in beer up to an oxygen content of 300ppb (Kyselová and Brányik, 2015). Although they can grow in full strength beer their growth rate is impaired, the lower the ABV the greater their growth rate is. This could lead to NABLABs being an ideal niche for them.

Megasphaera are strictly anaerobic Gram-negative cocci which can withstand iso-α acids, pH of 4.0 and ABVs of up to 4.2% (Juvonen, 2015). However, their growth is dramatically reduced at 2% ABV and anything higher will show little to no growth (Juvonen, 2015). They have been shown to tolerate oxygen up to 300ppb which makes them a viable spoilage organism for many modern breweries. Much like *Pectinatus spp.*, the spoilage effects of *Megasphaera spp.* are strong. The effects include: production of butyric (vomit), caproic (goaty), valeric (cheesy) compounds and significant turbidity (Juvonen, 2015). *Megasphaera* can be found in biofilms on stainless steel in bottling halls (Matoulková *et al.,* 2012). However, their relative susceptibility to the anti-microbial hurdles of a regular beer have so far prevented them from becoming a major beer spoiler. However, with the

increase in popularity for NABLABs especially in low dissolved oxygen packages such as keg, they now have a new potential spoilage niche (Paradh *et al.,* 2011).

1.8.6 Beer Spoilage Yeasts

Beer spoilage yeasts are any yeast that can negatively impact the beer and are not the desired production strain. The most common beer spoilage yeasts found in the brewing environment are the non-fermentative *Brettanomyces spp.* and *Pichia spp.* (Jevons and Quain, 2021). They can produce a range of off-flavours including barnyard, faecal, horsey and phenolic aromas, *Brettanomyces spp.* can also produce acetic acid and both cause significant turbidity (Campbell, 2003). Some strains of *Brettanomyces* and *Pichia* have been shown to have some resistance to commonly used preservatives in the food and beverage industry, including benzoic acid, sorbic acid and SO_2 (Aneja *et al*., 2014). Another beer spoilage yeast of concern is the brewing variant *Saccharomyces cerevisae var. diastaticus*, this strain possesses the gene *STA1* which allows it to produce extracellular glucoamylase (Krogerus and Gibson, 2020). This enzyme breaks down oligosaccharides and starch in beer into shorter chain sugars that the yeast can then ferment. So, if these yeasts contaminate a beer, they can cause it to ferment further, which not only significantly effects the flavour and alcohol content, but also produces CO_2 which can be disastrous in package. Excessive re-fermentation in pack has caused bottles to explode, leading to large recalls and lawsuits (Meier-Dörnberg *et al.,* 2018). The excess alcohol formation would also be a big problem for producers of NABLABs where the product could end up well above the stated ABV, which would cause major legal issues especially with regards to drink driving.

1.8.7 Pathogens

A pathogen is a microorganism that causes or can cause disease in a susceptible host (Pirofski and Casadevall, 2012). Food-borne pathogens are pathogens which are present in

food or drink and can cause disease when ingested, either when the pathogen establishes itself in the host or produces a toxin in the consumed food (Bintsis, 2017).

Escherichia coli 0157:H7 is a Gram-negative, rod shaped, facultative anaerobe. It is a shiga-toxin producing foodborne pathogen that can cause diarrhoea, haemorrhagic colitis and haemolytic-uremic syndrome (HUS) (Ameer *et al.,* 2021). Compared to other strains of *E. coli* it is highly acid resistant being able to survive at pH levels as low as 2.0 (Foster, 2004). Being Gram-negative, it is also resistant to Iso-α acids (Menz *et al.,* 2011). However, it is sensitive to ethanol > 2% ABV in beer but has been shown to grow in solution as high as 4% ABV in a 5.5 pH sweet wort which had ethanol added to it (Menz *et al.,* 2010). There have been multiple outbreaks of *E. coli* 0157:H7 mainly in raw or undercooked beef and vegetables contaminated with faeces (Pennington, 2014), but also in unpasteurised apple cider (Juice) which usually has a pH below 4.0 (Zhao *et al.,* 1993). There has been no evidence to show that *E. coli* 0157:H7 can grow in beers above 2% ABV. However, It has been shown to grow significantly in 0.5% ABV beer with a pH of 4.3 (Menz *et al.,* 2011). It also possesses the ability to ferment lactose, which is often used as an addition to increase the body and mouthfeel of NABLABs (Rahn *et al.,* 2012; Big Drop, 2022; Club Soda, 2022).

Salmonella Typhimurium is a Gram-negative, rod shaped, facultative anaerobe. It is a foodborne pathogen whose toxicity comes from its O-antigen lipopolysaccharide (LPS) outer membrane (Ashurst *et al.,* 2022). It causes gastroenteritis by damaging the microvilli in the intestine which leads to diarrhoea (Ashurst *et al.,* 2022). *S.* Typhimurium is resistant to Iso-α acids, tolerant to acid above a pH of 4.3, is sensitive to ABV > 2%. Although it exhibited the same ability to grow in up to 4% ABV 5.5 pH sweet wort as *E. coli* 0157:H7 (Menz *et al.,* 2010). It is one of the most common causes of food poisoning, causing over 1 million cases of food poisoning a year in the US alone (CDC, 2022). There is no evidence to

show that *S.* Typhimurium can grow in beers above 2% ABV but much like *E. coli* 0157:H7 it has been shown to grow in 0.5% ABV beer at a pH of 4.3 (Menz *et al.,* 2011).

Listeria monocytogenes is a Gram-positive, rod shaped, facultative anaerobe. It is a less common foodborne pathogen than *S.* Typhimurium. However, it has a high mortality rate around 15% with 260 deaths a year in the US (Rogalla and Bomar, 2021). Its virulence factors include: proteins to enable host cell attachment, actin polymerisation to help the bacteria move between cells, listeriolysin-O enabling the bacteria to escape from the host cells vacuole and the ability to grow at 4°C (Ashurst *et al.,* 2022). It is mostly found in cold deli meats, soft cheeses and raw sprouts (Rogalla and Bomar, 2021). Its ability to replicate at these low temperatures would be a serious issue for the brewing industry. Luckily, being Gram-positive means that it is susceptible to the anti-microbial action of isomerised-α acids >5 IBU (Menz *et al.,* 2010). It has been shown to be sensitive to ethanol but can resist up to 2% ABV in a pH 5.5 sweet wort. It was not able to grow in this sweet wort if the pH was 4.5 or below (Menz et al., 2011).

Clostridium botulinum is an obligate anaerobic, Gram-positive, rod shaped, spore-forming bacteria which can produce a neurotoxin called botulinum, which is the strongest toxin known to mankind (Masuyer and Stenmark, 2019). It can grow in the typical parameters of wort if allowed to cool to room temp and under anaerobic conditions. Boiling at 100°C for 15 minutes does denature the toxin, but it would not effectively kill any *C. botulinum* spores (Loutfy *et al.,* 2003). No resistance to isomerised-α acids has been observed, so even for a restricted fermentation low alcohol beer *C. botulinum* should not grow. Spores however could survive through the brewing process but if they are ingested no symptoms will occur, as usually *C. botulinum* will remain in its inactive spore form, apart from in rare cases (Jeffery and Karim, 2022).

Bacillus cereus is a facultatively anaerobic, Gram-positive, rod shaped, spore-forming, foodborne pathogen (McDowell *et al.,* 2021). Its pathogenicity is related to its exoenzyme toxin production, both in the food that it infects and in infection of the gastrointestinal tract (GI) (McDowell *et al.,* 2021). Not all *B. cereus* are pathogenic, some strains are highly pathogenic, and others are even used as probiotics (McDowell *et al.,* 2021). Currently, it is difficult to differentiate a pathogenic strain from a harmless one. Symptoms are mostly vomiting and nausea when the toxin is ingested and diarrhoea when the gastrointestinal (GI) tract is infected. Recently, *B. cereus* has been detected in fermented alcoholic beverages (Kim *et al*., 2014; Jeon *et al.,* 2015) and more concerningly a newly isolated strain has been shown to actively grow and spoil beer. It showed resistance to ethanol up to 6.7% ABV, iso-α acids to over 1g/ml and pH below 3.5 (Wang *et al*., 2017).

1.9 Microbiology of Draught Beer

Draught dispense has long had an issue of quality with improper temperature, over/under carbonation, haze, and off flavours and aromas produced by yeast and bacteria (Mallet *et al.,* 2018). The lines of draught dispense systems can be up to 120m long with many connecters to get the beer to the tap at the bar. The tap itself is one of the likely sources for contamination as it is open to the air and the microflora from the air, glassware, and bar staff (Quain, 2016). When this source of contamination is coupled with poor line cleaning practices the bacteria and yeast that have colonised the lines can persist and cause issues for subsequent beers. This can lead to consistently poor beer quality especially if the throughput on these lines is slow, which allows areas not properly cooled or insulated to warm up increasing the rate of spoilage (Mallet *et al.,* 2018).

The microbiome of draught beer lines has been assessed recently (Jevons and Quain, 2021; Bose *et al.,* 2021). The study identified *Brettanomyces* and *Acetobacter* species as the most common in the UK and found in all different types of draught beer tested. *Acetobacter* and *Saccharomyces spp.* were the most common in the US (Bose *et al.,* 2021). It was noted in both of these studies that the microflora found is somewhat determined by beer style.

Draught beer lines are a hospitable environment for beer spoilage bacteria. A constant supply of fresh beer, oxygen, and large surface area on which to grow especially when lines are poorly cleaned and irregularly replaced. This makes the lines and dispense system itself an ideal environment for biofilm formation (Hill, 2015; Quain, 2015). With improper cleaning these biofilms will be able to persist and quickly re-establish once beer is introduced to the line again. This is especially concerning for low alcohol beers as they may well already be more spoilable (Quain, 2021). With the removal of alcohol and greater concentration of fermentables they are a more hospitable environment than regular beer.

When biofilms are encountered with a changing external environment, they can detach in a process known as dispersion and become planktonic (Petrova and Sauer, 2017). This could mean that the introduction of a new beer, with a higher dissolved oxygen, sugar content, or lower pH, to a biofilm contaminated dispense system could signal the biofilm to start dispersion, possibly leading to rapid spoilage of an otherwise microbiologically stable beer (Quain, 2015). The formation of biofilms in draught dispense lines seems to be inevitable and the current recommended cleaning regime in the UK may not be sufficient to remove biofilms (Jevons and Quain, 2021). It is also known that the recommended guidelines are often not followed, and times between cleans are often extended, especially in times of economic struggle for publicans. Not removing the biofilm allows contamination of any beers subsequently served on those lines, which in turn will lead to further biofilm formation making it even harder to remove all biofilms.

In the UK, the standard keg dispense line cleaning regime recommended by the British Beer and Pubs Association (BBPA) involves introducing a suitable cleaning solution into the lines and 'steeping' for the manufacturers recommended time, pulling through some fresh cleaning solution periodically within this time (Wray, 2018) (BBPA, 2018). In the US, line cleaning operates by pumping the cleaning solution through the lines for 15 minutes adding a greater deal of mechanical action to the cleaning which may improve outcomes (Eßlinger, 2009). For beers that have the potential to be particularly susceptible to spoilage such as NABLABs, making sure that all biofilms are removed from the dispense system is of utmost importance. Especially regarding the possible infection with pathogenic organisms, which can under certain conditions survive in full strength beers at low temperatures. *E. coli* 0157:H7 has been shown to be able to form biofilms on stainless steel and glass, increasing the possibility of survival after line cleaning (Ryu *et al.,* 2004).

Biofilm formation also increases the possibilities of the bacteria gaining resistance to anti-microbials or cleaning agents, this could occur due to the diffusion gradient of the biofilm itself diluting the antimicrobial agent (Stewart, 2003). The microorganisms near the surface of the biofilm may be inactivated but those deep within may be exposed to the anti-microbials at sub-lethal concentrations, increasing the risk of resistance formation. In addition, some microorganisms dispersed from biofilms exhibit phenotypic changes which also increase their antimicrobial resistance (Petrova and Sauer, 2017). This, coupled with the fact that genetic exchange between bacteria is much more likely to happen in biofilms shows the importance of their removal and prevention.

As it would be incredibly difficult to exhibit the kind of control over line cleaning in trade as may be needed to prevent infection and spoilage of NABLABs from biofilms. It is imperative that we find a way of producing NABLABs which have a reduced spoilage potential and exhibit strong anti-pathogen activity particularly against *E. coli* 0157:H7 and *S.* Typhimurium.

1.10 NABLAB Microbiology

As the production and distribution of NABLABs is still fairly new there hasn't been a great deal of research into their spoilage. Most NABLABs have previously been packaged into bottles or cans and pasteurised, thus avoiding most spoilage issues. However, with the rise in demand for serving NABLABs on-trade, providing the beer in a commercially sterile state is only part of the solution (Drinks International, 2021; Quain, 2015). Draught dispense systems are not a sterile environment, and it seems that contamination of beer served through them is inevitable. This means that the rate at which a beer can spoil in this environment is critically important to maintaining its quality. A beer that has a greater number of microbiological hurdles should prevent or slow growth of spoilage microbes they come into contact with (Vriesekoop *et al.,* 2012). If the growth is slowed to a significant enough degree, the microbial contamination will not be visually or organoleptically evident within the time until the next clean. However, reducing the number of hurdles (i.e., removal of ethanol) could cause more rapid growth of these spoilers leading to spoilage of the beer within the time until the next clean. In addition, the greater the level of contamination the greater the chance for cleaning to be ineffective which then increases the risk of spoilage for the next beer (Quain, 2015).

1.10.1 NABLAB

Recently, it has been shown that the spoilage of NABLABs can be 2-5x greater than that of a 4.5% ABV premium lager (Quain, 2021). In this study 6 different alcohol-free beers and two low alcohol beers (0.5%) were challenged with microflora obtained from dispense lines. There was a large difference between the spoilage of the NABLABs. Contrary to the common assumption that the increased spoilage would be due to the lack of alcohol, it was observed that there was a much stronger correlation between spoilage and the level of 'fermentables' present, which also appears to explain why some NABLABs spoiled more

than others. To test the effect of ABV on spoilage Quain added ethanol into the NABLABs tested. The mean spoilage as measured (A_{660}) without alcohol was 1.289 and at 8% ABV was 0.974 which equates to a reduction of 24%. It has been noted that NABLABs will be at a much greater risk of spoilage by *Megasphaera* due to its low alcohol tolerance and increasing prevalence in the brewing industry (Suzuki, 2011), though no direct research exists.

The pathogens *E. coli* 0157:H7 and *S.* Typhimurium have been shown to grow in commercially available low alcohol beer by L'Anthoën and Ingledew and Menz *et al.* These are the only two studies on the growth of these pathogens in low alcohol beer and were published in 1996 and 2011 respectively. This is surprising given Menz *et al.* concluded 'pasteurization and pH values should be closely monitored, and the production of unpasteurized alcohol-free beer is not risk free' with L'Anthoën and Ingledew going as far to say, 'Draft AFB should not be manufactured in industry'. However, draught NABLABs are already being produced and served to customers (Beverage Daily, 2021). These studies did only each select one low alcohol beer to test, and from other research (Quain, 2021; Mallet *et al.,* 2018; Bose *et al.,* 2021) we know that the physical and chemical parameters of different beers vary greatly, and this affects the spoilers that can grow in them and the extent to which they can grow.

The beer tested by Menz *et al*. was only analysed for ABV, pH and IBU. However, L'Anthoën and Ingledew did analyse the sugar composition of 6 different low alcohol beers, although only one was used in the study (L'Anthoën and Inlgedew, 1996).

Table 2: Composition of regular and low alcohol beer used by L'Anthoën and Ingledew 1996

Beer	*Maltotriose g/100ml*	*Maltose g/100ml*	*Glucose g/100ml*	*Fructose g/100ml*	*ABV*	*pH*
Regular	0.32	0.18	0.00	0.00	3.84	4.26
Low Alcohol	0.72	2.92	0.7	0.14	0.4	5.25

As shown in Table 2, and Appendix 1 the chosen low alcohol beer has a high level of glucose and fructose, as well as an abnormally high pH when compared to the other beers analysed. These parameters are known to aid in the growth of *E. coli* (Kornberg and Lourenco, 2006).

The NABLAB industry has changed significantly in the past 30 years, there are now lower sugar alternatives of low alcohol beers available, especially with the improvement and industrialisation of alcohol removal processes such as vacuum distillation and reverse osmosis. These methods allow the beer to ferment fully, this means the amount of fermentable sugars such as glucose, fructose and maltose found in the final product should be significantly lower. A correlation between NABLABs produced by dealcoholisation and reduced spoilage has been observed (Quain, 2021). So, these pathogens need to be assessed again in current NABLABs to ascertain whether they are still a risk, which is particularly important if the industry wishes to continue serving NABLABs through draught dispense systems.

1.10.2 Fruit Juice

Fruit juices also suffer from microbial spoilage, they are often high in fermentable sugars and have a low pH (2.0-4.5). This makes them susceptible to growth of similar spoilage organisms that beers are, and with the lack of ethanol may also be similar to NABLABs

spoilage. The main microorganisms of concern for the fruit juice industry are yeast (Azeredo *et al.,* 2016). With the high sugar content of the fruit juice and yeasts high acid tolerance, spoilage is inevitable. Therefore, many fruit juices are pasteurised. The high sugar content and lack of other microbiological hurdles allows microorganisms to withstand heat to a greater degree (Rachon *et al.,* 2021). The yeasts found spoiling fruit juices are common to beer too, some of the most common being *Brettanomyces, Pichia, Saccharomyces, Candida* and *Rhodotorula* (Wareing and Davenport, 2007).

Moulds are also an issue for some fruit juices if they have sufficient oxygen content to support their growth. Some mould species such as *Byssochlamys fulva* and *Talaromyces* are heat resistant and can survive heating to 85°C for 4.5 minutes due to the production of heat resistant ascospores (Aneja *et al*., 2014). The bacteria that are commonly found spoiling fruit juice are also similar to those that we would see in beer, with the two major groups being Lactic acid bacteria and Acetic Acid bacteria. Unlike beer, the *Leuconostoc* species are a major spoiler in fruit juice, producing lactic acid, diacetyl and CO_2. They don't harbour either *horA* or *horC* genes so often aren't considered to be a beer spoiler. However, it has been shown that some strains of *L. mesenteroides* are able to grow in beer with an IBU of 25 (Ruiz *et al.* 2018). With reduced microbiological hurdles, *Leuconostoc* species may be able to survive and grow in NABLABs to a greater degree than they are able to in regular beer.

An emerging spoiler for fruit juices is *Alicyclobacillus* which is an aerobic, Gram-positive, rod shaped, thermoacidophilic, endospore forming bacteria (Ashurst, 2016; Sourri *et al.,* 2022). Its spores can survive pasteurisation at 95°C for 2 minutes. It spoils by producing guaiacol which gives a smoky, medicinal off flavour (Smit *et al.,* 2011). However, it doesn't produce significant turbidity or CO_2, so its spoilage is not easily visible. It grows optimally at 40-55°C and so is a more common spoilage organism in hotter climates, although some

strains do have much lower optimal growth temperatures. Despite being an aerobic organism, they can tolerate low dissolved oxygen levels and continue to produce off flavours, some strains are also facultatively anaerobic (Smit *et al.,* 2011). The conditions of a draught dispense system could harbour *Alicyclobacillus* if the beer were to be a low IBU NABLAB, especially considering its ability to survive pasteurisation and other harsh conditions by forming bacterial spores. Along with other spore formers *Alicyclobacillus* has previously been isolated from beer (Munford *et al.,* 2017).

1.11 Research Aims and Objectives

Due to the increase in the popularity of no and low alcohol beers and increased demand for them to be served on trade, a new beer spoilage niche has opened. The aims of this research are to investigate the apparent greater susceptibility to spoilage and pathogen growth of low alcohol beers compared to their full alcohol counterparts, and the effects of preservatives, chemical composition and pH on spoilage and pathogen growth in low alcohol beers. This will be accomplished by challenge testing a range of NABLABs and a full alcohol beer with beer spoilage microorganisms and food-borne pathogens. The beers will then be tested with sodium benzoate, potassium sorbate or sulphur dioxide to determine the effectiveness of these preservatives in NABLABs at preventing beer spoilage microorganism and food-borne pathogen growth. The compositional differences between the beers will also be tested to identify possible spoilage encouraging or preventing parameters.

This will provide support to brewers formulating new NABLABs, supplying information on what parameters they need to aim for to produce a product with a reduced susceptibility to spoilage, and which remove the possibility of pathogen growth. This will also aim to find a suitable method for brewers to serve NABLABs on trade through a regular dispense system by using preservatives. With a lower rate of infected kegs, more stable sensory characteristics and less ullaging, publicans and patrons alike will have an increased confidence in low alcohol keg products, which would hopefully aid in growing the market and preventing any damaging incidents to the industry.

The fundamental intention of this project is to aid breweries in determining consistent, economic, and practical methods for production and serving of safe, stable, and quality no and low products. As well as developing a method by which producers can test their products susceptibility to spoilage and pathogen growth

Chapter 2: Materials and Methods

2.1 Microorganisms

All spoilage organisms used were captured from the brewery environment. They were isolated by two rounds of streak plating. The isolated colonies were then transferred to agar slopes and sent for identification. The anaerobic spoilage organism was identified as *Lactobacillus brevis* by PALL Gene Disk RT-PCR (Performed by Murphys, UK). The aerobic organism was identified by 16s rDNA sequencing as *Rahnella spp*. The yeast was identified by ITS sequencing as *Pichia membranifaciens* (Performed by Campden BRI, UK). The pathogens *E. coli* 0157:H7 (shiga-toxin negative) NCTC 12900 and *Salmonella* Typhimurium NCTC 12023 were purchased directly from the NCTC (Salisbury, UK) as freeze-dried ampoules.

2.2 Pathogen Preparation

Nutrient broth was prepared by dissolving 4g of Nutrient Broth media (Neogen, USA) in 500ml of reverse osmosis (RO) water, this was then decanted into a 500ml Duran bottle and autoclaved at 121°C 15 PSI for 20mins. Pathogen ampoules were disinfected with 70% Isopropanol wipes (Vernacare, UK), the glass was then scored at the wool plug with a diamond glass cutter sterilized by soaking in 70% industrial methylated spirits (VWR, USA). The ampoule was then wrapped in multiple layers of 70% Isopropanol wipes and snapped at the score line. The wool plug was removed and 500µl of nutrient broth was added to the ampoule. The broth was mixed with the freeze-dried culture by carefully pipetting up and down a few times, the broth was then left for 5 minutes to allow the microorganisms to rehydrate. Once rehydrated, a sterile loop was used to transfer some inoculum to a nutrient agar (1.5%) slope. Slopes were incubated at 37°C for 48 hours and then stored at 4°C for a maximum of two weeks.

2.3 Culture Medium (Wort Broth)

The culture media for all microorganisms was a wort broth prepared with 50g of Cedarex Light malt extract (Muntons, UK) in 950g Reverse Osmosis water. The solution was boiled for 10 minutes to achieve hot break, then carefully decanted into a 1L Duran bottle and autoclaved at 121°C 15 PSI for 20mins. After autoclaving the wort broth was then allowed to settle for 24 hours. The resulting broth was then carefully decanted into a sterile 500ml Duran bottle making sure to leave any sediment behind. The finished broth had a present gravity of 1.017, a pH of 4.9 and absorbance of 0.1 at 660nm.

2.4 Inoculum Preparation

A 5ml aliquot of wort broth was transferred to a sterile 15ml centrifuge tube (Abdos, India). A sterile loop was used to move a small amount of the desired culture from the slope to the tube. The tube was sealed and mixed, for *Rahnella spp., L. brevis* and *P. membranifaciens* it was then incubated statically at 27°C for 24 hours. For *E. coli* 0157:H7 and *S.* Typhimurium incubation was at 37°C for 18 hours. In addition, 60µl of sterile 1% NaOH solution was added to the tube containing the wort broth to increase the pH to around 6.0 to allow for better growth of the pathogens.

2.5 Microbial Maintenance and Storage

WL Nutrient agar (Neogen, USA) was prepared using 40g media and 500ml RO water, Yeast and Mold agar (Neogen, USA) was prepared using 20.5g media and 500ml RO water, MRS agar (VWR, USA) was prepared using 33.7g media and 500ml RO water, Nutrient agar (Neogen, USA) was prepared using 11.5g media and 500ml RO water. All media were prepared on a Stuart Heat-Stir US 152-D (Cole-Parmer, UK) until fully dissolved and boiling. The media was then decanted into 500ml Duran bottles and autoclaved at 121°C 15 PSI for 15mins. After autoclaving agar was re-liquified in a microwave (if required), once at a

temperature of 45-50°C 15-20ml of liquid agar was poured into 35ml sterile transparent PP jars (Medfor, UK) in a laminar flow cabinet (Bass-Aire 03-HB, UK). Jars were then capped and left to set at a 45° angle. The WL nutrient agar was used for *Rahnella spp.*, Yeast and Mold agar for *P. membranifaciens*, MRS agar for *L. brevis* and Nutrient agar for *E. coli* 0157:H7 and *S.* Typhimurium. All prepared slopes were stored at 4°C.

2.6 Dosing (Calibration Curve)

Calibration curves were prepared for all the micro-organisms apart from the wild yeast which was manually counted by haemocytometer.

A 10ml aliquot of wort broth was added to a sterile 15ml centrifuge tube, the desired microorganism was then transferred to the wort by a sterile plastic loop. The culture was then incubated at 27°C for 24 hours for the beer spoilers and 37°C for 18 hours for the pathogens. The pathogens also received 120µl of 1% sterile NaOH to improve growth and so improve cell density allowing for more dilutions within a reliably measurable absorbance range. This inoculum was then used to prepare 2.0ml of the following dilutions with wort broth as the diluent. The dilutions for the beer spoilers were: 1:1.5, 1:2, 1:3, 1:4, 1:6 and 1:8. For pathogens dilutions were 1:1.5, 1:2, 1:3, 1:4, 1:5. A 1000µl sample of each dilution then had its absorbance measured at 660nm, blanked to uninoculated wort broth. Each dilution was then serially diluted with sterile 0.85% NaCl. The 10^{-4}, 10^{-5}, 10^{-6} dilutions had a 100µl sample taken which was spread plated onto the appropriate media and incubated. WLN for *Rahnella spp.* incubated at 27°C for 3-5 days, MRS for *L. brevis* incubated anaerobically for 7 days and Nutrient agar for *E. coli* 0157:H7 and *Salmonella* Typhimurium incubated at 37°C for 24-48 hours. Once incubated, plates were counted and CFU/ml determined.

The Slope, Intercept and R^2 were calculated so that the absorbance of the inoculums could be used to estimate the number of viable cells present in future inoculums.

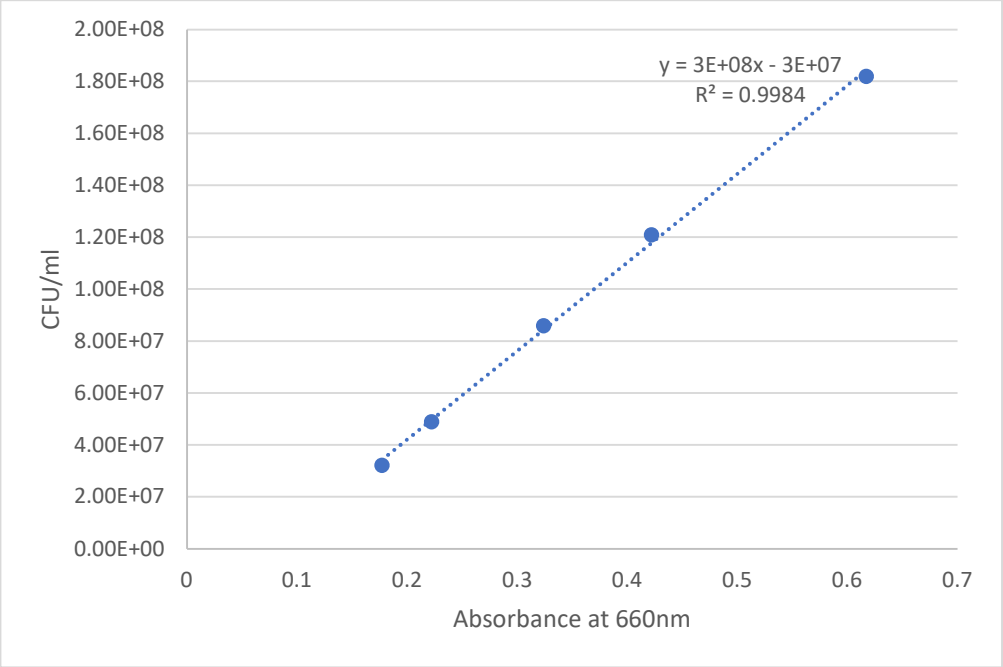

***Figure 9**: Calibration curve for **Rahnella spp.** Slope: 3.41x10⁸ Intercept: -2.61x10⁷ R²: **0.998**.*

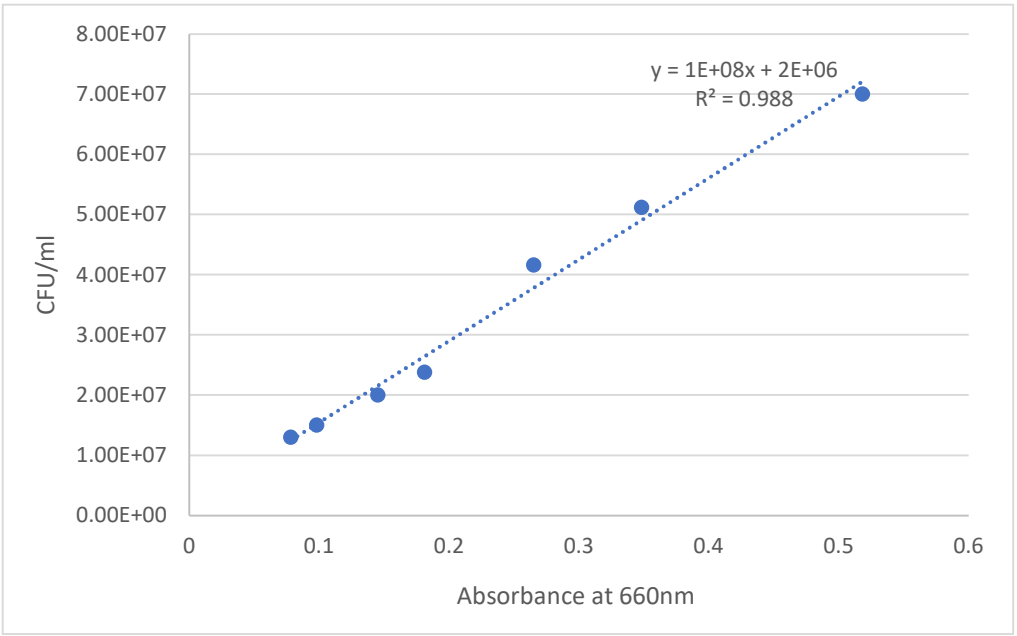

***Figure 10**: Calibration curve for **L. brevis** Slope: 1.35x10⁸ Intercept: 1.92x10⁶ R²: **0.988**.*

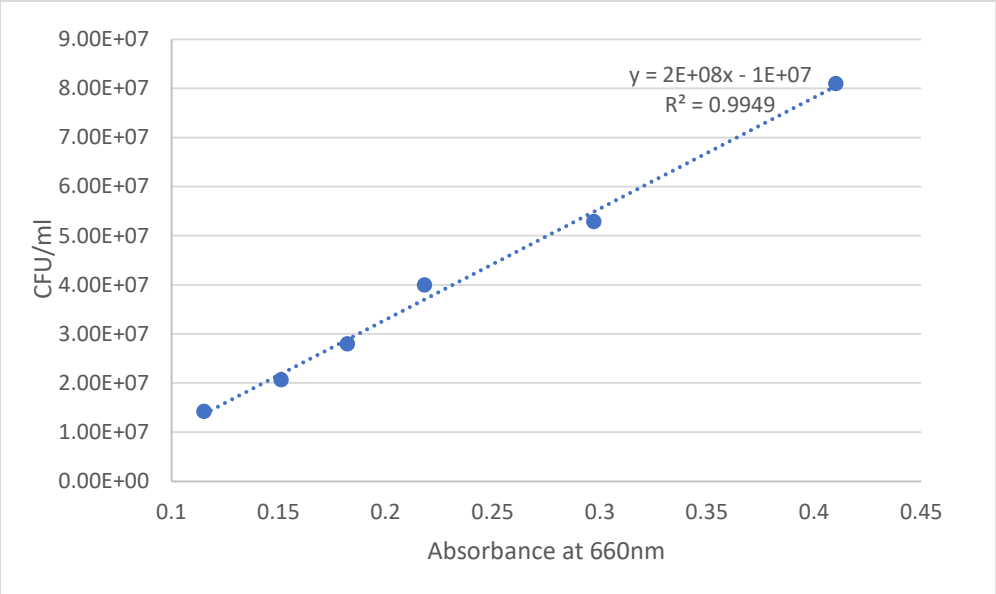

***Figure 11**: Calibration curve for **E. coli 0157:H7** Slope: 2.26x10^{8} Intercept: -1.23x10^{7} R^{2}: **0.995**.*

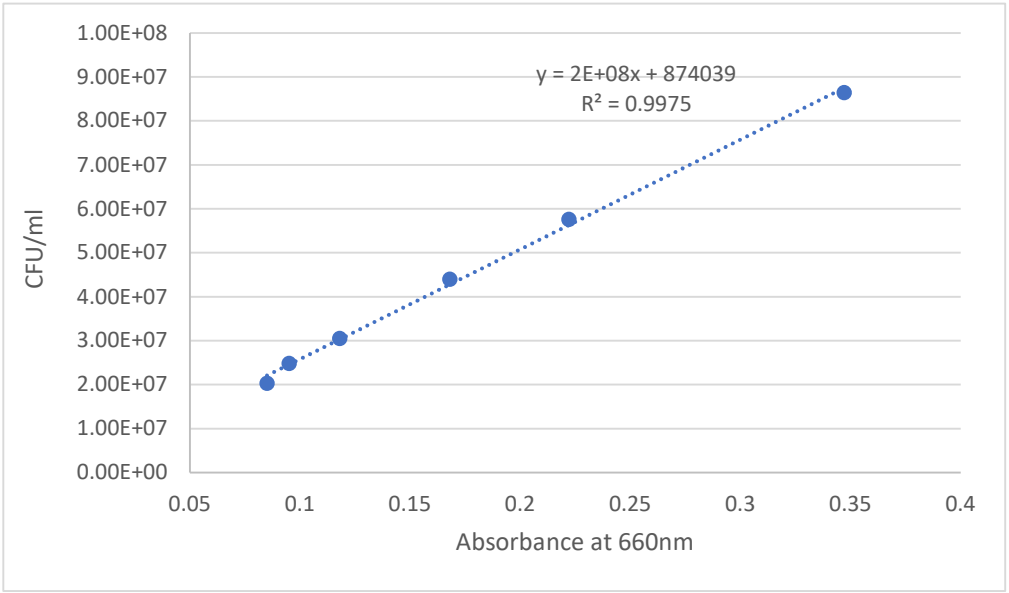

***Figure 12**: Calibration curve **S.** **Typhimurium** Slope: 2.50x10^{8} Intercept: 8.74x10^{5} R^{2}: **0.998**.*

The absorbance of the culture was measured at 660nm with the same spectrophotometer that the calibration curves were made on. For *Rahnella spp., E. coli* 0157:H7 and *S.* Typhimurium a Jenway 7200 visible spectrophotometer (Cole-Parmer, UK) was used and for *L. brevis* a Thermo Spectronic Genesys 10UV (Thermo Fisher, USA) was used. Both were blanked to uninoculated wort broth. The equation (Slope x A_{660} + Intercept) was used to determine CFU/ml. The dose per 100ml flask was then calculated by (Desired number of cells in 100ml/estimated CFU/ml) x 1000. This would give the required dose per 100ml flask in µl. If the calculated dose was under 10µl then a 1:10 dilution of the inoculum was performed to give a larger dose volume.

2.7 Yeast Cell Enumeration

Yeast cells were counted by haemocytometer due to inconsistent growth of *P. membranifaciens* on yeast and mould agar, and nutrient agar. Because of this dosing rates of *P. membranifaciens* will be stated in Cells/ml not CFU/ml.

A 100µl aliquot of prepared yeast inoculum was pipetted into a 15ml centrifuge tube, a 900µl aliquot of sterile 0.85% NaCl solution was also added to the tube to achieve a 1:10 dilution. Roughly 10µl was then loaded onto a haemocytometer (Hawksley, UK) and using a light microscope with a 10x ocular and 40x objective lens giving 400x magnification (Olympus, USA) the yeast cells in all 25 centre squares were counted. The number of cells per ml present in the sample was calculated using the equation (Total number of cells counted x Dilution Factor x 10^4 = Cells/ml).

2.8 Yeast Cell Viability

The yeast inoculums viability was assessed by methylene blue staining. A 0.02% methylene blue solution was introduced to yeast inoculum at a 1:1 ratio. The sample was then left for 5 minutes and then shaken before loading onto the haemocytometer. The number of

unstained cells were counted as live, and the number of blue stained cells were counted as dead. Viability was calculated using the equation: Live cells/(Live cells + Dead cells) = Viability %.

2.9 Solution Preparations

A 20% sodium benzoate solution was prepared by dissolving 19.96g of sodium benzoate (Murphy & Son LTD, UK) in 100ml sterile RO water.

A 10% sodium benzoate solution was prepared by dissolving 9.98g of sodium benzoate (Murphy & Son LTD, UK) in 100ml sterile RO water.

A 10% potassium sorbate solution was prepared by dissolving 9.98g of potassium sorbate (Youngs, UK) in 100ml sterile RO water.

A pre-prepared 30% solution of sulphur dioxide was used to add sulphites, Salicon (Murphy & Son LTD, UK).

A 3% solution of sulphur dioxide was prepared by diluting 10ml 30% Salicon (Murphy & Son LTD, UK) in 90ml of sterile RO water.

A pre-prepared 80% solution of lactic acid was used for acidification (Murphy & Son LTD, UK).

A 1% NaOH solution was prepared by dissolving 1g of NaOH (VWR, USA) in 100ml sterile RO water.

A 50% Lactose solution was prepared using 50g Lactose (Youngs, UK) and 50ml RO water, the solution was heated and stirred until the lactose was fully dissolved. The resulting solution was autoclaved at 121°C, 15 PSI for 15 minutes.

A 0.85% NaCl solution was prepared by weighing out 4.25g of NaCl (BDH, UK) on a balance (FX-3000i, A&D, USA) 500ml of RO water was added and the solution stirred until the NaCl was fully dissolved. The resulting solution was autoclaved at 121°C, 15 PSI for 15 minutes.

The malt extract (ME) was prepared using 100g of Muntons Cedarex light (Muntons, UK) and made up to 400g with reverse osmosis water. The malt extract was dissolved by heating and stirring (Stuart Heat-stir US152D, Cole-Parmer, UK), the resulting solution was then decanted into a 500ml Duran and autoclaved at 121°C, 15 PSI for 15 minutes.

Methylene blue stain (0.02%) was prepared by weighing out 50mg of Methylene blue (BDH, UK) on a balance (AA-200, Denver instrument Co, USA). The methylene blue was suspended in 50ml of RO water, 10g of pre-weighed (FX-3000i, A&D, USA) tri-sodium citrate (Vickers, UK) was then added to the methylene blue suspension and placed on a stirrer (Stuart Heat-stir US152D, Cole-Parmer, UK) until fully dissolved. The solution was then made up to 500ml with RO water in a 500ml Volac (John Poulten LTD, UK), which was inverted 3-5 times to mix. The prepared stain was then decanted into a clean brown glass bottle to protect the stain from UV exposure.

2.10 Present Gravity and Alcohol by Volume

A 100ml sample was decanted from the bottles (room temperature) through a Grade 6 qualitative filter paper (Sartorius, Germany) to degas the sample and remove any large particulate matter. A 50ml sample of the filtrate was taken and run through an Anton Paar DMA 5000 M (Anton Paar, Austria) which measured the density of the sample by oscillating u-tube method. The sample was also passed through the Alcolyzer Beer ME (Anton Paar, Austria) which measured the alcohol by volume of the sample by Near-infrared analysis (NIR).

2.11 pH

A 50ml sample of room temperature beer was decanted through a Grade 6 qualitative filter paper (Sartorius, Germany) to degas the sample. The pH probe was calibrated to 4.0 and 7.0 buffer solutions (SLS, UK). The pH and automatic temperature correction probe were submersed in the sample, this was then allowed to settle for 5 mins until the pH reading on the meter (Eutech pH 700, Thermo Fisher, USA) had become stable.

2.12 International Bitterness Units (IBU)

A 50ml sample of room temperature beer was filtered as previously mentioned. The sample was allowed to fully filter through before taking any filtrate. To a 50ml conical flask 10ml of filtered sample was added, then 3ml of 1M hydrochloric acid (VWR, USA) was added to the flask to acidify the sample. Then 20ml of 2,2,4-Trimethylpentane (Vickers, UK) was added to the conical flask. The flask was capped and then shaken vigorously for 2 minutes. The flask was then allowed to settle for 20 minutes to allow for separation of the layers. A spectrophotometer (Thermo Spectronic Genesys 10UV, Thermo Fisher, USA) was then blanked using 2,2,4-Trimethylpentane at 275nm using a 10mm quartz cuvette. The top layer of the sample was then carefully decanted into another 10mm quartz cuvette and its absorbance measured. The number of IBUs was determined by the equation (IBU = A_{275} x 50).

2.13 Carbohydrate Analysis

Carbohydrate analysis was kindly performed by Research Fellow Dr. Joshua Reid at University of Nottingham.

The sugars glucose, fructose, maltose and maltotriose were quantified using High Performance Anion-Exchange Chromatography (HPAEC). Using a Dionex ICS 6000 with a CarboPac PA210 column (250mm x 4mm) and guard (50mm x 4mm). The mobile phase of

the HPAEC composed of four eluents at varying ratios and were all prepared using ultrapure water obtained using a Suez Select Fusion ultrapure water deionisation unit, degassed under vacuum and with a sonicating water bath. The four eluents were, 100% H_2O, 100 mM NaOH, 200 mM NaOH and 100 mM NaOH + 500 mM NaOAc. The column chamber was equilibrated to 30°C. In all samples, a sample loop volume of 25.0 µL was used with a push-partial injection mode, with 2.5 µL of sample used per injection.

Samples of degassed beer were diluted at a ratio of 1:100 in 10 mM NaOH (100 µL in 10 mL total volume) and agitated using a bench top vortex. Due to the higher concentration of certain sugars, Brand 2 0.5% was diluted at a ratio of 1:1000 in 10 mM NaOH (10 µL in 10 mL total volume). Samples were then filtered through a 0.45µm filter into a glass vial with split septum cap, equilibrated to 4°C and placed into the autosampler. Standard solutions of glucose, fructose, maltose and maltotriose were prepared at concentrations from 2.00 – 0.10 mg L^{-1} in 10 mM NaOH and were used for quantification of the sugars in each beer.

2.14 Elemental Analysis

Elemental analysis was kindly performed by Research Fellow Dr. Joshua Reid at University of Nottingham.

Multi-element analysis was undertaken by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) on the iCAP-Q instrument (Thermo-Fisher Scientific, USA) with a 'Flatopole collision cell' (charged with helium gas). Internal standards were introduced to the sample stream via a T-piece and included Sc (50 µg L^{-1}), Ge (20 µg L^{-1}) Rh (10 µg L^{-1}) and Ir (5 µg L^{-1}) in the matrix of 2% HNO_3. External calibration standards were all in the range 0 – 100 µg L^{-1} (ppb). Samples were introduced via an autosampler (Cetac ASX-520, USA) through a concentric glass venturi nebuliser (Thermo-Fisher Scientific, USA). Sample processing was undertaken using 'Qtegra software' (Thermo-Fisher Scientific, USA).

Trace elements quantified were: Mn, Fe, Cu, Zn, Sr, Ba

The major alkali and alkali-earth elements, Ca, Mg, Na and K, were run at ppm concentrations with a separate range of multi-element calibration standards. These were assayed at the same time as the trace elements and under the same conditions.

2.15 Challenge Testing

The flasks for the challenge testing were prepared by using clean and dry 250ml Erlenmeyer flasks, plugging tightly with cotton wool and covering the top with foil. These were then autoclaved at 121°C 15 PSI for 15mins.

Before decanting the beer bottles Brand 1 0.5%, Brand 2 0.5% and Brand 1 4.5% were inverted, and the top of the bottle stood in 70% industrial methylated spirits for 2 minutes to sterilize. Any excess was wiped with a 70% Iso-propanol wipe. As Brand 3 0.5% contained sediment, the bottles were allowed to settle. A 500ml Beaker was sterilized with 70% industrial methylated spirit. The settled bottles were carefully poured into the sterilized beaker leaving any sediment in the bottle. All bottles were opened with a bottle opener sterilized in 70% industrial methylated spirits for 2 minutes.

2.16 Preservative Dosing for Spoilage Trials

For spoilage trials (Section 2.17) preservatives were dosed into the 100ml of beer immediately before inoculation with the solutions described in Section 2.9. The 20% sodium benzoate solution was dosed at 100µl/100ml and 10% potassium sorbate solution at 200µl/100ml with a P200 pipette (Sartorius, Germany) to achieve close to 200ppm. The 30% sulphur dioxide solution was dosed at 6.65µl/100ml with a P10 pipette (Sartorius, Germany) to achieve close to 20ppm.

2.17 Spoilage Trials

Sterilised flasks were filled with 100ml beer sample, any preservatives and the inoculum. The flasks were thoroughly swirled to mix. A 1000µl sample of each flask was taken and its absorbance measured at 660nm (Jenway 7200 visible spectrophotometer, Cole-Parmer, UK) blanked with RO water. After 48 hours the flasks were swirled thoroughly to suspend any sediments. A sample of 1000µl was taken and the absorbance checked with the same

method as detailed above. Every 24 hours after this the flasks were checked again in the same way until day seven. *P. membranifaciens* growth was not determined by viable counts to keep the results consistent with the other organisms, to avoid diluting the sample to take the measurement, and to simplify the method.

Each spoilage trial consisted of three flasks of one variable run in parallel, each trial was repeated in the same way on three separate occasions giving a total of nine biological replicates and three technical replicates.

2.18 Pathogen Trials

2.18.1 Initial Survival Trials

Sterilised flasks were filled with 100ml beer sample, and 10^3 CFU/ml inoculum. The flasks were thoroughly swirled to mix. A 100µl sample was taken from each flask and spread plated onto nutrient agar. The plates were left to dry for 10 minutes and then incubated at 37°C for 24 hours. The number of colonies formed were counted and the result multiplied by ten to achieve the starting CFU/ml. The flasks were incubated statically at room temperature for 7 days. On the seventh day the flasks were swirled thoroughly to homogenise the sample. A 100µl aliquot was taken and spread plated, incubated and counted as previously described.

2.18.2 Growth Trials

For assessment of preservatives Brand 1 0.5% was adapted to cause more rapid spoilage. This was achieved by adding 5ml of ME and 1 ml of 1% NaOH into 100ml of Brand 1 0.5%. A sample of the modified Brand 1 0.5% (MB1 0.5%) was analysed after this addition. PG: 15.04 ABV: 0.37% pH: 4.44

Sugar composition was calculated from Muntons Cedarex light (Muntons, UK) certificate of analysis (Table 5) (Murphy & Son 2022).

Sterilised flasks were filled with 100ml Modified Brand 1 0.5% (MB1 0.5%) sample, any preservative and 10^3 CFU/ml pathogen. The flasks were thoroughly swirled to mix. A 100µl aliquot was taken and spread plated, incubated, and counted as previously described. After 72 hours of room temperature incubation the flasks were thoroughly swirled to homogenise the sample. A 100µl aliquot was taken and serially diluted, spread plated, incubated, and counted as previously described.

2.18.3 Preservative Sensitivity Trial

To assess any sensitivity to preservatives should a pathogen not grow in the Modified Brand 1 0.5% solution, sterilised flasks were filled with 100ml Brand 1 0.5% sample, any preservative and 10^3 CFU/ml pathogen. The flasks were thoroughly swirled to mix. A 100µl aliquot was taken and spread plated, incubated, and counted as previously described. After 72 hours of room temperature incubation the flasks were thoroughly swirled to homogenise the sample. A 100µl aliquot was taken and serially diluted, spread plated, incubated, and counted as previously described.

2.19 Preservative Dosing and pH Adjustments for OmniLog® Trial

The OmniLog® trials were dosed with an additional 10% preservative as the sample medium of 90µl was inoculated with 10µl bacterial inoculum, leading to 10% dilution in preservative concentration. 30ml samples of partially degassed Brand 1 0.5% were decanted into 35ml sterile transparent PP jars (Medfor, UK) and preservatives dosed accordingly to achieved desired concentrations (Table 3a). The pH of samples was adjusted to either Low (3.8) or High (4.55) using 80% lactic acid or 1% NaOH, Med pH (4.15) was the pH in the beer as received.

Table 3a: Dosing of lactic acid, sodium hydroxide and preservatives in each 30ml sample for OmniLog® trials samples

	Lactic acid 80%	NaOH 1%	Sodium Benzoate 10%	Potassium Sorbate 10%	Sulphur Dioxide 3%
1	33µl	0	0	0	0
2	0	0	0	0	0
3	0	540µl	0	0	0
4	33µl	0	16.5µl	0	0
5	0	0	16.5µl	0	0
6	0	540µl	16.5µl	0	0
7	33µl	0	33µl	0	0
8	0	0	33µl	0	0
9	0	540µl	33µl	0	0
10	33µl	0	66µl	0	0
11	0	0	66µl	0	0
12	0	540µl	66µl	0	0
13	33µl	0	0	16.5µl	0
14	0	0	0	16.5µl	0
15	0	540µl	0	16.5µl	0
16	33µl	0	0	33µl	0
17	0	0	0	33µl	0
18	0	540µl	0	33µl	0
19	33µl	0	0	66µl	0
20	0	0	0	66µl	0
21	0	540µl	0	66µl	0
22	33µl	0	0	0	11µl
23	0	0	0	0	11µl
24	0	540µl	0	0	11µl
25	33µl	0	0	0	22µl
26	0	0	0	0	22µl
27	0	540µl	0	0	22µl
28	33µl	0	0	0	55µl
29	0	0	0	0	55µl
30	0	540µl	0	0	55µl
31	33µl	0	33µl	33µl	0
32	0	0	33µl	33µl	0
33	0	540µl	33µl	33µl	0
34	33µl	0	33µl	33µl	22µl
35	0	0	33µl	33µl	22µl
36	0	540µl	33µl	33µl	22µl
37	33µl	0	66µl	0	22µl
38	0	0	66µl	0	22µl
39	0	540µl	66µl	0	22µl
40	33µl	0	0	66µl	22µl
41	0	0	0	66µl	22µl
42	0	540µl	0	66µl	22µl
43	33µl	0	16.5µl	16.5µl	0
44	0	0	16.5µl	16.5µl	0
45	0	540µl	16.5µl	16.5µl	0
46	0	0	0	0	0

Table 3b: Sample list for OmniLog® trials. Benz = sodium benzoate. Sorb = potassium sorbate. SO_2 = Sulphur dioxide. Low pH (3.8), Med pH (4.15), High pH (4.55)

1	Low pH	13	Low pH 50ppm Sorb	25	Low pH 20ppm SO_2	37	Low pH 200ppm Benz + 20ppm SO_2
2	Med pH	14	Med pH 50ppm Sorb	26	Med pH 20ppm SO_2	38	Med pH 200ppm Benz + 20ppm SO_2
3	High pH	15	High pH 50ppm Sorb	27	High pH 20ppm SO_2	39	High pH 200ppm Benz + 20 SO_2
4	Low pH 50ppm Benz	16	Low pH 100ppm Sorb	28	Low pH 50ppm SO_2	40	Low pH 200ppm Sorb + 20ppm SO_2
5	Med pH 50ppm Benz	17	Med pH 100ppm Sorb	29	Med pH 50ppm SO_2	41	Med pH 200ppm Sorb + 20ppm SO_2
6	High pH 50ppm Benz	18	High pH 100ppm Sorb	30	High pH 50ppm SO_2	42	High pH 200ppm Sorb + 20ppm SO_2
7	Low pH 100ppm Benz	19	Low pH 200ppm Sorb	31	Low pH 100ppm Benz + 100ppm Sorb	43	Low pH 50ppm Benz + 50ppm Sorb
8	Med pH 100ppm Benz	20	Med pH 200ppm Sorb	32	Med pH 100ppm Benz + 100ppm Sorb	44	Med pH 50ppm Benz + 50ppm Sorb
9	High pH 100ppm Benz	21	High pH 200ppm Sorb	33	High pH 100ppm Benz + 100ppm Sorb	45	High pH 50ppm Benz + 50ppm Sorb
10	Low pH 200ppm Benz	22	Low pH 10ppm SO_2	34	Low pH 100ppm Benz + 100ppm Sorb + 20ppm SO_2	46	Brand 1 4.5% Control
11	Med pH 200ppm Benz	23	Med pH 10ppm SO_2	35	Med pH 100ppm Benz + 100ppm Sorb + 20ppm SO_2		
12	High pH 200ppm Benz	24	High pH 10ppm SO_2	36	High pH 100ppm Benz + 100ppm Sorb + 20ppm SO_2		

2.20 OmniLog® Trial

The OmniLog® plates were loaded and run by Research Associate Dr. Adriano Gigante at University of Nottingham

To each prepared medium (Table 3b) Tetrazolium (2,3,5-Triphenyltetrazolium chloride) (Sigma Aldric, UK) was added to achieve a final concentration of 0.02% (w/V) before loading 90µL to each designated well on Biolog half-volume clear plates (Technopath Distribuition, Ireland). Both microorganisms (*P. membranifaciens* and *L. brevis*) were grown in degassed Brand 1 0.5% for around 48h at 27°C until OD= 0.5 was reached. A 10µL aliquot of that liquid culture was pipetted into each well. To the negative control 10µL of plain degassed beer was added. Plates were incubated statically at 20°C for 144h in the Omnilog®(Technopath Distribuition, Ireland). The OmniLog® camera measures the colour level of the static plates on a proprietary scale and was expressed as Biolog Units, readings were taken every 30 minutes.

2.21 Statistical Analysis

Spoilage, pathogen and OmniLog® challenge test trials were analysed in Graph pad prism, data sets were first tested for normality by Kruskal-Wallis test. If passed normality check the data was analysed using Welch and Brown-Forsythe ANOVA with 95% Confidence intervals. Significance was determined as $P<0.05$. Where One-way ANOVA couldn't be used due to non-normal distributions or too few variables a two-tailed T-test was used.

Chapter 3: Results

3.1 Beer Analysis Results

3.1.1 Introduction

As described in Section 1.3, the chosen method for producing a NABLAB can have a significant effect on the chemical composition of the final beer. A number of these differences are from parameters which contribute to the microbiological hurdles of beer, changing or removing any of them could have significant consequences for spoilage (Vriesekoop *et al.,* 2012).

3.1.2 Results

The present gravity (PG) and alcohol by volume (ABV) of each of the beers was measured as described in Section 2.10, pH as described in Section 2.11 and IBU as described in Section 2.12. As shown in Table 4, the lowest PG and ABV was found in Brand 2 0.5% which is produced by restricted fermentation, it also had the highest pH. The highest PG was measured in Brand 3 0.5% which is produced by vacuum distillation. Brand 3 0.5% had the lowest pH and lowest IBU. The IBU of the other three beers was close with only a 4 IBU difference between Brand 2 0.5% and Brand 1 4.5%.

Table 4: Analysis of Present Gravity, Alcohol By Volume, pH and International Bitterness Units of beer samples

Beer	*Description*	*Production Method*	*Present Gravity*	*Alcohol by Volume*	*pH*	*IBU*
Brand 1 4.5%	Pale Ale	Normal Fermentation	1005.88	4.42%	4.41	32
Brand 1 0.5%	Pale Ale	Reverse Osmosis	1013.74	0.46%	4.26	30
Brand 2 0.5%	Amber Ale	Restricted Fermentation	1004.68	0.42%	4.46	28
Brand 3 0.5%	Lager	Vacuum Distillation	1015.48	0.51%	4.19	18

Carbohydrate composition of the beers was analysed by HPAEC as described in section 2.13 apart from MB1 0.5% which was calculated as described in Section 2.18.2. Of the unmodified beers shown in Table 5 Brand 1 0.5% had the highest glucose and fructose concentration, which could potentially lead to greater spoilage by providing a more readily available carbon source for spoilage microorganisms. Brand 3 0.5% had the lowest glucose concentration but had the highest maltose concentration. Brand 1 0.5% and Brand 3 0.5% had similarly high maltotriose concentrations which could aid in the beers organoleptic properties. MB1 0.5% is higher in all tested sugars than all of the other tested beers, but had similar levels of sugars to NABLABs tested by L'Anthoën and Ingledew, (1996) and Quain, (2021) (Appendix 1).

*Table 5: Sugar composition analysis of beer samples *indicates calculated result from certificate of analysis of malt extract*

Beer	*Glucose g L^{-1}*	Fructose g L^{-1}	*Maltose g L^{-1}*	*Maltotriose g L^{-1}*
Brand 1 4.5%	0.14	0.98	1.07	1.24
Brand 1 0.5%	0.52	1.02	1.74	2.29
Brand 2 0.5%	0.05	0.07	0.27	0.51
Brand 3 0.5%	0.03	0.67	2.59	2.23
MB1 0.5%	3.52*	1.035*	20.74*	6.54*

The Elemental analysis was performed by ICP-MS as described in Section 2.14. As shown in Table 6 Brand 1 0.5% contained the highest amount of Sodium (Na), Magnesium (Mg), Sulphur (S) and Calcium (Ca). Brand 3 0.5% had the highest concentration of Phosphorous (P) and Potassium (K). The trace elements in Table 7 show Brand 1 0.5% had the highest concentration of Iron (Fe), Strontium (Sr) and Barium (Ba). Brand 2 0.5% had the highest Manganese (Mn) and Copper (Cu). Brand 3 0.5% had the highest Zinc (Zn).

Table 6: Analysis for major alkali and alkali-earth elements in beer samples by Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Beer	Na (mg/L)	Mg (mg/L)	P (mg/L)	S (mg/L)	K (mg/L)	Ca (mg/L)
Brand 1 4.5%	63.3	100.9	133.7	165.8	546.7	172.8
Brand 1 0.5%	171.1	119.2	134.8	316.4	356.3	664.9
Brand 2 0.5%	53.1	45.1	54.1	118.9	238.0	141.5
Brand 3 0.5%	15.7	98.2	286.3	146.7	652.5	67.5

Table 7: Analysis of trace elements in beer samples by Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Beer	Mn (µg/L)	Fe (µg/L)	Cu (µg/L)	Zn (µg/L)	Sr (µg/L)	Ba (µg/L)
Brand 1 4.5%	295.5	36.8	32.4	9.6	862.5	84.0
Brand 1 0.5%	284.5	108.7	37.4	8.7	5840.7	206.8
Brand 2 0.5%	317.6	65.3	57.8	10.9	1015.4	65.5
Brand 3 0.5%	83.5	18.3	49.0	14.5	119.8	22.4

3.2 The Growth of Beer Spoilage Microorganisms in NABLABs Compared to a Full Alcohol Counterpart

3.2.1 Introduction

With a significant reduction in ethanol content, one of the main microbiological hurdles, NABLABs are expected to be more susceptible to the currently described beer spoilage organisms. Additionally, they may also provide an environment for emerging beer spoilers, such as *Pectinatus* and *Megasphaera*, or even previously undescribed beer spoilers.

Previous research has described that NABLABs can suffer from greater spoilage than their full alcohol counterparts (Quain, 2021). It has been shown that the removal of ethanol does play a part in this increase in spoilage susceptibility (Quain, 2021). However, it was concluded that another major factor was the level of 'fermentable' sugars (glucose, fructose, maltose), which NABLABs had in higher concentrations than the regular beers tested as shown in Section 3.1 and Appendix 1 (Quain, 2021; L'Anthoën and Ingledew, 1996). The macro and micronutrient composition of full alcohol beers varies greatly between styles, and this is also true for NABLABs as shown in Section 3.1 (Quain, 2021; L'Anthoën and Ingledew, 1996). One reason for this is the many different production methods used for NABLABs, being a new area of innovation there is currently no agreed upon best method.

Individual microorganisms growth in NABLABs has previously not been assessed, and so it is not clear whether all of the common beer spoilage organisms grow preferentially in NABLABs compared to their full alcohol counterparts. To be able to assess the effectiveness of preservatives in NABLABs, it was important to first determine whether the selected organisms do grow more readily in NABLABs than regular beers.

This section assesses the ability of three spoilage organisms isolated from a brewery environment to grow in three 0.5% ABV beers (2 ales, 1 lager) and a 4.5% ABV ale. This is to be able to compare the differences between NABLABs of different production methods (Reverse osmosis, restricted fermentation, and vacuum distillation), compositional parameters (Section 3.1) and spoilage organisms.

The spoilage organisms are:

- ***Pichia membranifaciens**:* This is a beer spoilage yeast commonly found in finished beer that is exposed to oxygen such as draught dispense lines and cask served ales. It produces turbidity, thick pellicles and off flavours such as ethyl acetate (pear drops) (Campbell, 2003).

- ***Levilactobacillus brevis**:* This is the most common cause of beer spoilage incidents (Back, 1994; Suzuki, 2015), it can be found throughout the whole brewing process and through to package and dispense. It can produce large amounts of lactic acid and cause significant turbidity (Suzuki, 2015).

- ***Rahnella spp.***: Is often not considered a beer spoiler as it can only survive the early stages of fermentation and is sensitive to ethanol, and so could possibly grow in NABLABs. It can persist within a yeast culture, especially if the yeast is a top cropping ale variety, which gives it the ability to contaminate subsequent batches if the yeast is reused. It can produce large amounts of diacetyl and DMS (Van Vuuren and Priest, 2003).

3.2.2 Growth Curves

Each beer was inoculated with 10^3 Cells/ml *P. membranifaciens,* 10^3 CFU/ml *L. brevis* or *Rahnella spp.* And incubated at room temperature as described in Section 2.16**.** Results are the turbidity of the beer measured as A_{660nm}. Samples were taken at inoculation and then after two days every day until the seventh day. Error bars for x and y axis are ± standard deviation. X error bars are included as not every sample was measured at the same time into incubation. As can be seen in Figure 13 there was little difference between all the beers after 48 hours but following that all the NABLABs started to see higher absorbances than Brand 1 4.5% caused by *P. membranifaciens* growth. With *L.* brevis (Figure 14) it was not until around 120hrs into incubation that the absorbance for Brand 1 0.5% started to increase at a greater rate than the other beers. For *Rahnella spp.* Figure 15 shows a linear increase in turbidity for Brand 1 0.5% and Brand 2 0.5%. Brand 1 4.5% showed little signs of increasing turbidity and although Brand 3 0.5% did show some increase this was not much more than Brand 1 4.5%.

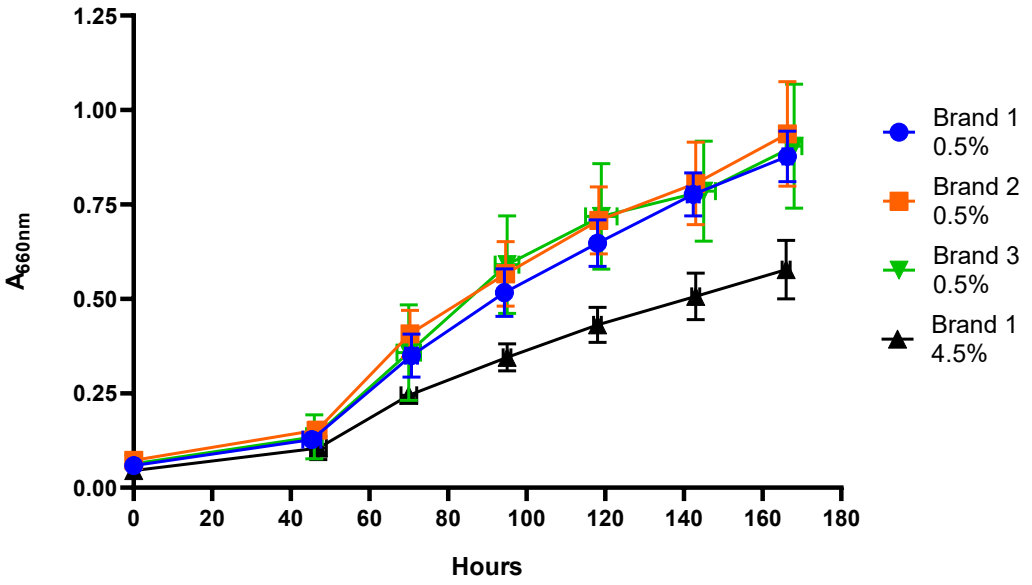

Figure 13: Growth curves of P. membranifaciens in the tested beers 'as received' as measured by A_{660nm} Results are the means of nine replicates, error bars are ± standard deviation.

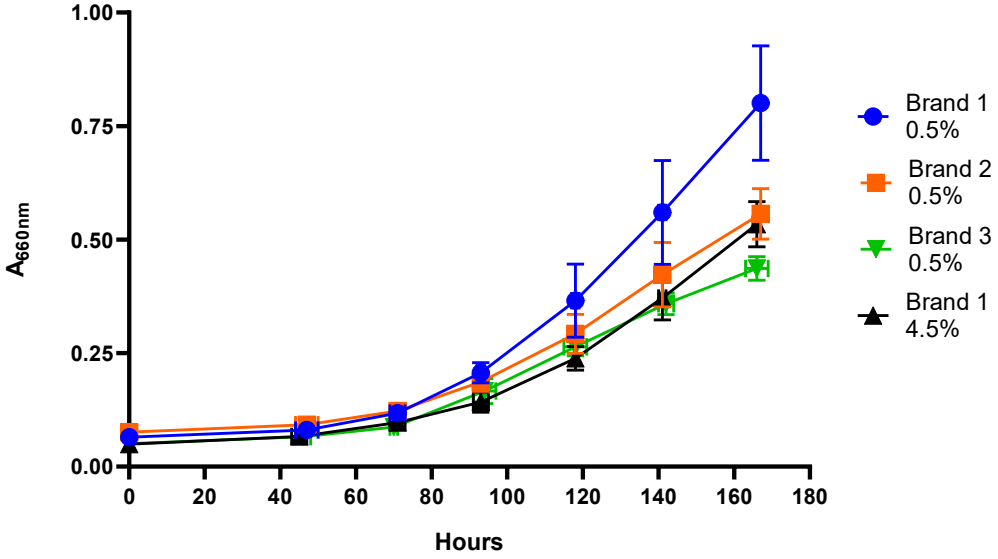

Figure 14: Growth curves of L. brevis in the tested beers 'as received' as measured by A_{660nm} Results are the means of nine replicates, error bars are ± standard deviation.

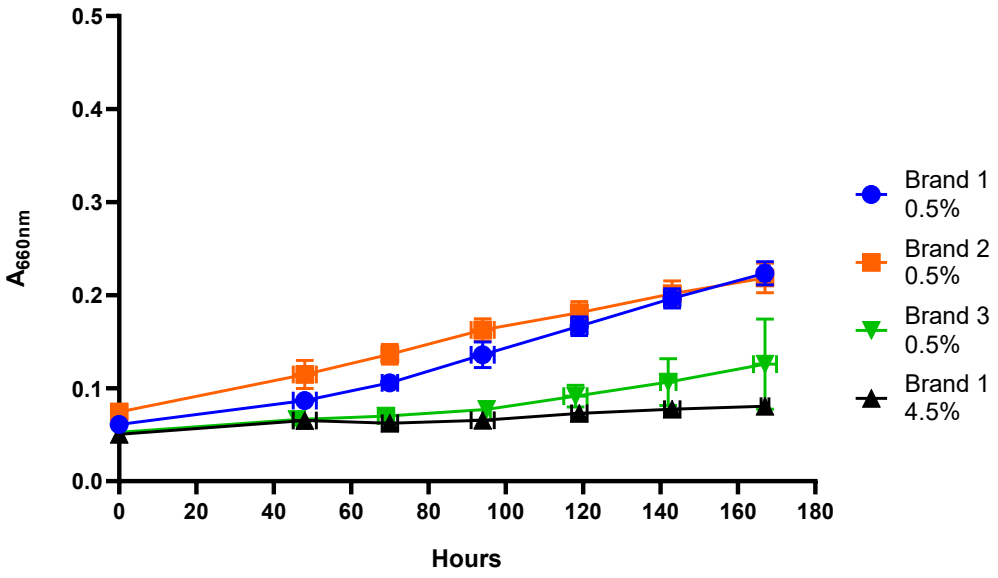

Figure 15: Growth curves of Rahnella spp. in the tested beers 'as received' as measured by A_{660nm} Results are the means of nine replicates, error bars are ± standard deviation.

3.2.3 Day Seven Results

Each beer was inoculated with 10^3 Cells/ml of *P. membranifaciens* and incubated at room temperature as described in Section 2.16**.** Results are the turbidity of the beer measured as Δ A_{660nm} after 7 days. A higher turbidity at A_{660nm} indicates greater microbial cell density. In Figure 16 it is shown that Brand 1 0.5% (Δ A_{660nm} = 0.818), Brand 2 0.5% (Δ A_{660nm} = 0.863) and Brand 3 0.5% (Δ A_{660nm} = 0.841) all had a significantly higher Δ A_{660nm} than that of Brand 1 4.5% (Δ A_{660nm} = 0.531) after 7 days incubation. This indicates that *P. membranifaciens* grows more readily in NABLABs compared to a regular 4.5% ABV beer. All the NABLAB brands tested had similar growth kinetics when challenged with *P. membranifaciens* over 7 days (Figure 13).

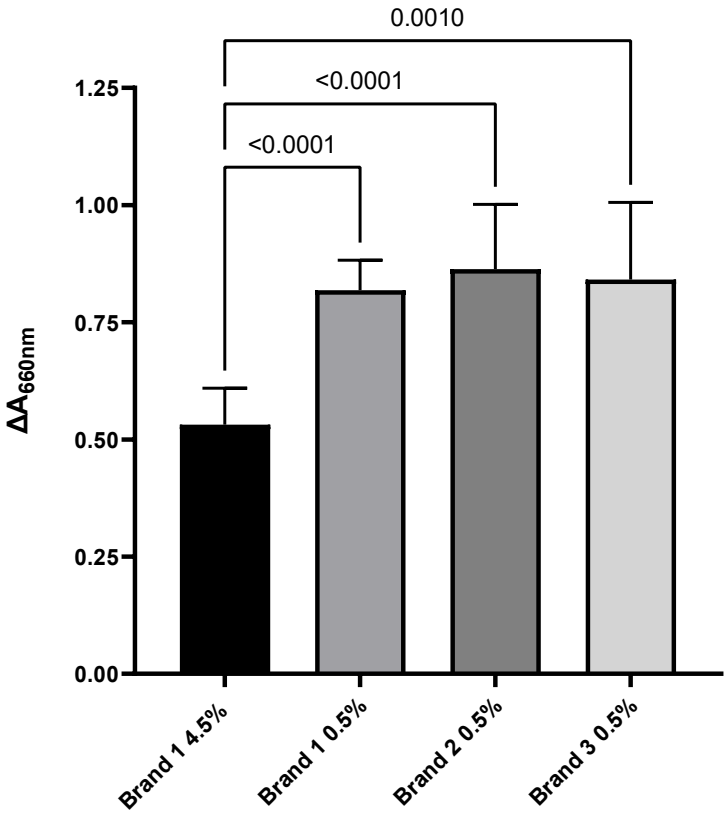

Figure 16: Δ A_{660nm} after 7 days incubation at room temperature of Low alcohol beers inoculated with 10^3 Cells/ml P. membranifaciens, compared to Brand 1 4.5%. Results are the means of nine replicates, error bars are ± standard deviation

Each beer was inoculated with 10^3 CFU/ml *L. brevis* and incubated at room temperature as described in Section 2.16**.** Results are the turbidity of the beer measured as Δ A_{660nm} after 7 days. In Figure 17 it is shown that Brand 1 0.5% (Δ A_{660nm} = 0.735) has a significantly higher Δ A_{660nm} when compared to Brand 1 4.5% (Δ A_{660nm} = 0.484) (p = 0.0004) indicating greater growth of *L. brevis.* Conversely, Brand 3 0.5% showed a significantly lower Δ A_{660nm} than Brand 1 4.5% (Δ A_{660nm} = 0.386) (p = 0.0006) suggesting less growth. Brand 2 0.5% showed no significant difference between itself and Brand 1 4.5%. This suggests that the removal of ethanol is not always the deciding factor when it comes to *L. brevis* growth, and the differences observed must be due to other factors. However, this does not appear to be related to hop resistance as Brand 1 0.5% which had the highest Δ A_{660nm} after 7 days, also has higher IBUs than both Brand 2 0.5% and Brand 3 0.5% (Table 4).

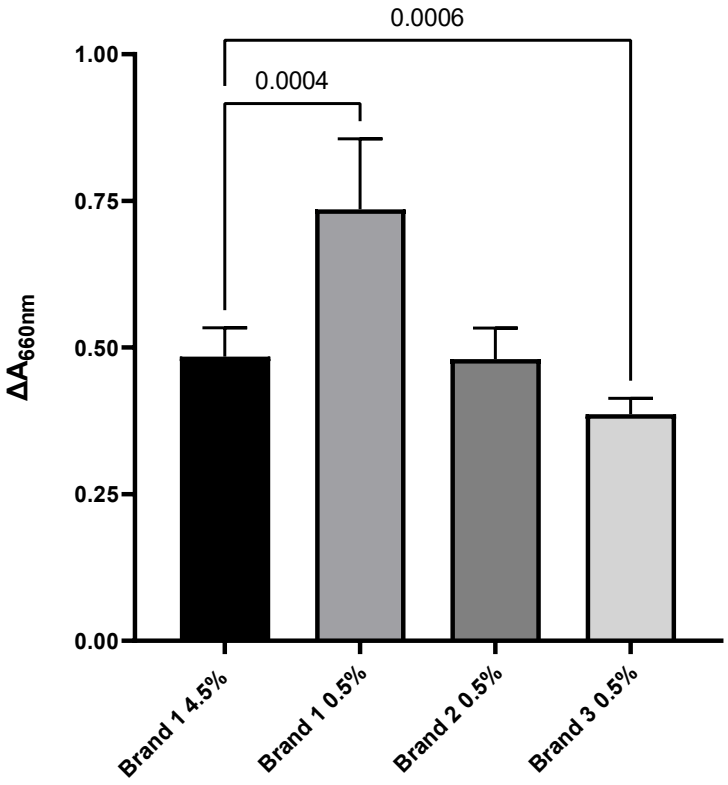

Figure 17: Δ A_{660nm} after 7 days incubation at room temperature of Low alcohol beers inoculated with 10^3 CFU/ml L. brevis, compared to Brand 1 4.5%. Results are the means of nine replicates, error bars are ± standard deviation

Each beer was inoculated with 10^3 CFU/ml *Rahnella spp.* And incubated at room temperature as described in Section 2.16**.** Results are the turbidity of the beer measured as Δ A_{660nm} after 7 days. In Figure 18 it can be seen that Brand 1 0.5% (Δ A_{660nm} = 0.162) and Brand 2 0.5% (Δ A_{660nm} = 0.144) show a significantly higher Δ A_{660nm} than Brand 1 4.5%. Brand 3 0.5% (Δ A_{660nm} = 0.057) shows a smaller but still significant increase in Δ A_{660nm} over Brand 1 4.5%. This suggests that *Rahnella spp.* grows in the three NABLABs. Although there was a small increase in the Δ A_{660nm} of Brand 1 4.5% this is most likely due to oxidation of the beer over the seven-day incubation period rather than growth of *Rahnella spp.*, as this can increase $A_{660nm.}$ This is shown happening in all beers when tested uninoculated in Figure 19**.** Similarly, when looking at the results in Figure 19 it appears that Brand 3 0.5% did not have significant growth of *Rahnella spp.* As its uninoculated absorbance was similar to the *Rahnella spp.* inoculated one.

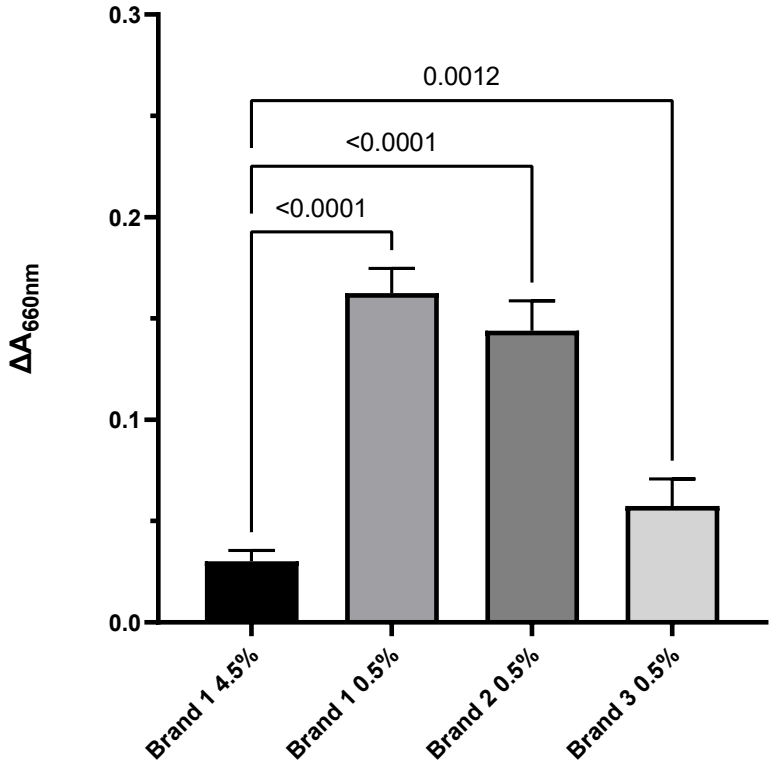

Figure 18: Δ A_{660nm} after 7 days incubation at room temperature of Low alcohol beers inoculated with 10^3 CFU/ml Rahnella spp, compared to Brand 1 4.5%. Results are the means of nine replicates, error bars are ± standard deviation

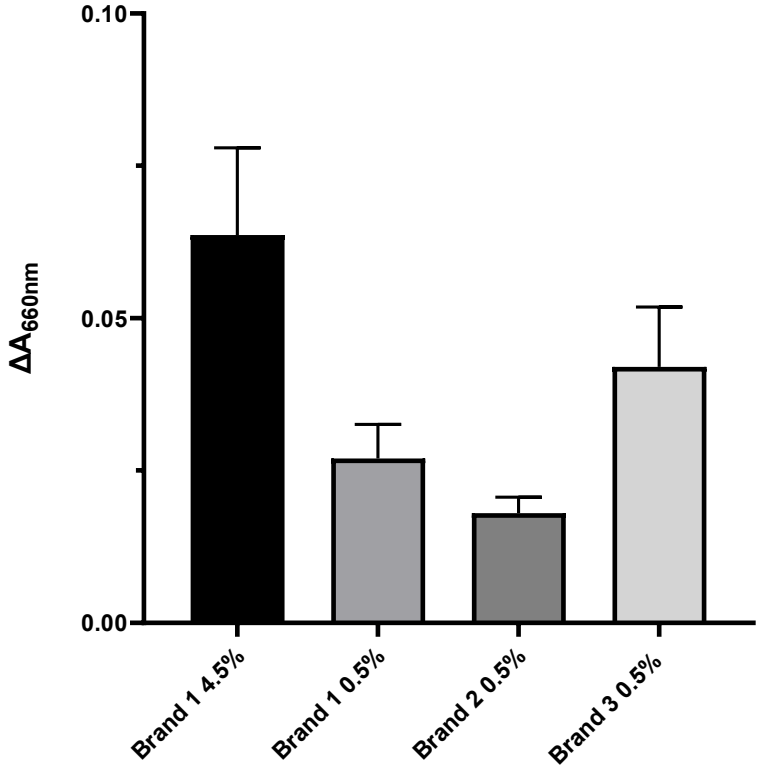

Figure 19: Δ A_{660nm} after 7 days incubation at room temperature of Low alcohol beers (Uninoculated)

3.2.4 Discussion

The previous understanding of NABLABs susceptibility to spoilage was that the removal of alcohol would cause NABLABs to become a more readily spoiled medium, regardless of the organisms they would be exposed to. This was backed up by Quain (2021) where the vast majority of NABLABs tested spoiled significantly more than the regular lagers tested. The methods in this study however were slightly different, with Quain's likely having a lower dissolved oxygen content in the beer. In addition, it was also performed at 30°C, so this may have played a part in the differing results. However, the results presented in Section 3.2 show that NABLABs don't always spoil more than a 4.5% ABV beer. In full alcohol beers different styles seem to have differing microbiomes, indicating that the differing environments select for specific beer spoilers (Jevons and Quain, 2021; Bose *et al.,* 2021), a similar effect can be seen here in the NABLABs tested.

The common draught beer spoilage yeast *P. membranifaciens* grew preferentially, and to a similar extent, in all the NABLABs tested compared to Brand 1 4.5%. This suggests that the low ethanol content in the NABLABs was the primary driver for the difference. This is interesting as *P. membranifaciens* can easily withstand ethanol concentrations up to 11%, so it may not be expected that 4.5% ethanol play much of a role in preventing its growth (Campbell and Msongo, 1990). However, it has been previously noted that their cellular function may be affected at higher ethanol concentrations (Caballero *et al.,* 2016). Some of the differences being observed could be due to the sugar composition of the beers rather than ethanol. However, *P. membranifaciens* can only ferment glucose (Kurtzman *et al.,* 2010). Brand 1 0.5% had a higher glucose concentration (0.52g L^{-1}) Section 3.1 (Table 5) than the other beers, yet despite these differences all the NABLABs saw *P. membranifaciens* growth to a similar degree. Even though *P. membranifaciens* can ferment glucose it has been shown to do so poorly, but it can metabolise other compounds commonly found in beers such as glycerol (Kurtzman *et al.,* 2010). Glycerol is formed as a by-product of fermentation and although the beers tested were analysed for glycerol it was not quantified. Highest to lowest in glycerol was; Brand 3 0.5%, Brand 1 4.5% and Brand 1 0.5% were very similar and then much lower was Brand 2 0.5%. Those which undergo a full fermentation will generally have a higher concentration (Nordström, 1968). Considering these factors Brand 2 0.5% would be expected to spoil the least, however, this was not the case. This suggests that in this situation the concentration of fermentable sugars is not playing a large role in the spoilage of the challenged beers. These trials were performed aerobically, which is important to consider as *P. membranifaciens* can also use ethanol as a carbon source via oxidation into acetic acid (Saez *et al.,* 2011). So, when there are limited fermentable sugars available (Table 5) and sufficient oxygen present, such as in this trial setup (Section 2.16), *P. membranifaciens* could be able to utilize this source in Brand 1 4.5%

that would not be readily available in the NABLABs. Despite this, the NABLABs were still a better medium for the growth of *P. membranifaciens.*

One of the most economically important spoilage organisms in the brewing industry is *L. brevis* as it is responsible for a large percentage of beer spoilage incidences (Suzuki, 2015). Consequently, it is essential to understand how it reacts to the new NABLAB niche. Contrary to the general consensus and previous research (Quain, 2021), the results produced in this section show that NABLABs aren't always a better medium for the growth of beer spoilers. Brand 1 0.5% did indeed show a greater level of *L. brevis* growth than its full alcohol equivalent, but the other NABLABs showed similar or lower, in the case of Brand 3 0.5%, levels of spoilage (Figure 17). As Brand 1 0.5% is the dealcoholized version of Brand 1 4.5% it could be assumed that ethanol is the driving factor in the growth differences as they are the same brand, and if Brand 2 0.5% and Brand 3 0.5% had full alcohol equivalents they would show less growth than each of them respectively. However, the production processes of NABLABs have a large effect on the final composition of the beer. For example, Brand 1 0.5% is brewed to a similar recipe to Brand 1 4.5% but has a slightly lower ABV after fermentation and a greater concentration of dry hop additions. As the alcohol is being removed anyway, aiming for a lower ABV after fermentation saves on malted barley costs. These savings can be used to increase the dry hopping rate to attempt to achieve the same hop aroma in the final beer, as some amount of volatile aroma compounds are often lost through all dealcoholisation processes (Andrés-Iglesias *et al.,* 2014)**.** Brand 2 0.5% has a statement of 'No More than 0.5% ABV' this is likely the reason that it has the lowest ABV as the producer would target a specification below 0.5% to ensure compliance with this statement. Brand 1 0.5% and Brand 3 0.5% only stated '0.5% ABV' and so from batch to batch are likely to vary both above and below the stated 0.5% ABV. So, essentially even if aiming for a similar end-product albeit without the alcohol, it is

likely that the beers compositions are significantly different in many ways and should essentially be treated as different beers (Section 3.1).

One of the other major hurdles to consider when the spoilage potential of *L. brevis* is being assessed is the iso-α acid content of the beers. As *L. brevis* is Gram-positive, it is sensitive to the antimicrobial action of these hop compounds (Suzuki, 2011). The growth seen in Figure 14 shows that this strain of *L. brevis* is hop resistant and most likely has one of or both *horA* and *horC* genes. However, this resistance is finite and so a higher concentration of iso-α acids will still inhibit the growth of a hop resistant bacteria even if they are expressing these genes (Suzuki *et al.,* 2006)**.** In this case, Brand 1 4.5%, Brand 1 0.5% and Brand 2 0.5% all have similar IBUs (Section 3.1 Table 4). In fact, the beer with the lowest IBU, Brand 3 0.5%, also showed the least growth of *L. brevis,* indicating that the concentration of iso-α acids is not greatly influencing these results. A factor which could affect the ability of *L. brevis* to resist the antimicrobial effects of hops is the Mn^{2+} and Mg^{2+}content of the beer (Section 1.6.3 Figure 5)**.** These divalent cations are important in maintaining regular cellular function and so when iso-α acids remove them from the cell its function may be impaired (Suzuki, 2011). A greater concentration of these divalent cations should help *L. brevis* to resist iso-α acids effects. Additionally, increasing the concentration of Mn^{2+} and Mg^{2+} has been shown to not only increase growth of *Lactobacillus* species but also increase their lactic acid production which would lead to greater spoilage effects (Lew *et al.,* 2014; Ciosek *et al.,* 2020). Interestingly the Mn^{2+} content of Brand 1 4.5%, Brand 1 0.5% and Brand 2 0.5% are all comparable (284.5-317.6 µg L^{-1}) with Brand 3 0.5% being much lower (83.5 µg L^{-1}). This could be a contributing factor to why Brand 3 0.5% saw the lowest growth of *L. brevis,* but it does also have the lowest concentration of iso-α acids, which would usually indicate an environment that would be easier for *L. brevis* to grow in. However, as mentioned previously Mn^{2+} concentration can positively influence the growth of *Lactobacillus* species irrespective of iso-α acid concentration. The Mg^{2+} content is similar in

Brand 1 0.5% (119.2 mg/L), Brand 1 4.5% (100.9 mg/L) and Brand 3 0.5% (98.2 mg/L) but is lower in Brand 2 0.5% (45.1 mg/L) which could be contributing to the lower growth of *L. brevis* in Brand 2 0.5% when compared to Brand 1 0.5%.

However, what is more likely is that the effect is due to the sugar composition of the beers. In fact, the glucose concentration trends in a similar way to *L. brevis* growth (Table 8).

Table 8: Measured concentration of glucose and fructose in each beer and mean Δ A_{660nm} of L. brevis after 7 days incubation at room temperature in each beer

Beer	Glucose (g L^{-1})	Fructose (g L^{-1})	Δ A_{660nm} after 7 days
Brand 1 0.5%	0.52	1.02	0.700
Brand 2 0.5%	0.05	0.07	0.460
Brand 3 0.5%	0.03	0.67	0.390
Brand 1 4.5%	0.14	0.98	0.470

L. brevis is known to be able to utilize glucose and so it is no surprise that a beer with a higher glucose concentration is a preferable medium for its growth (Ciosek *et al.,* 2020). Brand 1 0.5% is produced in a unique reverse osmosis process which consists of a concentration of the retentate but without a full dilution, so the dealcoholized volume at the end of production is down to roughly half of the starting volume. This is to aid in concentration of compounds contributing to mouthfeel and aroma, however it also concentrates other compounds that can't pass through the RO membrane such as glucose, fructose and maltose which could have a significant effect on spoilage. Reducing the glucose content by modifying the recipe, using a full dilution reverse osmosis, changing yeast strain or increasing the length of fermentation time could be a viable method to reduce the spoilage of this NABLAB by *L. brevis*. The fructose content of the beers was also analysed, the beer with the highest fructose concentration was Brand 1 0.5% which showed the greatest growth of *L. brevis*. It has been previously shown that *L. brevis* can

readily ferment fructose in beer and so it is likely that this is also aiding the growth of *L. brevis* in these beers (Ciosek *et al.,* 2020; Nsogning *et al.,* 2018). However, Brand 3 0.5% was higher in fructose (0.67g L^{-1}) than Brand 2 0.5% (0.07g L^{-1}) yet saw less *L. brevis* growth, so there may still be other factors affecting the growth of *L. brevis.*

In other *Lactobacillus* species it has been shown that Ca^{2+} can cause morphological changes that can improve viability in cryostorage of cultures, it is also a common addition to lactobacillus growth media (Wright and Klaenhammer, 1981). Similarly to glucose concentration, Ca^{2+} is highest in Brand 1 0.5% and lowest in Brand 3 0.5% (Section 3.1 Table 5; Table 6) and so the higher Ca^{2+} concentration may be aiding in *L. brevis* growth**.** It was recently shown that greater zinc concentrations can decrease the production of lactic acid by *L. brevis,* which is often related to growth and metabolism (Ciosek *et al.,* 2020). Brand 3 0.5% had the lowest growth of *L. brevis* and had a zinc concentration of 14.5 μg/L whereas the other three beers varied from 8.7-10.9 μg/L. Possibly indicating that a higher zinc concentration may be inhibiting the growth of *L. brevis.*

There are many microorganisms that can spoil full alcohol beers. Although compared to other food and drinks, beer is often considered stable (Vriesekoop *et al.,* 2012). However, this cannot be said for NABLABs, and there is a risk of overlooking spoilage organisms that can't spoil beer when assessing NABLABs, one example of that is *Rahnella spp*. This bacterium is not particularly common but can be found in breweries in early-stage fermentation and yeast slurry where it can be re-pitched into the next beer causing repeated contaminations (Van Vuuren and Priest, 2003). Previously, it has not been implicated in many spoilage incidents in the brewery although it can produce diacetyl and DMS in large quantities (Van Vuuren and Priest, 2003). It is generally not considered as a spoilage risk to finished beer. However, as the results in Figure 18 show *Rahnella spp.* was able to grow in Brand 1 0.5% and Brand 2 0.5%, although not to the same extent that *L. brevis* and *P. membranifaciens* could. It did not appear however to grow in Brand 3 0.5%

and Brand 1 4.5%. As *Rahnella spp.* is sensitive to ethanol it was expected that it would not grow in Brand 1 4.5%. However, after observing growth in both Brand 1 0.5% and Brand 2 0.5% it is interesting that it could not grow in Brand 3 0.5%. Brand 3 0.5% does have the lowest concentration of glucose but is very similar to Brand 2 0.5%. It may be that there is a lack of a specific micronutrient, in elemental analysis Brand 3 0.5% had the lowest concentrations of: Na, Ca, Mn, Fe, Sr and Ba. Or that it is sensitive to others as Brand 3 0.5% was highest in concentrations of: P, K and Zn, which can exhibit anti-microbial properties (McDevitt *et al.,* 2011). Nevertheless, the growth of *Rahnella spp.* In two of the NABLABs tested does demonstrate the need to consider atypical beer spoilage organisms in the spoilage of NABLABs. Interestingly, the *Rahnella spp.* used was originally isolated from an alcoholic beer in a conditioning tank with an ABV of 4.3%. Although it is unlikely to grow in this environment especially considering the low temperatures, it was still viable and so could be introduced into a NABLAB if there was cross-contamination. It is often found in water supplies and so this suggests that the importance of the sterility of water in the brewery should be even greater when producing NABLABs.

3.3 The Effect of Preservatives on the Growth of Beer Spoilage Microorganisms in NABLABs

3.3.1 Sodium Benzoate Introduction

Ethanol acts as a preservative in beer, and so after its removal perhaps it would be advisable to replace it with another preservative (Vriesekoop *et al.,* 2012). However, current regulations on additions to beer are much tighter than those for soft drinks, likely owing to beer previously being relatively microbiologically stable without the aid of additional preservatives (Vriesekoop *et al.,* 2012). One of the only preservatives permitted for use in NABLABs in keg is sodium benzoate at a maximum limit of 200ppm (EU Regulation 1129, 2011).

Sodium benzoate is commonly used in acidic food and drink as a microbiostatic agent. It can prevent the growth of yeast, moulds and bacteria (Lück and Jager, 1997). Its effectiveness is heavily dependent on the pH of the substrate it is protecting because the main antimicrobial component is the undissociated benzoic acid molecule. The lower the pH the greater the concentration of the undissociated acid molecule (Lück and Jager, 1997). Sodium benzoate has a pKa of 4.19. The NABLABs tested here all have pH levels equal to or greater than the pKa, which could significantly impact the effectiveness of this preservative.

Table 9: pH of the three NABLABs used in these trials

Beer	**pH**
Brand 1 0.5%	4.26
Brand 2 0.5%	4.46
Brand 3 0.5%	4.19

This Section assesses the effectiveness of a 200ppm dose of sodium benzoate in each NABLAB in preventing growth of the three beer-spoilage organisms: *P. membranifaciens, L. brevis* and *Rahnella spp.*

3.3.2 Day Seven Results

Each beer was dosed with 200ppm sodium benzoate solution as described in section 2.16 and then inoculated with 10^3 Cells/ml of *P. membranifaciens* and incubated at room temperature as in Section 2.17**.** Results are the turbidity of the beer measured as Δ A_{660nm} after 7 days. As seen in Figure 20 the addition of 200ppm sodium benzoate significantly reduced the Δ A_{660nm} of Brand 1 0.5% (-0.276) ($p < 0.0001$), Brand 2 0.5% (-0.227) ($p = 0.0030$) and Brand 3 0.5% (-0.359) ($p = 0.0004$) as compared to the results without the sodium benzoate addition. The beer with the highest pH (Brand 2 0.5%) showed the smallest reduction.

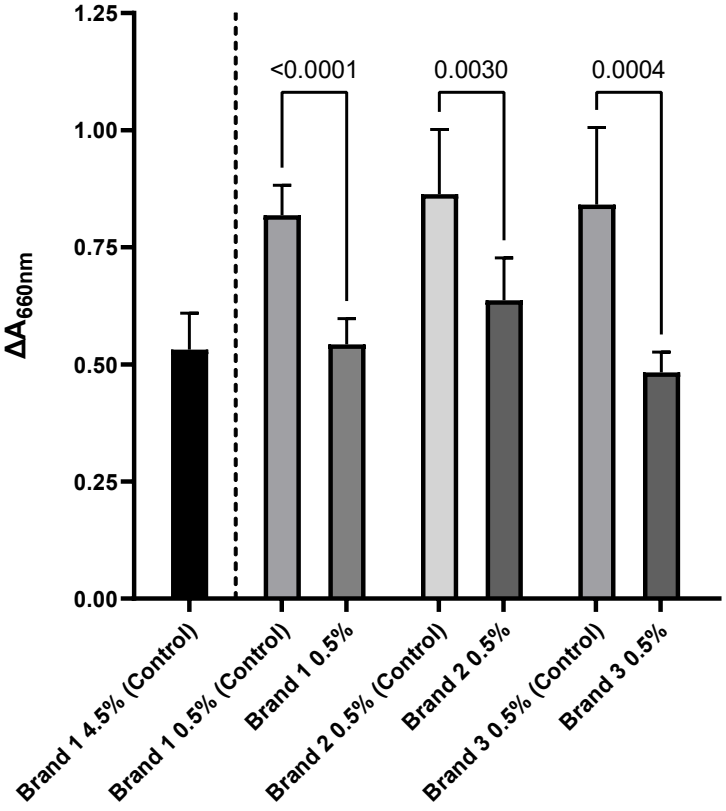

Figure 20: Δ A_{660nm} after 7 days incubation at room temperature of Low alcohol beers inoculated with 10^3 Cells/ml P. membranifaciens. Control samples are 'as received', test samples are dosed with 200ppm sodium benzoate. Comparisons are between the 'as received' control and sodium benzoate dosed test samples. Results are the means of nine replicates, error bars are ± standard deviation

Each beer was dosed with 200ppm sodium benzoate solution as described in Section 2.16 and then inoculated with 10^3 CFU/ml of *L. brevis* and incubated at room temperature as described in Section 2.17. Results are the turbidity of the beer measured as ΔA_{660nm} after 7 days. As shown in Figure 21 Brand 1 0.5% and Brand 2 0.5% both saw a large decrease in the ΔA_{660nm} (-0.334) and (-0.287) reductions respectively ($p < 0.0001$) as compared with the results without a sodium benzoate addition. Brand 3 0.5% saw a smaller yet sill significant decrease in ΔA_{660nm} (-0.059) ($p = 0.0064$). Despite the pH of Brand 3 0.5% being the lowest of the NABLABs and so potentially having the greatest concentration of undissociated benzoic acid it showed the least reduction in ΔA_{660nm}.

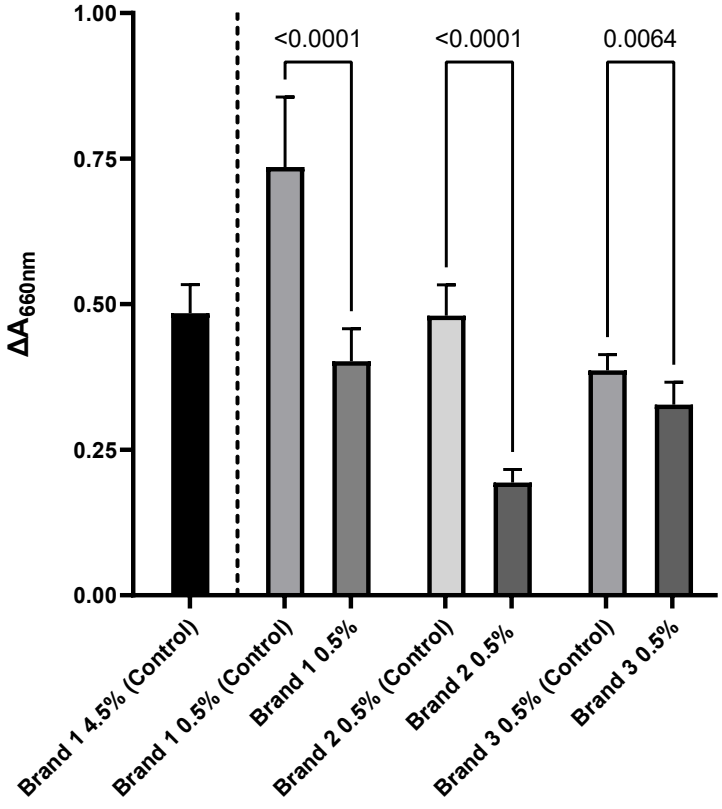

Figure 21: Δ A_{660nm} after 7 days incubation at room temperature of Low alcohol beers inoculated with 10^3 CFU/ml L. brevis. Control samples are 'as received', test samples are dosed with 200ppm sodium benzoate. Comparisons are between the 'as received' control and sodium benzoate dosed test samples. Results are the means of nine replicates, error bars are ± standard deviation

Each beer was dosed with 200ppm sodium benzoate solution Section 2.16 and then inoculated with 10^3 CFU/ml of *Rahnella spp.* and incubated at room temperature as described in Section 2.17. Results are the turbidity of the beer measured as ΔA_{660nm} after 7 days. As seen here in Figure 22 Brand 1 0.5% showed the largest reduction in ΔA_{660nm} (-0.089) ($p < 0.0001$), with Brand 2 0.5% also showing a significant reduction (-0.046) ($p < 0.0001$) when compared to the control without the sodium benzoate addition. Brand 3 0.5% however, showed no significant difference in ΔA_{660nm}. Compared to *P. membranifaciens* and *L. brevis, Rahnella spp.* showed a much lower ΔA_{660nm} indicating less growth. It is possible that the ΔA_{660nm} seen for Brand 3 0.5% is due to oxidation and not growth (Figure 19).

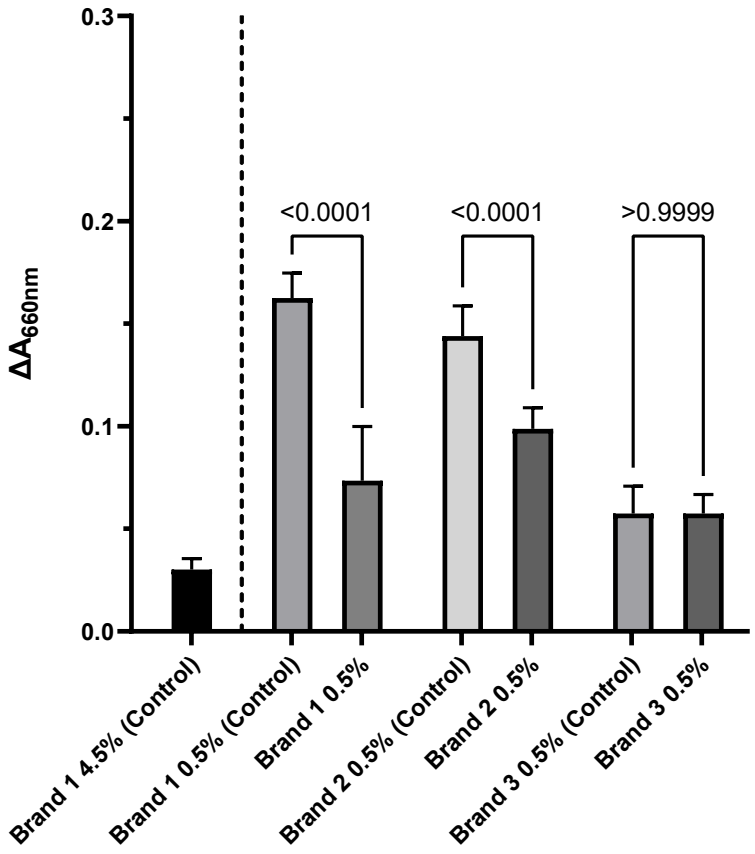

Figure 22: Δ A_{660nm} after 7 days incubation at room temperature of Low alcohol beers inoculated with 10^3 CFU/ml Rahnella spp. Control samples are 'as received', test samples are dosed with 200ppm sodium benzoate. Comparisons are between the 'as received' control and sodium benzoate dosed test samples. Results are the means of nine replicates, error bars are ± standard deviation

3.3.3 Potassium Sorbate Introduction

Potassium sorbate is currently not permitted for use in NABLAB keg products (EU Regulation 1129, 2011). However, it is extensively used in many foods and soft drinks, as well as other alcoholic beverages. It is regularly used in wine and cider making as a fermentation stopper at concentrations of 1-200ppm (Catherine *et al.,* 2014). Similar to sodium benzoate, potassium sorbate exhibits microbiostatic effects via its undissociated acid form, which is dependent on the pH of the substrate (Catherine *et al.,* 2014; Lück and Jager, 1997). However, it has a pKa of 4.69 which is much higher than that of sodium benzoate (NCBI, 2022). This means that it could be a better preservative option for the range of pH that beers usually occupy. On the contrary, it has been shown that it doesn't significantly prevent malolactic fermentation in wine, and so despite the pKa advantage may not be as effective of an agent against bacteria as sodium benzoate (Catherine *et al.,* 2014).

This section assesses the effectiveness of a 200ppm dose of potassium sorbate in each NABLAB in preventing growth of the three beer-spoilage organisms: *P. membranifaciens, L. brevis* and *Rahnella spp.*

3.3.4 Day Seven Results

Each beer was dosed with 200ppm potassium sorbate solution as described in Section 2.16 and then inoculated with 10^3 Cells/ml of *P. membranifaciens* and incubated at room temperature as in Section 2.17. Results are the turbidity of the beer measured as Δ A_{660nm} after 7 days. Shown in Figure 23 all brands of NABLAB showed a significant decrease in the Δ A_{660nm} (-0.422), (-0.478) and (-0.421) respectively ($p < 0.0001$) in samples that were dosed with potassium sorbate. There was very little difference between the Δ A_{660nm} of all brands in Figure 23 unlike Figure 20.

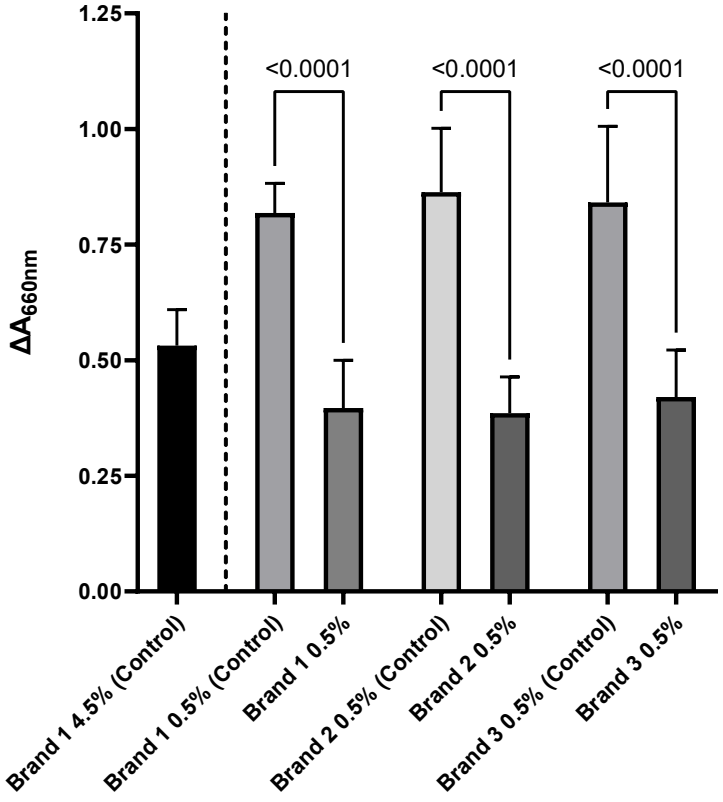

Figure 23: Δ A_{660nm} after 7 days incubation at room temperature of Low alcohol beers inoculated with 10^3 Cells/ml P. membranifaciens. Control samples are 'as received', test samples are dosed with 200ppm potassium sorbate. Comparisons are between the 'as received' control and potassium sorbate dosed test samples. Results are the means of nine replicates, error bars are ± standard deviation

Each beer was dosed with 200ppm potassium sorbate solution as described in Section 2.16 and then inoculated with 10^3 CFU/ml of *L. brevis* and incubated at room temperature as described in Section 2.17**.** Results are the turbidity of the beer measured as Δ A_{660nm} after 7 days. As seen in Figure 24 Brand 1 0.5% showed a significant decrease in Δ A_{660nm} (-0.266) ($p = 0.0003$) as did Brand 2 0.5% (-0.184) ($p < 0.0001$) over the no potassium sorbate control. Although Brand 3 0.5% did show a significant decrease in the Δ A_{660nm} (-0.07) ($p = 0.0013$) it was much smaller than the other two brands. A similar occurrence appeared in Figure 21, so for *L. brevis* it seems there may be other factors than just pH at play which determine the effectiveness of these two preservatives.

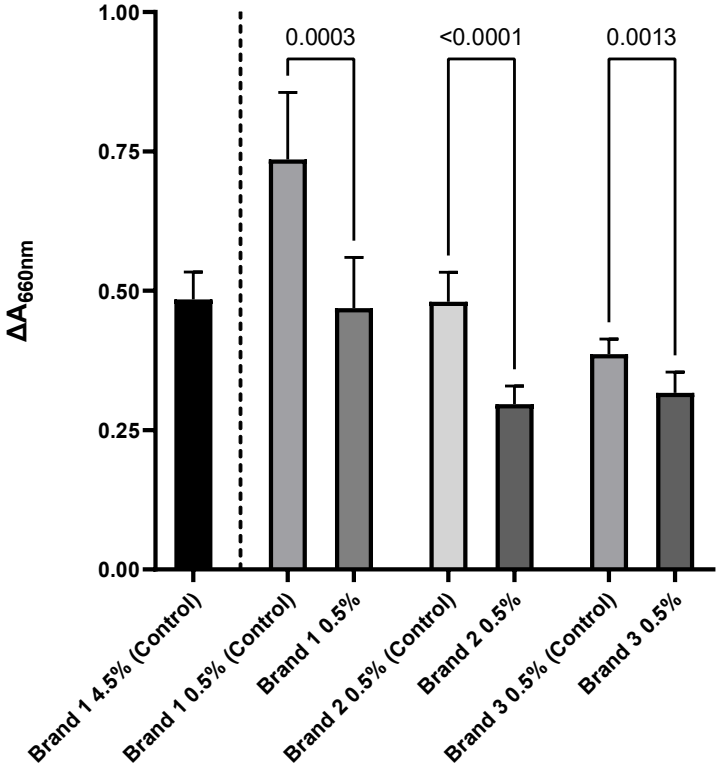

Figure 24: Δ A_{660nm} after 7 days incubation at room temperature of Low alcohol beers inoculated with 10^3 CFU/ml L. brevis. Control samples are 'as received', test samples are dosed with 200ppm potassium sorbate. Comparisons are between the 'as received' control and potassium sorbate dosed test samples. Results are the means of nine replicates, error bars are ± standard deviation

Each beer was dosed with 200ppm potassium sorbate solution as described in Section 2.16 and then inoculated with 10^3 CFU/ml of *Rahnella spp.* and incubated at room temperature as in Section 2.17. Results are the turbidity of the beer measured as ΔA_{660nm} after 7 days. Shown here in Figure 25 Brand 1 0.5% and Brand 2 0.5% show a significant decrease in ΔA_{660nm} (-0.071) and (-0.039) respectively ($p < 0.0001$) when compared to the control without potassium sorbate. Brand 3 0.5% did show a significant reduction in ΔA_{660nm} (-0.02). This possibly indicates that there was some growth of *Rahnella spp.* in Brand 3 0.5% control, but at such a low absorbance the limitations of the method may be impacting the results.

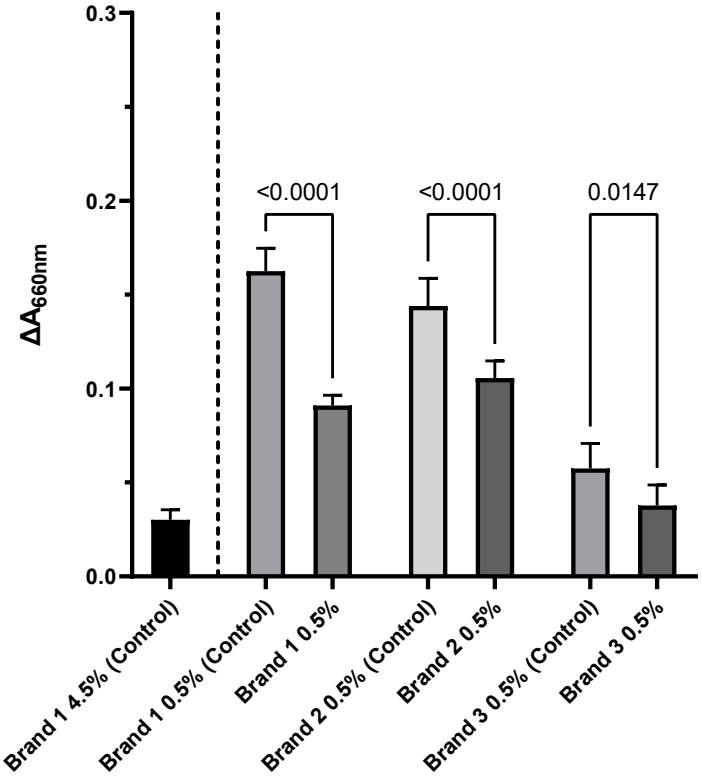

Figure 25: Δ A_{660nm} after 7 days incubation at room temperature of Low alcohol beers inoculated with 10^3 CFU/ml Rahnella spp. Control samples are 'as received', test samples are dosed with 200ppm potassium sorbate. Comparisons are between the 'as received' control and potassium sorbate dosed test samples. Results are the means of nine replicates, error bars are ± standard deviation

3.3.5 Sulphur Dioxide Introduction

Sulphur dioxide is found in a wide array of food products, employed as an antioxidant and antimicrobial (Garcia-fuentes *et al.,* 2015). It is the only preservative permitted for use in NABLAB keg other than sodium benzoate, at a maximum rate of 20ppm (EU Regulation 1129, 2011). However, in wine it is permitted up to 350ppm, but is commonly used at lower concentrations depending on the pH of the wine (Butzke, 2010). It is an allergen and so must be labelled as such at concentrations exceeding 10ppm (Guido, 2016). The ability of sulphur dioxide to exhibit anti-microbial activity is dependent on the pH of the substrate, operating in a three-way equilibrium reaction (Illet, 1995). Molecular sulphur dioxide is the most antimicrobial, Bisulfite shows some antimicrobial activity, and the sulfite species shows almost no activity as shown in Section 1.7.1 (Figure 6). Sulphur dioxide is effective against Gram-negative bacteria, shows some effect against Gram-positives and little effect against yeasts (Illet, 1995).

This section assesses the effectiveness of a 20ppm dose of sulphur dioxide in each NABLAB in preventing growth of the three beer-spoilage organisms: *P. membranifaciens, L. brevis* and *Rahnella spp.*

3.3.6 Day Seven Results

Each beer was dosed with 20ppm sulphur dioxide solution as described in Section 2.16 and then inoculated with 10^3 CFU/ml of *P. membranifaciens* and incubated at room temperature as in Section 2.17. Results are the turbidity of the beer measured as Δ A_{660nm} after 7 days. The addition of 20ppm sulphur dioxide caused significant pellicle formation on some samples (Figure 26). This interfered with absorbance measurements causing significant variation between samples. This is further discussed in Section 5.1**.**

Figure 26: Pellicle formation of P. membranifaciens in Brand 2 0.5% (Top) and Brand 3 0.5% (Bottom) when dosed with 20ppm sulphur dioxide

Each beer was dosed with 20ppm sulphur dioxide solution as described in Section 2.16 and then inoculated with 10^3 CFU/ml of *L. brevis* and incubated at room temperature as in Section 2.17. Results are the turbidity of the beer measured as Δ A_{660nm} after 7 days. As shown in Figure 27 the addition of 20ppm sulphur dioxide had no significant effect on the Δ A_{660nm} as compared to the controls without sulphur dioxide.

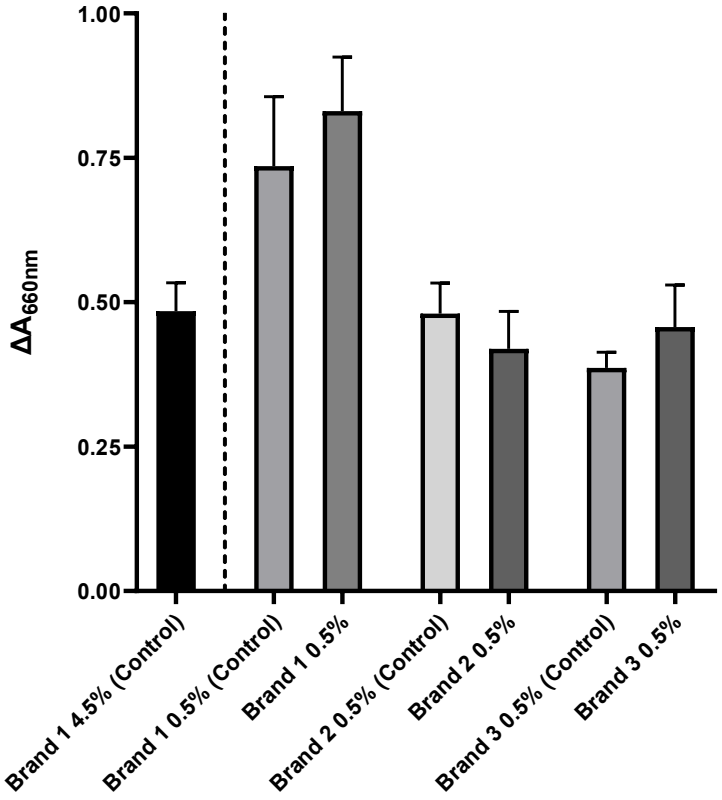

Figure 27: Δ A_{660nm} after 7 days incubation at room temperature of Low alcohol beers inoculated with 10^3 CFU/ml L. brevis. Control samples are 'as received', test samples are dosed with 20ppm sulphur dioxide. Comparisons are between the 'as received' control and sulphur dioxide dosed test samples. Results are the means of nine replicates, error bars are ± standard deviation

Each beer was dosed with 20ppm sulphur dioxide solution as described in Section 2.16 and then inoculated with 10^3 CFU/ml of *Rahnella spp.* and incubated at room temperature as described in Section 2.17. Results are the turbidity of the beer measured as ΔA_{660nm} after 7 days. Shown here in Figure 28 Brand 1 0.5% and Brand 2 0.5% show a significant decrease in ΔA_{660nm} (-0.138) ($p < 0.0001$) and (-0.036) ($p = 0.0004$) respectively. Brand 1 0.5% shows a greater decrease than sodium benzoate (Figure 22) and is similar to potassium sorbate (Figure 25) and when compared to (Figure 19) it is likely that no growth occurred. Brand 2 0.5% had a reduction that was similar to those produced by sodium benzoate (Figure 22) and potassium sorbate (Figure 25). Brand 3 0.5% did show a significant reduction in ΔA_{660nm} (-0.021). However, as previously described the increase in ΔA_{660nm} could be due to oxidation, and so the addition of 20ppm sulphur dioxide may have been able to reduce the effect of oxidation on the sample.

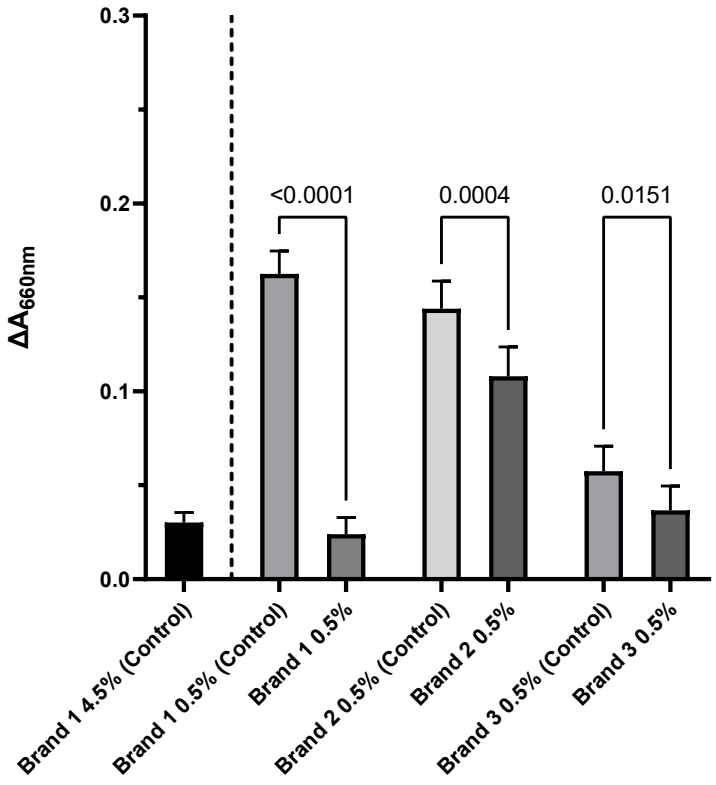

Figure 28: ΔA_{660nm} after 7 days incubation at room temperature of Low alcohol beers inoculated with 10^3 CFU/ml Rahnella spp. Control samples are 'as received', test samples are dosed with 20ppm sulphur dioxide. Comparisons are between the 'as received' control and sulphur dioxide dosed test samples. Results are the means of nine replicates, error bars are ± standard deviation

3.3.7 Preservative Comparisons Introduction

As sodium benzoate has a low pKa and lower consumer acceptance than potassium sorbate, it would be logical to switch to using potassium sorbate in NABLAB keg if it is more effective (Olmo *et al.,* 2017). However, potassium sorbate is currently not permitted for use in NABLAB keg despite it being widely used in many other food and drink, and also being permitted in keg beers which have had fruit added after fermentation (EU Regulation 1129, 2011).

This section is designed to assess the antimicrobial differences between sodium benzoate and potassium sorbate in each NABLAB against the three beer-spoilage organisms: *P. membranifaciens, L. brevis* and *Rahnella spp.* to determine which is the more effective antimicrobial in NABLABs under these specific test conditions.

3.3.8 Day Seven Results

Here sodium benzoate and potassium sorbate, both at a dose of 200ppm, are compared in each NABLAB when challenged with 10^3 Cells/ml *P. membranifaciens***.** Results are the turbidity of the beer measured as Δ A_{660nm} after 7 days. As shown in Figure 29 the potassium sorbate dosed Brand 1 0.5% and Brand 2 0.5% have significantly lower Δ A_{660nm} (-0.146) ($p = 0.0083$) and (-0.251) ($p < 0.0001$) respectively when compared to sodium benzoate. The largest difference is seen with Brand 2 0.5% which may be due to its high pH reducing the effectiveness of sodium benzoate. Brand 3 0.5% had a lower average Δ A_{660nm} however this was not significant. So, for Brand 1 0.5% and Brand 2 0.5% these results suggest that potassium sorbate is more effective at inhibiting the growth of *P. membranifaciens*.

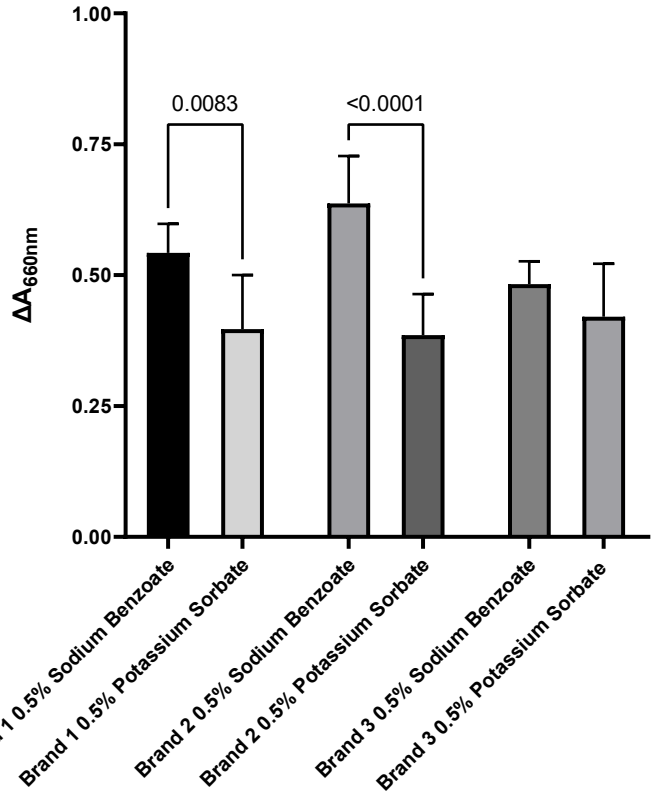

Figure 29: Δ A_{660nm} after 7 days incubation at room temperature of Low alcohol beers inoculated with 10^3 Cells/ml P. membranifaciens. Comparisons are between sodium benzoate and potassium sorbate dosed test samples. Results are the means of nine replicates, error bars are ± standard deviation

Here sodium benzoate and potassium sorbate, both at a dose of 200ppm, are compared in each NABLAB when challenged with 10^3 CFU/ml *L. brevis***.** Results are the turbidity of the beer measured as ΔA_{660nm} after 7 days. As seen in Figure 30 Brand 2 0.5% showed a significantly lower ΔA_{660nm} (-0.103) ($p < 0.0001$) when dosed with sodium benzoate when compared to potassium sorbate. However, Brand 1 0.5% and Brand 3 0.5% showed no significant differences between the two preservatives.

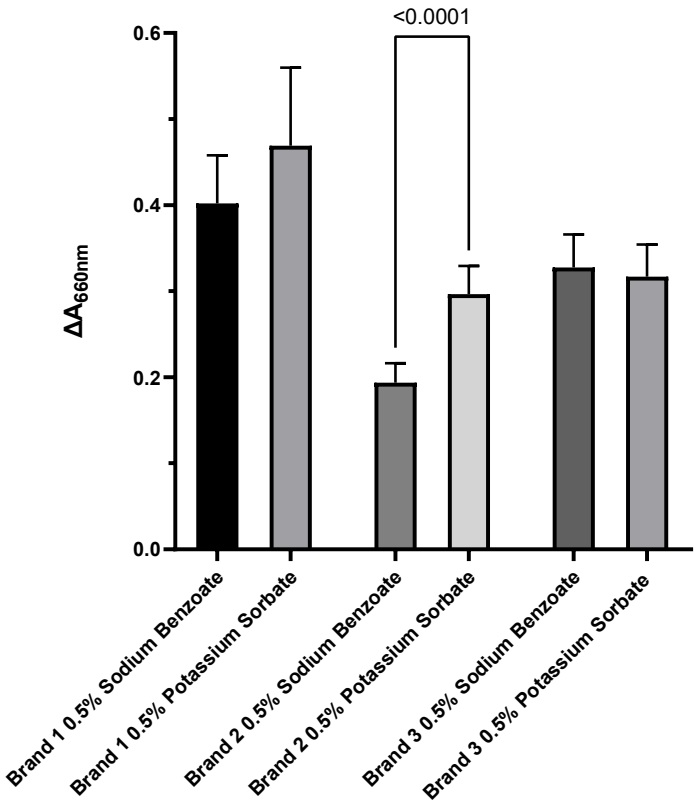

Figure 30: Δ A_{660nm} after 7 days incubation at room temperature of Low alcohol beers inoculated with 10^3 CFU/ml L. brevis. Comparisons are between sodium benzoate and potassium sorbate dosed test samples. Results are the means of nine replicates, error bars are ± standard deviation

Here sodium benzoate and potassium sorbate, both at a dose of 200ppm, are compared in each NABLAB when challenged with 10^3 CFU/ml *Rahnella spp***.** Results are the turbidity of the beer measured as Δ A_{660nm} after 7 days. Shown here in Figure 31 there were no significant differences in Δ A_{660nm} between sodium benzoate and potassium sorbate for Brand 1 0.5% and Brand 2 0.5%. Brand 3 0.5% did have a significantly lower Δ A_{660nm} but this difference is small and as mentioned in Section 3.4.2 is likely not due to growth.

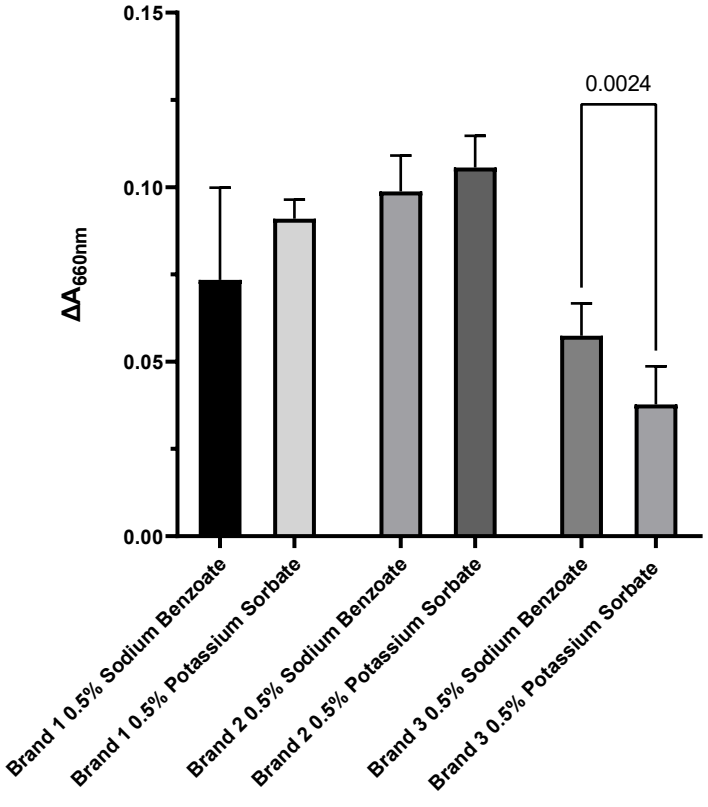

Figure 31: Δ A_{660nm} after 7 days incubation at room temperature of Low alcohol beers inoculated with 10^3 CFU/ml Rahnella spp. Comparisons are between sodium benzoate and potassium sorbate dosed test samples. Results are the means of nine replicates, error bars are ± standard deviation

3.3.9 Discussion

The three preservatives, sodium benzoate, potassium sorbate and sulphur dioxide, have been used in many different foods and beverages to prevent spoilage (Garcia-fuentes *et al.,* 2015; Catherine *et al.,* 2014; Lück and Jager, 1997). However, their use has been limited in beer due to its inherent microbiological stability and restrictive legislation surrounding preservative use in beer (EU Regulation 1129, 2011). NABLABs, despite emulating regular beer, must be treated as a separate case due to removal of ethanol, possible increase in fermentable sugar content and pH, amongst other compositional changes. The results here show that all the preservatives tested could be utilized to stabilise NABLABs and so brewers should consider using them to improve quality outcomes.

Sodium benzoate showed desirable microbiostatic effects against *P. membranifaciens* in all the NABLABs tested. The smallest reduction in ΔA_{660nm} by sodium benzoate was in Brand 2 0.5%, which had the highest pH of the NABLABs at 4.46. As mentioned in Section 1.7.3 and seen in Figure 8 the pH of a solution affects the amount of undissociated benzoic acid available. So, Brand 2 0.5% would have had a lower undissociated benzoic acid concentration which could have led to the lower microbiostatic effects observed. However despite this, the reduction in ΔA_{660nm} for Brand 2 0.5% (0.227) (Figure 20) was still large enough that the use of 200ppm sodium benzoate in a higher pH NABLAB could still be recommended as a viable preservative against the growth of *P. membranifaciens.* For NABLABs with a lower pH than Brand 2 0.5%, 200ppm sodium benzoate should be even more effective and so it could be possible to reduce the dose while still receiving the desirable level of spoilage prevention.

Although sodium benzoate is most commonly used to inhibit the growth of yeast and moulds it has also been shown to exhibit microbiostatic effects on some bacteria (Olmo *et*

al., 2017). At a concentration of 200ppm, sodium benzoate was effective at inhibiting the growth of *L. brevis* in every NABLAB showing a significant decrease in Δ A_{660nm}. However, Brand 3 0.5% showed a much smaller reduction (0.059) over the control than Brand 1 0.5% (0.334) and Brand 2 0.5% (0.287) (Figure 21). When in its undissociated form benzoic acid can act as a nitrogen binding agent, and so there is a possibility that this could reduce its effectiveness if Brand 3 0.5% had a higher nitrogen content (de las Heras *et al.,* 2017). However, this reduced effectiveness would be expected to be observed for *P. membranifaciens* as well if this was the case. The degree to which the growth of *L. brevis* was inhibited in Brand 2 0.5% is surprising given its high pH. As *L. brevis* did not grow as well in the controls of Brand 2 0.5% and Brand 3 0.5% compared to Brand 1 0.5%, there is a possibility of starvation of essential compounds for growth such as nitrogen, therefore influencing the sensitivity of *L. brevis* to sodium benzoate. Brand 2 0.5% did have the lowest Mg^{2+} levels and as previously mentioned in Section 3.2.4 they are known to be important for normal cellular function of *L. brevis* (Suzuki, 2011). Interestingly, when in the presence of Mg^{2+}, benzoic acid can form magnesium dibenzoate. Which due to the charge on Mg^{2+} requires two benzoic acid molecules to form (NCBI, 2022). As we know that the undissociated form provides the most anti-microbial activity, transformation of benzoic acid into magnesium dibenzoate could reduce antimicrobial activity, especially given that it would take up two benzoic acid molecules per Mg^{2+} ion.

For *Rahnella spp.* a significant reduction in Δ A_{660nm} was seen for Brand 1 0.5% and Brand 2 0.5% showing that sodium benzoate can exhibit broad spectrum antimicrobial activity against different types of microorganisms; eukaryotic yeast, and prokaryotes both Gram-positive and Gram-negative. It was more effective at reducing *Rahnella spp.* growth in Brand 1 0.5% which again could be due to the lower pH. Brand 3 0.5% saw no significant effect ($p > 0.9999$) further adding to the evidence that the resulting increase in absorbance was due to confounding factors of the method such as oxidation of the beer. The mean Δ

A_{660nm} for the control was 0.0575 and the sodium benzoate dosed 0.0574, the uninoculated results for Brand 3 0.5% had a mean of 0.042.

Potassium sorbate showed strong microbiostatic effects against *P. membranifaciens* showing large reductions in ΔA_{660nm} for all brands (Figure 23). It is commonly used as a fermentation stopper in wine and cider making, so this level of effectiveness against *P. membranifaciens* was to be expected (Catherine *et al.,* 2014). Potassium sorbate also relies on the undissociated acid form for microbial inhibition much like sodium benzoate. However, it has a higher pKa, meaning that it has a higher concentration of its undissociated acid form compared to sodium benzoate when at the same pH (Catherine *et al.,* 2014). Therefore, it may be a more suitable preservative for NABLABs, these results support that showing that it is an effective agent in controlling *P. membranifaciens* growth.

It has been previously reported that potassium sorbate does not have a significant effect on the growth of some *Lactobacillus* species (Catherine *et al.,* 2014). However, in this case there was a significant reduction in ΔA_{660nm} in all brands (Figure 24). Being one of the most damaging and frequent spoilers to beer, inhibition of *L. brevis* is crucially important to the brewing industry (Suzuki, 2015). Potassium sorbate doesn't have the negative connotation of carcinogenic benzene attached to it like sodium benzoate. So, being more acceptable to consumers and still performing similarly makes potassium sorbate a good candidate for approval for use in NABLABs (Olmo *et al.,* 2017). However, it has been shown previously that there are some microorganisms that can metabolise potassium sorbate. Not only does this reduce the concentration of the antimicrobial, and so allow sorbic acid sensitive microbes to grow, but the metabolisation process can produce off flavours which are detectable at very low concentrations (Described in Section 1.7.2). This would be unacceptable in NABLABs as the preservative is specifically being used to prevent these issues. More research as to which microbes can metabolise potassium sorbate may need to

be carried out before it could be recommended over sodium benzoate. It may be the case that no beer spoilage microbes can metabolise potassium sorbate. However, as previously mentioned the NABLAB category has presented a new niche for microorganisms previously not described which may well be able to metabolise it.

Potassium sorbate also showed similar results to sodium benzoate in the inhibition of *Rahnella spp.* growth (Figure 25). Showing its broad-spectrum effects, which are incredibly important when being used to prevent spoilage in systems such as draught dispense, where the product is likely to come into contact with many different types of microorganisms in varying conditions (Quain, 2015).

Although sulphur dioxide is commonly used in food and drink as an anti-oxidant and antimicrobial, it is often used at much higher concentrations than tested here. The 20ppm concentration used is the maximum legal limit permitted in NABLAB keg products in the UK, although 50ppm is permitted in cask beers (EU Regulation 1129, 2011). This concentration is clearly too low to inhibit *P. membranifaciens,* which is logical as yeast produce sulphites as an intermediate of sulphur assimilation (Feldmann and Branduardi, 2012). It also had no significant effect on *L. brevis* (Figure 27), in fact the anti-oxidant effect could possibly improve growth due to the reduction in dissolved oxygen (Guido, 2016). Other *Lactobacillus* species have also been shown to have resistance to sulphur dioxide at low levels (Quiros *et al.,* 2012). As seen in Figure 27 the sulphur dioxide dosed samples had a slightly higher mean than the control although not significant it would be interesting to see if a slightly higher dose of sulphur dioxide could make this difference significant. However, in the case of *Rahnella spp.* 20ppm sulphur dioxide was enough to give the biggest reduction in ΔA_{660nm} of all the preservatives tested in Brand 1 0.5% (0.138) (Figure 28). The same couldn't be said for Brand 2 0.5% which showed a much smaller reduction (0.036). Again, much like sodium benzoate and potassium sorbate, sulphur dioxide's

antimicrobial activity is heavily dictated by the pH of the solution (Illet, 1995). Though, in this case it is in a three-way equilibration reaction as described in Section 1.7.1. Here, the difference between a pH of 4.25 (Brand 1 0.5%) and 4.46 (Brand 2 0.5%) appears to affect the *Rahnella spp.* Inhibition ability of sulphur dioxide. However, sulphur dioxide can also bind to carbonyl compounds in beer effectively sequestering anti-microbially active sulphite species, thus reducing the concentration of antimicrobial sulphites**.** It strongly binds acetaldehyde, which is a compound formed in fermentation and by other contaminating microbes such as *Acetobacter* and *Zymomonas* (Lisanti *et al.,* 2019; Pardah, 2015)*.* In addition, it can bind other carbonyl compounds such as pyruvate, however these are more easily reversible (Lisanti *et al.,* 2019). So, Brand 2 0.5% may well have a higher acetaldehyde, especially considering that NABLABs produced by restricted fermentations are known to be high in aldehydes (Gernat *et al.,* 2019). This could be affecting the concentration of free sulphur dioxide leading to its inferior inhibition of *Rahnella spp*. The reduction in ΔA_{660nm} for Brand 3 0.5% indicates that some of the ΔA_{660nm} seen in the control is down to oxidation, as sulphur dioxide is a potent anti-oxidant and was able to reduce the ΔA_{660nm} to lower than the control (Figure 28).

If trying to inhibit the growth of *P. membranifaciens,* potassium sorbate was a more effective preservative than sodium benzoate for Brand 1 0.5% and Brand 2 0.5%. The ΔA_{660nm} difference between potassium sorbate and sodium benzoate was largest for Brand 2 0.5% (0.251) (Figure 29). This beer has a higher pH at 4.46, and so these results suggest that potassium sorbate is a better choice of preservative than sodium benzoate, especially for NABLABs that may have a higher pH due dealcoholisation or restricted fermentation (Muller *et al.,* 2020*;* Branyik, 2012). The pH can be adjusted in NABLABs with the use of citric, lactic or other weak acids, so in terms of preservative effectiveness reducing the pH as low as possible would be advantageous. However, adding too much of these acids will induce a sour taste and spoil the organoleptic properties of the beer (Siebert, 1999). So, a

careful balance must be implemented between having a low pH, to aid in preservative effectiveness, but not going too low as to negatively affect the organoleptic properties of the beer. There is no sense in creating an unspoilable product that no customer is willing to consume.

However, when dosed with *L. brevis* the same trend wasn't observed. Brand 2 0.5% had a significantly lower ΔA_{660nm} when dosed with sodium benzoate compared to potassium sorbate, this is despite the lower quantity of undissociated acid. Brand 1 0.5% did have a lower mean with sodium benzoate but was not significant. This suggests that sodium benzoate could be better than potassium sorbate at inhibiting the growth of *L. brevis,* and possibly other Gram-positive bacteria. This could be due to the differing mechanisms of action of the two preservatives (Section 1.7.2; 1.7.3). In wine, potassium sorbate has been shown to be ineffective at sufficiently inhibiting acetic acid bacteria and preventing excessive malolactic fermentation (Catherine *et al.,* 2014), which also suggests that potassium sorbate may not be the most effective against bacteria. Alternatively, its lower antimicrobial activity could have been caused by 105tabilize105tion of potassium sorbate by *L. brevis* (Catherine *et al.,* 2014; Chisholm and Samuels, 1992).

3.4 Does the Addition of Preservatives to a NABLAB Reduce the Growth of Beer Spoilage Microorganisms to a Similar Level to that of its Full Alcohol Counterpart?

3.4.1 Introduction

Despite being known as a microbially stable product regular beers found in trade can still be subject to spoilage (Quain, 2015). They have been served through the same dispense systems for many years, and so, brewers, publicans and consumers have become accustomed to how spoilable these products are. With NABLABs however, the possibility for an increased spoilage rate could turn customers and publicans away from serving NABLABs through traditional dispense systems. One of the goals of this research was to ascertain whether preservatives could be used to 106tabilize NABLABs, so that they could be served in the traditional dispense systems with a similar spoilage rate as their full alcohol counterparts.

In this section the spoilage of Brand 1 4.5% was compared with its dealcoholized equivalent Brand 1 0.5% with addition of preservatives, against the three beer-spoilage organisms: *P. membranifaciens, L. brevis* and *Rahnella spp.*

3.4.2 Day Seven Results

Here Brand 1 0.5% was dosed with either 200ppm sodium benzoate or 200ppm potassium sorbate and compared to Brand 1 4.5%. Each dosed with 10^3 Cells/ml *P. membranifaciens*. Results are the turbidity of the beer measured as ΔA_{660nm} after 7 days. Sulphur dioxide was not included due to the pellicle formation interfering with the results as demonstrated in Section 3.3.6. As shown in Figure 32 there is no significant difference in ΔA_{660nm} between the Brand 1 4.5% control and Brand 1 0.5% dosed with 200ppm sodium benzoate. When Brand 1 0.5% is dosed with potassium sorbate the ΔA_{660nm} is significantly lower than that of Brand 1 4.5% (-0.135) ($p = 0.0133$). These results indicate that 200ppm of sodium benzoate or potassium sorbate in Brand 1 0.5% would bring spoilage by *P. membranifaciens* down to a level equal to or better than a full alcohol beer.

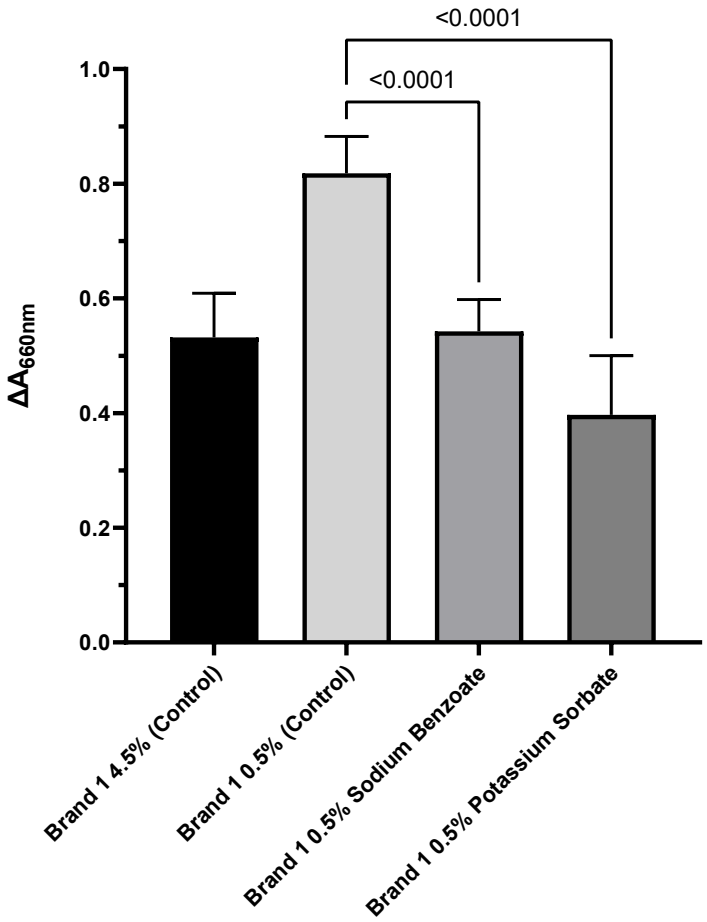

Figure 32: Δ A_{660nm} after 7 days incubation at room temperature of Low alcohol beers and alcoholic control inoculated with 10^3 Cells/ml P. membranifaciens. Comparisons are between preservative dosed test samples and a full alcohol control. Results are the means of nine replicates, error bars are ± standard deviation

Here Brand 1 0.5% was dosed with either 200ppm sodium benzoate, 200ppm potassium sorbate or 20ppm sulphur dioxide and compared to Brand 1 4.5% each dosed with 10^3 CFU/ml *L. brevis*. Results are the turbidity of the beer measured as Δ A_{660nm} after 7 days. Shown here in Figure 33 when dosed with 200ppm sodium benzoate Brand 1 0.5% had a significantly lower Δ A_{660nm} (-0.103) ($p = 0.0125$) when compared to brand 1 4.5%. When dosed with 200ppm potassium sorbate there was no significant difference between Brand 1 0.5% and Brand 1 4.5%. Sulphur dioxide dosed Brand 1 0.5% had a significantly higher Δ A_{660nm} (+0.346) ($p < 0.0001$) than Brand 1 4.5%. So, these results suggest that for *L. brevis* 200ppm sodium benzoate and 200ppm potassium sorbate would bring spoilage down to a level equal to or better than a full alcohol beer.

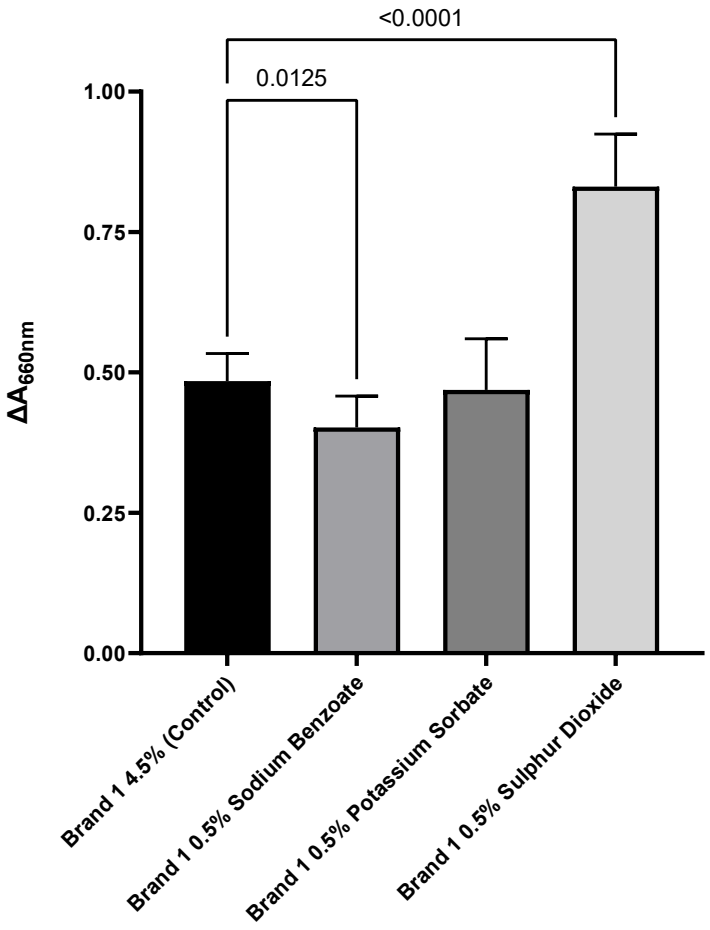

Figure 33: Δ A_{660nm} after 7 days incubation at room temperature of Low alcohol beers and alcoholic control inoculated with 10^3 CFU/ml L. brevis. Comparisons are between preservative dosed test samples and a full alcohol control. Results are the means of nine replicates, error bars are ± standard deviation

Here Brand 1 0.5% was dosed with either 200ppm sodium benzoate, 200ppm potassium sorbate or 20ppm sulphur dioxide and compared to Brand 1 4.5% each dosed with 10^3 CFU/ml *Rahnella spp*. Results are the turbidity of the beer measured as Δ A_{660nm} after 7 days. As seen in Figure 34 Brand 1 0.5% dosed with 200ppm sodium benzoate has a significantly higher Δ A_{660nm} ($p = 0.0028$), as does 200ppm potassium sorbate ($p < 0.0001$) when compared to Brand 1 4.5%. When 20ppm of sulphur dioxide was used in Brand 1 0.5% there were no significant differences in Δ A_{660nm} compared to Brand 1 4.5%. This suggests that for *Rahnella spp.* sodium benzoate and potassium sorbate are not effective at reducing spoilage to the levels of Brand 1 4.5%. However, 20ppm of sulphur dioxide did reduce spoilage to comparable levels.

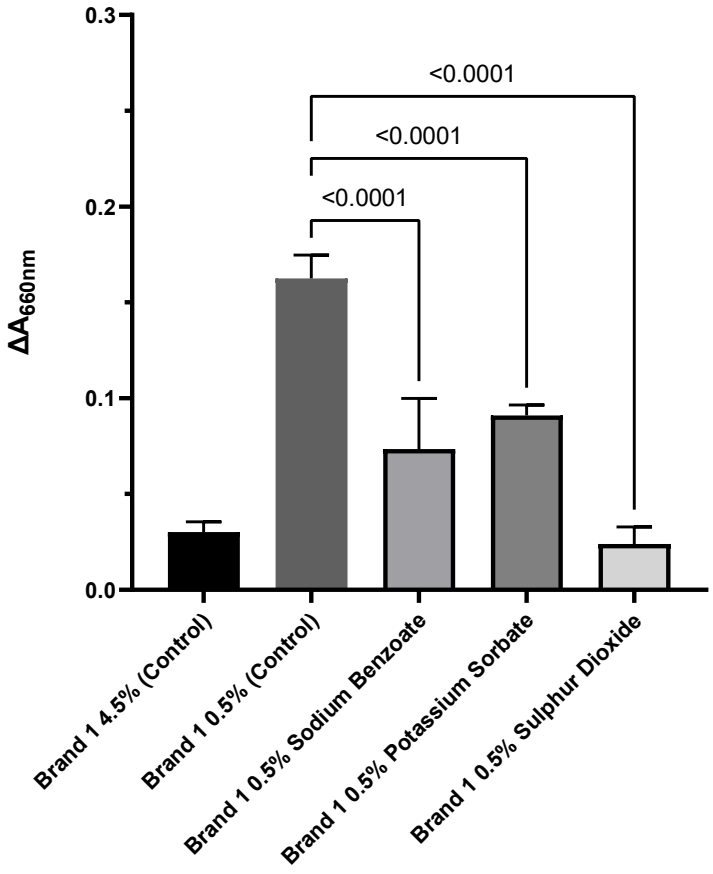

Figure 34: Δ A_{660nm} after 7 days incubation at room temperature of Low alcohol beers and alcoholic control inoculated with 10^3 CFU/ml Rahnella spp. Comparisons are between preservative dosed test samples and a full alcohol control. Results are the means of nine replicates, error bars are ± standard deviation

3.4.3 Discussion

The effectiveness of each in Brand 1 0.5% was compared to its alcoholic counterpart Brand 1 4.5% without any preservatives. This was to determine whether the addition of these preservatives could reduce the level of spoilage of Brand 1 0.5%. Ideally, to a comparable level to its full alcohol counterpart that is served in keg, on-trade, through normal dispense systems in the UK. For *P. membranifaciens* and *L. brevis* both sodium benzoate (200ppm) and potassium sorbate (200ppm) were able to bring the spoilage of Brand 1 0.5% in-line with or better than Brand 1 4.5% (Figure 32 and Figure 33). For *Rahnella spp.,* growth was reduced but not to the level of Brand 1 4.5%. Although potassium sorbate and sodium benzoate couldn't reduce *Rahnella spp.* growth to the desired level, just 20ppm of sulphur dioxide completely inhibited its growth (Figure 34), although this was not the case for Brand 2 0.5% (Figure 28). This means that these preservatives could well allow the serving of NABLABs on regular dispense systems.

As presented here and in previous research, NABLABs can be more susceptible to spoilage depending on their composition and the beer spoilage organisms in question (Quain, 2021). So, a method needs to be developed to be able to reduce spoilage, allowing NABLABs to be served consistently on-trade. Ideally, this method would bring NABLABs susceptibility to spoilage in line with full alcohol beers. In addition, it would be advantageous to be able to keep all other cellar management the same, as to increase uptake of keg NABLABs and simplify the care of the product for the publican.

There are some methods which have already been employed to try and solve the issue of NABLABs increased spoilage. Much like regular beers, pasteurisation and sterile filtration are used to ensure that the beer in package is free from beer spoilage organisms. Unfortunately, the environment of a draught dispense system is far from sterile (Quain, 2015, Bose *et al.,* 2021). With improper cleaning and management, draught systems are

often consistently contaminated which can lead to biofilm formation allowing spoilage organisms to survive through cleaning protocols (Hill, 2015; Quain, 2015). So, to combat this issue some brewers are utilizing custom dispense systems. Heineken released their 0.0% ABV lager on-trade by using the BLADE ® dispense system which is a bar-top standalone unit that uses small 8L plastic kegs (Heineken, 2021). The advantage of this system is that each keg has a sterile beer tube attached which goes directly from the keg to the tap. The BLADE® also keeps the entire keg at 3°C which will dramatically slow the growth of any beer spoilage organisms if the keg were to become contaminated (Membré *et al.,* 2005). Diageo have developed a similar system, but it uses pint sized cans instead. They can be stored in the fridge and then dispensed immediately, meaning the footprint of the unit can be much smaller (Independent, 2021). Both systems have smaller package sizes, this is advantageous as current demand for NABLABs on-trade is still lower than regular beer (IWSR, 2021). This means that it takes longer to get through a standard 30L keg. As it takes longer to empty a package there is an increasing risk of a spoilage incident, and so reducing the size of package reduces the amount of time the beer is 'open' and so potentially lowers the risk of becoming contaminated with beer spoilage microorganisms. Recently however, Heineken have switched to using 30L kegs of 0.0% lager, preserving it by maintaining cool cellar and line temperatures, which will slow the growth of any beer spoilage microorganisms (Beverage Daily, 2021).

These methods are possible for large breweries, however the capital expenditure and operating expenditures are high as a new dispense system must be created and maintained separately to the already present draught dispense system. As the NABLAB market is growing much faster than the alcoholic beer market many brewers, of all sizes, may want to start producing NABLABs. Especially as demand continues to increase for a high quality low or no alcohol alternative on-trade (IWSR, 2021). So, a low-cost method for ensuring safety and stability of NABLABs in keg is needed for these breweries to be able to take part

in this growing sector. This is where commonly used food and drink preservatives could help. Currently, the options are limited for NABLAB in keg with only sodium benzoate at maximum 200ppm and sulphur dioxide at maximum 20ppm permitted for use. These don't seem like obvious choices given the low dose of sulphur dioxide and with both being regarded as inefficient antimicrobials within the normal pH range of beer, of which can be even higher in NABLABs (Muller *et al.,* 2020; Branyik, 2012). Sulphur dioxide is also an allergen that must be declared at over 10ppm, possibly reducing the number of customers able to drink the product. Potassium sorbate is a popular choice of preservative for many foods and drinks. It has the advantage of having a greater percentage of undissociated acid at the standard pH range of beer compared to sodium benzoate or sulphur dioxide (Sofos and Busta, 1981).

3.5 Survival of Food-borne Pathogens *E. coli* 0157:H7 and *S.* Typhimurium in NABLABs and a Full Alcohol Counterpart

3.5.1 Introduction

The anti-microbial hurdles of beer don't only prevent against spoilage of the product, they also inhibit the growth of any food-borne pathogens (Vriesekoop, 2012; Menz *et al.,* 2011). The removal of ethanol, and differences in other hurdles caused by the varying production methods, makes NABLABs sufficiently different that their susceptibility to food-borne pathogens must be individually assessed. Previous research has shown that *E. coli* 0157:H7 and *S.* Typhimurium can grow in NABLABs (L' Anthoën and Ingledew, 1996; Menz *et al.,* 2011). However, the NABLAB market is rapidly expanding, and many production methods used now weren't available 10 years ago, so it is to be expected that the beers being produced now are different to those being produced in 1996 and 2011 (Appendix 1). Due to the differences in their composition, it is important to test multiple NABLABs to ascertain the factors which make a NABLAB more susceptible to growth of food-borne pathogens (L' Anthoën and Ingledew, 1996; Quain, 2021).

In this section the survival of *E. coli* 0157:H7 and *S.* Typhimurium was assessed in Brand 1 4.5%, Brand 1 0.5%, Brand 2 0.5% and Brand 3 0.5%.

3.5.2 Results

Here Brand 1 0.5%, Brand 1 4.5%, Brand 2 0.5% and Brand 3 0.5% were dosed with 10^3 CFU/ml *E. coli* 0157:H7 as described in Section 2.17.1. Results are CFU/ml at inoculation and after 7 days incubation as described in Section 2.17.1. Shown here in Figure 35 Brand 1 0.5% and Brand 2 0.5% achieved a significant reduction in viable *E. coli* 0157:H7 to 3.80 x 10^2 CFU/ml and 4.71 x 10^2 CFU/ml respectively ($p < 0.0001$). Brand 3 0.5% saw a smaller yet still significant reduction to 8.88 x 10^2 CFU/ml ($p = 0.0363$). Reduction in viable cells continued when tested at 14 and 21 days with no viable *E. coli* 0157:H7 recovered in Brand 1 0.5% and Brand 2 0.5% after 21 days. In Brand 3 0.5% only 1.33 x 10^1 CFU/ml remained after 21 days (Three replicates only, data not presented in Figure 35). Brand 1 4.5%, seen in Figure 36 showed the largest reduction in viable *E. coli* 0157:H7 after 7 days (1.33 x 10^2 CFU/ml) ($p < 0.0001$).

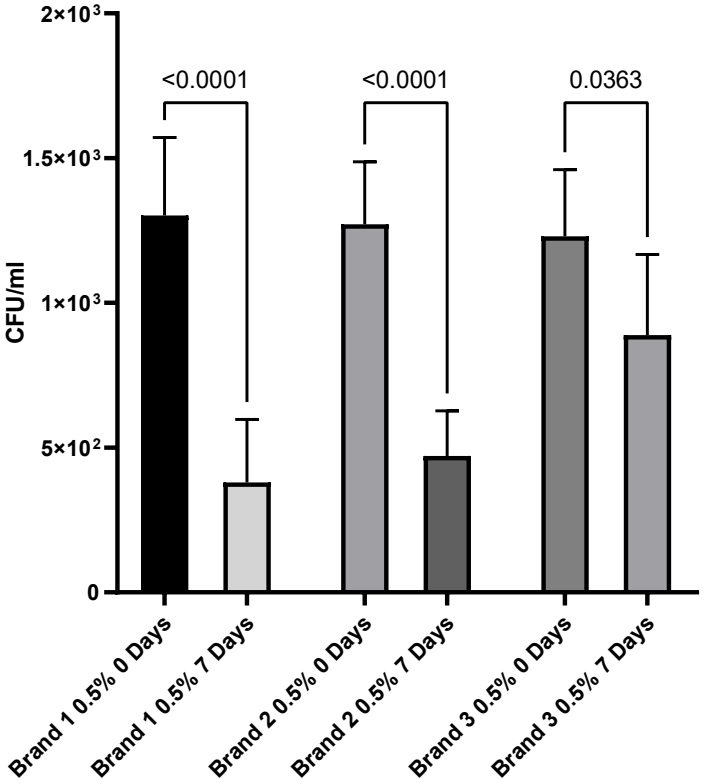

Figure 35: Survival of 10^3 CFU/ml E. coli 0157:H7 in low alcohol beers incubated at room temperature for 7 days. CFU/ml was determined at inoculation (0 days) and after 7 days by spread plating. Results are the means of nine replicates, error bars are ± standard deviation

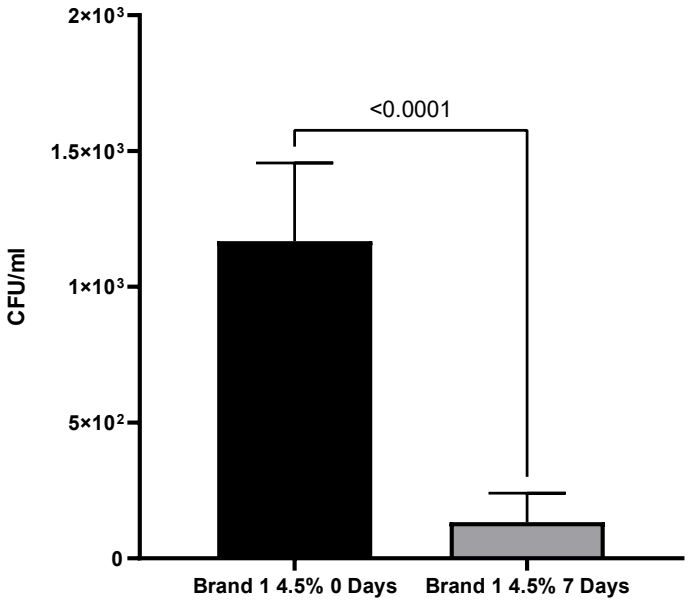

Figure 36: Survival of 10^3 CFU/ml E. coli 0157:H7 in Brand 1 4.5% incubated at room temperature for 7 days. CFU/ml was determined at inoculation (0 days) and after 7 days by spread plating. Results are the means of nine replicates, error bars are ± standard deviation

Here Brand 1 0.5%, Brand 1 4.5%, Brand 2 0.5% and Brand 3 0.5% were dosed with 10^3 CFU/ml *S.* Typhimurium as described in Section 2.17.1. Results are CFU/ml at inoculation and after 7 days incubation as described in Section 2.17.1. As seen in Figure 37 Brand 1 0.5% showed a significant reduction in viable *S.* Typhimurium with only 2.2 CFU/ml remaining after 7 days incubation ($p < 0.0001$). In Brand 3 0.5% (Figure 38) there was also a significant reduction in viable *S.* Typhimurium ($p = 0.0016$) however there were still 7.1 x 10^2 CFU/ml remaining after the incubation period. So, the trial was extended to 14 days where no viable cells could be recovered (Figure 39). As seen in Figure 40 Brand 1 4.5% showed a significant reduction in *E. coli* 0157:H7 viable cells after 7 days with only 1.33 x 10^2 CFU/ml ($p < 0.0001$) remaining. A similar effect was seen in Figure 40 for *S.* Typhimurium (2.4 x 10^2 CFU/ml) ($p = 0.0021$). For Brand 2 0.5% when plated as in Section 2.17.1 after the 7-day incubation period the plates showed innumerable viable cells of *S.* Typhimurium indicating strong growth with > 10^4 CFU/ml. Interestingly, after 3 days the viable count of *S.* Typhimurium in Brand 2 0.5% had dropped from 9.2 x 10^2 CFU/ml to 4.63 x 10^2 CFU/ml.

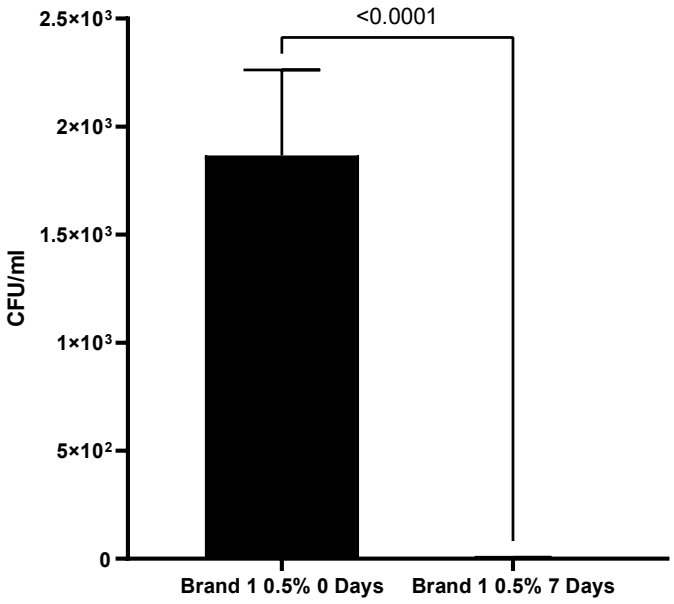

Figure 37: Survival of 10^3 CFU/ml S. Typhimurium in Brand 1 0.5% incubated at room temperature for 7 days. CFU/ml was determined at inoculation (0 days) and after 7 days by spread plating. Results are the means of nine replicates, error bars are ± standard deviation

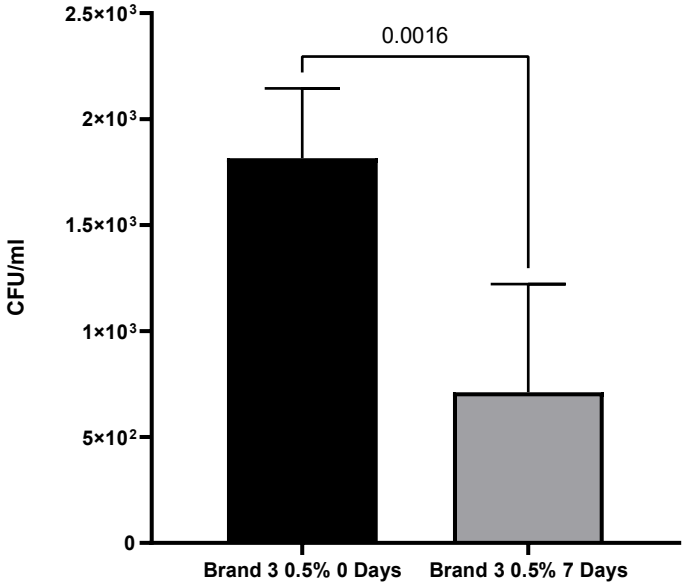

Figure 38: Survival of 10^3 CFU/ml S. Typhimurium in Brand 3 0.5% incubated at room temperature for 7 days. CFU/ml was determined at inoculation (0 days) and after 7 days by spread plating. Results are the means of nine replicates, error bars are ± standard deviation

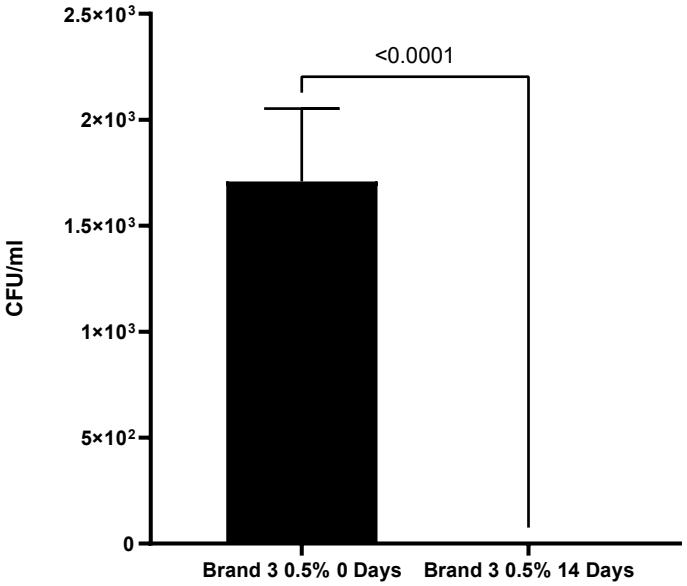

Figure 39: Survival of 10^3 CFU/ml S. Typhimurium in Brand 3 0.5% incubated at room temperature for 7 days. CFU/ml was determined at inoculation (0 days) and after 14 days by spread plating. Results are the means of nine replicates, error bars are ± standard deviation

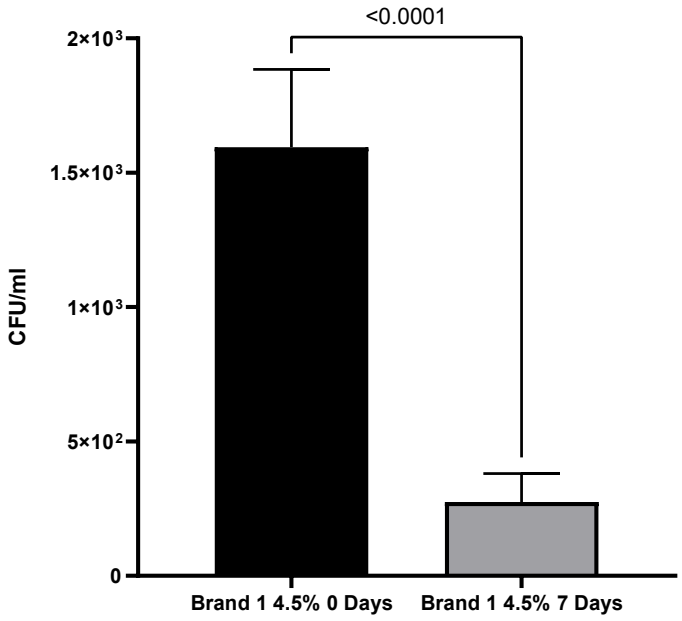

Figure 40: Survival of 10^3 CFU/ml S. Typhimurium in Brand 1 4.5% incubated at room temperature for 7 days. CFU/ml was determined at inoculation (0 days) and after 7 days by spread plating. Results are the means of nine replicates, error bars are ± standard deviation

3.5.3 Discussion

Previous research from L'Anthoën and Ingledew and Menz *et al.* had shown that *E. coli 0157:H7* and *S.* Typhimurium can grow in low alcohol beer. However, there are now new and improved production methods for NABLABs which is likely to have significantly changed the underlying parameters of the NABLABs produced today (Muller *et al., 2020;* Salanta *et al.,* 2020; Bellut and Arendt, 2019; Blanco *et al.,* 2016; Branyik *et al.,* 2012). The market for NABLABs is also growing rapidly so the importance for understanding the potential for growth of pathogens has never been greater (IWSR, 2021).

In the three NABLABs tested in this study, the growth of *E. coli* 0157:H7 was not observed after 7 days (Figure 35). In addition, the number of viable cells continued to drop at 14 and 21 days (Section 3.9.2). This shows that not all NABLABs are susceptible to the growth of *E. coli* 0157:H7, and so there must be some differences other than the alcohol content that are contributing to the growth of *E. coli* 0157:H7 in some NABLABs. Although the beer tested by Menz *et al.* was only analysed for ABV, pH and IBU, L'Anthoën and Ingledew had more comprehensive analysis on the NABLAB that they showed to grow both *E. coli* 0157:H7 and *S.* Typhimurium (Table 10).

Table 10: ABV, pH and sugar composition of 'AFB' used in L'Anthoën and Ingledew, 1996 and the NABLAB used in this study

	Glucose (g/100ml)	Fructose (g/100ml)	Maltose (g/100ml)	Maltotriose (g/100ml)	ABV	pH
AFB	0.7	0.14	2.92	0.72	0.4	5.25
Brand 1 0.5%	0.052	0.102	0.174	0.229	0.46	4.26
Brand 2 0.5%	0.005	0.007	0.027	0.051	0.42	4.46
Brand 3 0.5%	0.003	0.67	0.259	0.223	0.51	4.19

The beer used by L'Anthoën and Ingledew had a much higher concentration of glucose, fructose, maltose, maltotriose and pH. Although known as an acid resistant food-borne pathogen, *E. coli* 0157:H7 has been shown to have its growth in a NABLAB prevented when the pH was reduced to 4.0 (Xiong *et al.,* 2021; Menz *et al.,* 2011). Conversely, growth was aided when the pH was brought up to 5.0 (Menz *et al.,* 2011). Brand 2 0.5% has a pH of 4.46 which is in the range which *E. coli* 0157:H7 has been shown to grow previously, but in this beer it could not grow. As described earlier sugar composition plays a strong role in spoilage of NABLABs (Quain, 2021), and as *E. coli* 0157:H7 is known to utilize glucose, its concentration may well affect their ability to grow (Kornberg and Lourenco, 2006). The lack of growth however, is likely not solely attributed to one specific beer parameter, the many microbiological hurdles that can still remain in NABLABs could be contributing to preventing the growth of *E. coli* 0157:H7. Interestingly, when *E. coli* 0157:H7 was dosed into Brand 1 4.5% there were still some viable cells after the 7 days incubation (Figure 39) despite the 4.5% ethanol content.

When *S.* Typhimurium was tested in Brand 1 0.5% and Brand 3 0.5% there were greater viable cell reductions than was observed for *E. coli* 0157:H7, showing a lower resistance to the conditions of these NABLABs. However, in Brand 2 0.5% *S.* Typhimurium grew after 7 days to > 10^4 CFU/ml. interestingly, the viable cell count had dropped to 4.6 x 10^2 CFU/ml from a 9.6 x 10^2 CFU/ml inoculation after 3 days, showing that although it did manage to grow it was not rapid and many cells died in the process of adapting to the environment. This growth was not rapid and so later trials use Brand 1 0.5% that has been modified to allow faster growth of *S.* Typhimurium. The rate at which growth can occur is important to consider as even if a product does support the growth of pathogens, if they cannot grow within the time they are in favourable growth conditions (e.g. between serves from a draught dispense line) then it is unlikely that the risk of growth will be high. Despite this, it is always favourable to produce a product that can resist growth of foodborne pathogens

to prevent outlier contamination cases due to poor hygienic practice, cellar management, and differing growth abilities of the many different variants of *S.* Typhimurium*, E. coli* and other foodborne pathogens. Of the parameters analysed, it seems that the higher pH of Brand 2 0.5% was what allowed *S.* Typhimurium to grow, as Brand 1 0.5% has much more available glucose but *S.* Typhimurium could still not grow. Interestingly, Brand 2 0.5% had very little in the way of glucose, fructose or maltose but yet *S.* Typhimurium was still able to grow. It appears that it was able to utilize other compounds as its carbon source, such as amino acids or organic acids (Gutnik *et al.,* 1969). *S.* Typhimurium will preferentially use glucose as its sole carbon source if enough is present but will be able to switch to other sources once the glucose is fully utilized possibly leading to a diauxic growth pattern (Kenyon *et al.,* 2005). These results show again that all NABLABs are not created equally, and different organisms have different growth requirements. Ideally, each product should be challenge tested against the most likely spoilers and food-borne pathogens, to assess its microbiological stability before attempting to serve on-trade. If a NABLAB was to perform poorly, then preservative additions or re-formulation to reduce pH or fermentable sugars should be implemented to improve outcomes.

3.6 Effectiveness of Preservatives in Inhibiting Food-borne Pathogen Growth in a Modified NABLAB

3.6.1 Introduction

Preservatives are not solely used to prevent spoilage of food and drink, they are also regularly used to reduce or eliminate the risk of foodborne pathogen growth (Msagati, 2012). They help to extend the shelf-life and reduce the risk of severe illness or death of the consumer. Sodium benzoate, potassium sorbate and sulphur dioxide have all shown anti-microbial activity towards *E. coli* and *S.* Typhimurium as well as other food-borne pathogens (Ceylan *et al.,* 2004; Basaran-Akgul *et al.,* 2009; Banks and Board, 1982; Zhao *et al.,* 1993; Sofos and Busta, 1981; Baik *et al.,* 1996). However, they have not been assessed in low or no alcohol beers. As previously described in Section 1.7 the pH of the solution each of these preservatives is in affects their antimicrobial activity. Additionally, for sulphur dioxide the permitted maximum legal limit in NABLAB keg in the UK is 20ppm which is lower than most research into its effectiveness against food-borne pathogens (EU Regulation 1129, 2011; Basaran-Akgul *et al.,* 2009). Therefore, we must directly assess the ability of these preservatives to perform in NABLABs, especially considering that *E. coli* 0157:H7 and *S.* Typhimurium have been shown to grow in NABLABs with the latter growing in Brand 2 0.5% in Section 3.9.2.

To increase the rate at which pathogens would grow, Brand 1 0.5% was modified with a small addition of malt extract (ME) solution as described in Section 2.18.2 and its pH raised to 4.44. The ME increased fermentable sugar to levels comparable to some of the beers analysed by Quain (2021) and L'Anthoën and Ingledew (1996) (Appendix 1). Brand 1 0.5% was used as it is the dealcoholized counterpart of Brand 1 4.5%, it is brewed in a very similar way until it is run through the reverse osmosis process. The method of modifying a

beer in which the pathogens didn't grow to then allow them to grow, aids in understanding that factors which effect the growth and survival of the food-borne pathogens *E. coli* 0157:H7 and *S.* Typhimurium.

3.6.2 Results

Here Brand 1 0.5% was modified with an addition of malt extract and its pH raised to 4.44 (MB1 0.5%) as prepared in Section 2.18.2. It was dosed with 10^3 CFU/ml *S.* Typhimurium as described in Section 2.6. Results are CFU/ml at inoculation and after 72 hours incubation as described in Section 2.18.2. As presented in Figure 41 the MB1 0.5% control showed significant growth of *S.* Typhimurium with 4.16 x 10^6 CFU/ml increase in viable cells in 72 hours at 20°C (p = 0.0179). All the preservative dosed samples showed no growth of *S.* Typhimurium. Conversely, they all showed a significant reduction in viable cells with sulphur dioxide being the largest showing a 1.1 x 10^3 CFU/ml reduction (p <0.0001). Sodium benzoate had a 7.8 x 10^2 CFU/ml reduction (p = 0.0055) and potassium sorbate a 6.6 x 10^2 CFU/ml reduction (p = 0.0414).

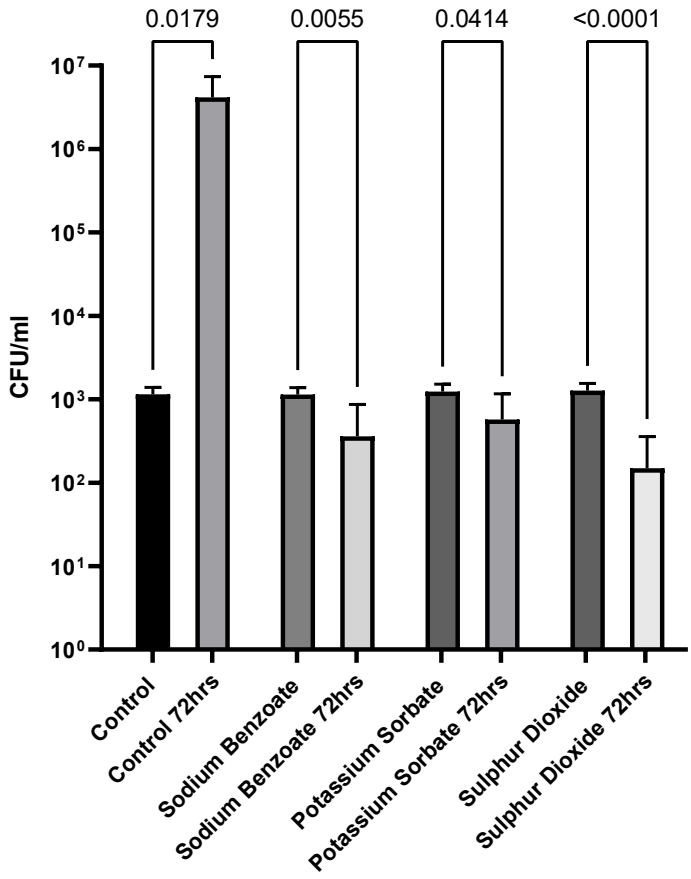

Figure 41: Growth of 10^3 CFU/ml S. Typhimurium in MB1 0.5%, as received control and preservative dosed samples incubated at room temperature for 72hrs. CFU/ml was determined at inoculation (0 days) and after 72hrs by spread plating. Results are the means of nine replicates, error bars are ± standard deviation

E. coli 0157:H7 did not grow in MB1 0.5% and so its sensitivity to preservatives in 72hrs was assessed instead. This was performed in unmodified Brand 1 0.5% to give a greater chance for the preservatives to reduce the number of viable cells of *E. coli* 0157:H7.

Here Brand 1 0.5% (un-modified) was dosed with 10^3 CFU/ml *E. coli* 0157:H7 as described in Section 2.18.3. Results are CFU/ml at inoculation and after 72 hours incubation as described in Section 2.18.3. As shown in Figure 42 the addition of 200ppm sodium benzoate, 200ppm potassium sorbate or 20ppm sulphur dioxide had no significant effect on the survivability of *E. coli* 0157:H7 in Brand 1 0.5% compared to the control without any preservative.

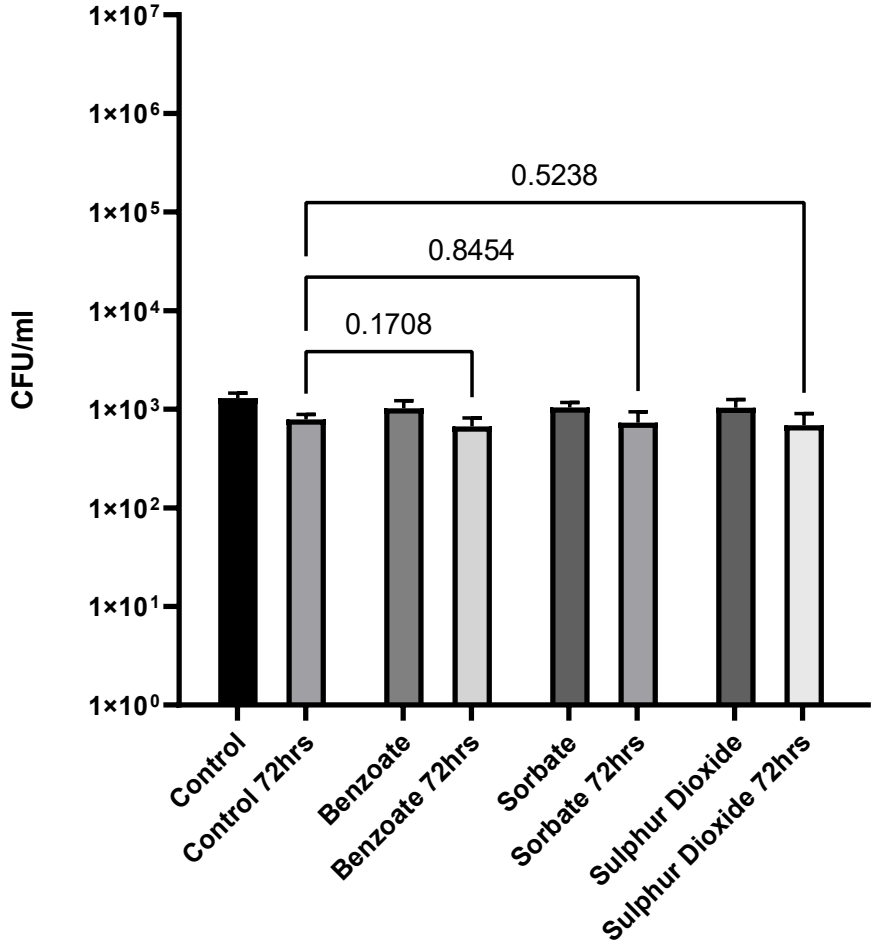

Figure 42: Survival of 10^3 CFU/ml E. coli 0157:H7 in Brand 1 0.5%, as received control and preservative dosed samples incubated at room temperature for 72hrs. CFU/ml was determined at inoculation (0 days) and after 72hrs by spread plating. Results are the means of nine replicates, error bars are ± standard deviation

3.6.3 Discussion

Ideally, all NABLABs produced would be resistant to the growth of food-borne pathogens without the need for addition of preservatives. However, as shown previously (Menz *et al.,* 2011; L'Anthoën and ingledew, 1996) they are susceptible. On the contrary, results produced in this work (Figures 35, 36, 37) show that some are able to resist the growth of the food-borne pathogens *E. coli* 0157:H7 and *S.* Typhimurium. For those that cannot, or have not been proven to, resist pathogens, preservatives should be used to inhibit their growth. As shown in Figure 41 each preservative tested was very effective at preventing the growth of *S.* Typhimurium. This was performed in a beer that was modified to allow pathogens to grow more rapidly than any of the beers tested. This had reduced microbiological hurdles with a higher pH and an increase in fermentables from the addition of malt extract, which added in more available glucose, fructose and maltose as well as other nutrients such as free amino nitrogen (FAN) (Murphy & Son, 2022). This allowed the rapid growth of *S.* Typhimurium in a beer that it previously showed no growth in Figure 36. This adds to the theory that simple sugar content and pH are critical factors in allowing *S.* Typhimurium to be able to grow in some NABLABs and not others. Lowering fermentable sugars and keeping pH as low as is reasonably achievable should be the goal for the development of food-borne pathogen resistant NABLABs. However, if this is not possible, or the pathogen in question is utilising other carbon sources in the beer, as seemed to be the case for *S.* Typhimurium in Brand 2 0.5%, then sodium benzoate, potassium sorbate (if permitted) or sulpur dioxide should be used.

The modified Brand 1 0.5% did not allow *E. coli* 0157:H7 to grow so it was not able to assess the growth prevention effects of the preservatives. Some short assays were completed on other beers, and also lactose dosed and pH modified Brand 1 0.5% in which *E. coli* 0157:H7 was able to grow. These were not further investigated for reasons explained in Section 5.1. Instead, *E. coli* 0157:H7 was assesed for its sensitivity to these preservatives

in Brand 1 0.5% after 72hrs. There was no signifcant difference between the samples dosed with preservatives and the control, indicating that none of the additions increased the rate at which *E. coli* 0157:H7 would be killed in Brand 1 0.5%. This does not mean however that these preservatvies would be ineffective at preventing the growth of *E. coli* 0157:H7. Sodium benzoate and potassium sorbate are considered microbiostatic agents. Which means they inhibit the growth of microorganisms but don't necessarily kill cells directly (Mohammadzadeh-Aghdash *et al.,* 2019).

Chapter 4. Results of OmniLog® Trials to Assess the Effect of pH and Preservative Concentration on *P. membranifaciens* and *L. brevis*

4.1 OmniLog® Spoilage Trials at Three pH Levels

4.1.1 Introduction

The pH of a beer is one of the most important antimicrobial hurdles (Vriesekoop *et al.,* 2012). It is routinely measured throughout the brewing process and can be adjusted in a number of ways (Bamforth, 2006). This makes it a good candidate for adjustment to improve microbiological outcomes. However, not all microorganisms are sensitive to a pH that is practical and can still be deemed organoleptically acceptable by the consumer. Yeast for example are highly acid resistant being able to survive down to a pH of 2.0 (Simpson and Hammond, 1989). *Lactobacillus* species are also acid tolerant to a pH of 2.0-3.0 (Jin *et al.,* 1998). Despite this, a lower pH may still slow their ability to grow as the lower the pH gets the harder it is for microorganisms to be able to maintain their desired intracellular pH (Suzuki, 2015).

To be able to assess this many variables the OmniLog® system was used. This uses up to fifty 96-well plates and utilises cells NADH production as a reporter. It does this by using a tetrazolium dye which is reduced by NADH causing it to form a strong colour, the more an organism grows in the well the greater the reduction, the stronger the colour. This colour change is measured and logged by the OmniLog® at regular intervals over the incubation period (Cruz *et al.,* 2021).

Rahnella spp. Was omitted from these trials due to its poor growth in the beers. Unfortunately, the use of *E. coli* 0157:H7 (Non-STEC) and *S.* Typhimurium was intended for these trials but the risk assessment application was rejected.

In this section three samples of Brand 1 0.5% were used at Low (3.8), Med (4.15) and High (4.55) pH, and Brand 1 4.5% (4.41 pH) was used as the full alcohol control. *P. membranifaciens* and *L. brevis* were dosed into each variable as described in Section 2.20 and incubated for 6 days at 20°C. Results are trials in triplicate with ± standard deviation, presented as Biolog Units every 3.5 hours, although measurements were taken every 30 minutes.

4.1.2 Results

Here Brand 1 0.5% Low, Med and High pH samples and Brand 1 4.5% were inoculated with *P. membranifaciens* or *L. brevis* as described in Section 2.20. As seen in Figure 43 the values for all of the Brand 1 0.5% samples were much higher than the Brand 1 4.5% control which matches the trend seen in the spoilage trials (Figure 13; Figure 16). Interestingly, the low pH sample seemed to show the most growth. Shown in Figure 44 there seems to be no clear difference between the growth of *L. brevis* in any of the differing pH conditions. The growth in Brand 1 4.5% was also similar to that of Brand 1 0.5%. In the spoilage trials Brand 1 0.5% saw greater growth of *L. brevis* than Brand 1 4.5%. However, most of this difference was seen after 7 days (Figure 14; Figure 17). Despite this the curve shown in Figure 44 appears to be flattening for all samples after the incubation period, so it seems unlikely that a greater difference would have arisen had this trial been extended.

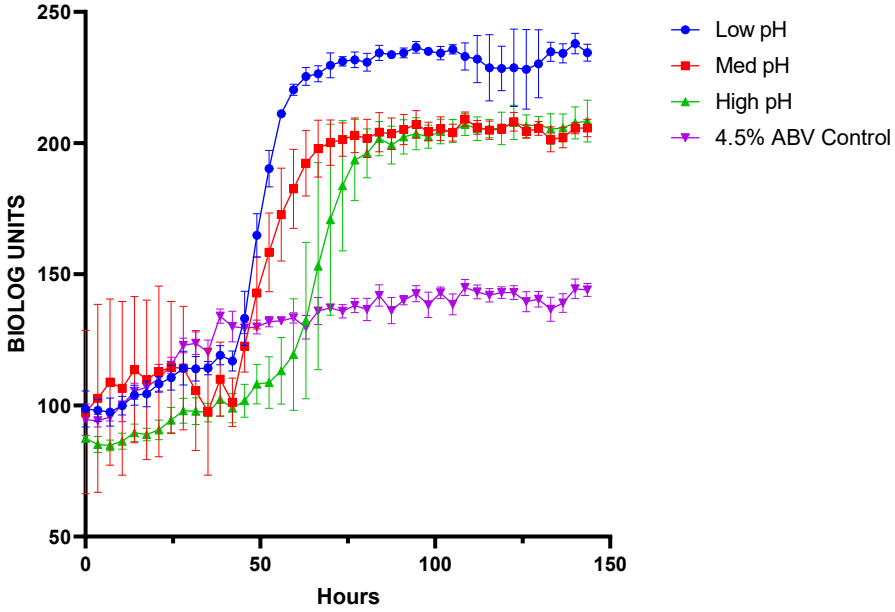

Figure 43: Growth of P. membranifaciens in Brand 1 4.5% and Brand 1 0.5% at Low (3.8), Med (4.15) and High (4.55). Results are means of three replicates, error bars are ± standard deviation

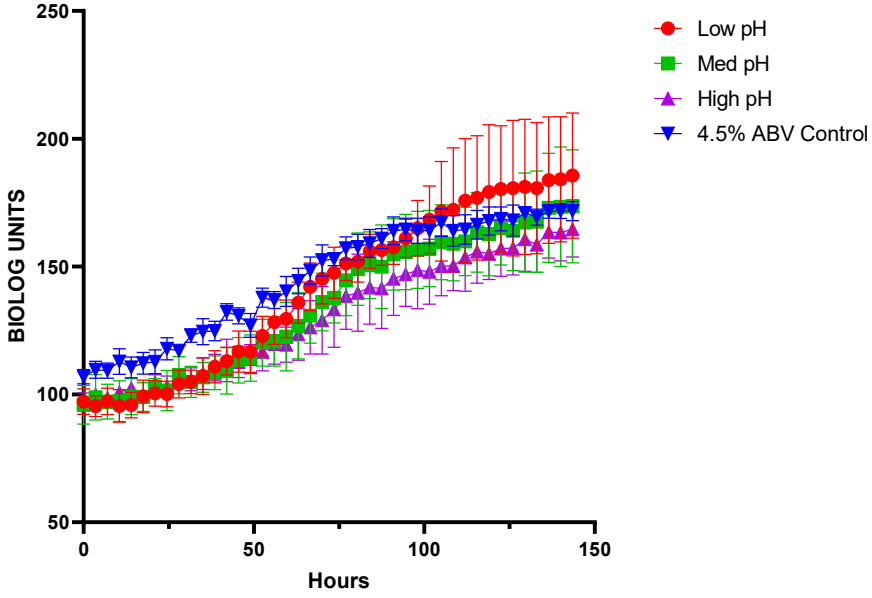

Figure 44: Growth of L. brevis in Brand 1 4.5% and Brand 1 0.5% at Low (3.8), Med (4.15) and High (4.55). Results are means of three replicates, error bars are ± standard deviation

4.2 Omnilog® Spoilage Trials to Assess Preservative Effectiveness in Reducing Brand 1 0.5% Spoilage to a Level Comparable to Brand 1 4.5%

4.2.1 Introduction

As mentioned previously in Sections 1.10.1 and 3.4.3 to be able to serve NABLABs via a regular draught dispense system their potential to be spoilt should be similar to or better than their full alcohol counter parts. The results in section 3.8 showed that 200ppm of sodium benzoate or potassium sorbate were effective at inhibiting *P. membranifaciens* and *L. brevis* in Brand 1 0.5%.

In this section the highest dose of each preservative was compared to Brand 1 0.5% control and Brand 1 4.5% in a similar way to section 3.8. In addition, a higher dose of 50ppm sulphur dioxide was tested as there wasn't a growth inhibiting effect seen at 20ppm in section 3.8. This was to ascertain whether the addition of these preservatives brought the *P. membranifaciens* and *L. brevis* spoilage of Brand 1 0.5% to a comparable level with Brand 1 4.5% when assessed by Omnilog®.

4.2.2 Results

Here Brand 1 0.5% Med pH was dosed with 200ppm sodium benzoate, potassium sorbate or 50ppm sulphur dioxide with Brand 1 4.5% as full alcohol control. These were inoculated with *P. membranifaciens* or *L. brevis* as described in Section 2.20. As shown in Figure 45 the addition of sodium benzoate reduced the degree to which *P. membranifaciens* grew but not to the level of Brand 1 4.5%. Seen here in Figure 46 the addition of potassium sorbate to Brand 1 0.5% greatly reduces the growth of *P. membranifaciens* and to a level lower than Brand 1 4.5%. In Figure 47 there were no clear differences between any of the variables tested, indicating that in this instance even though sodium benzoate didn't reduce the growth of *L. brevis* this was not needed to match the spoilage of Brand 1 4.5%. As seen in Figure 48 there was a slight indication that potassium sorbate may be inhibiting the growth of *L. brevis,* but this is not clear. The growth however, was below that of Brand 1 4.5%. Despite the increase in dose from previous trials, sulphur dioxide was not seen to be effective at inhibiting *P. membranifaciens* or *L. brevis* as shown in Figure 49 and (Figure 50).

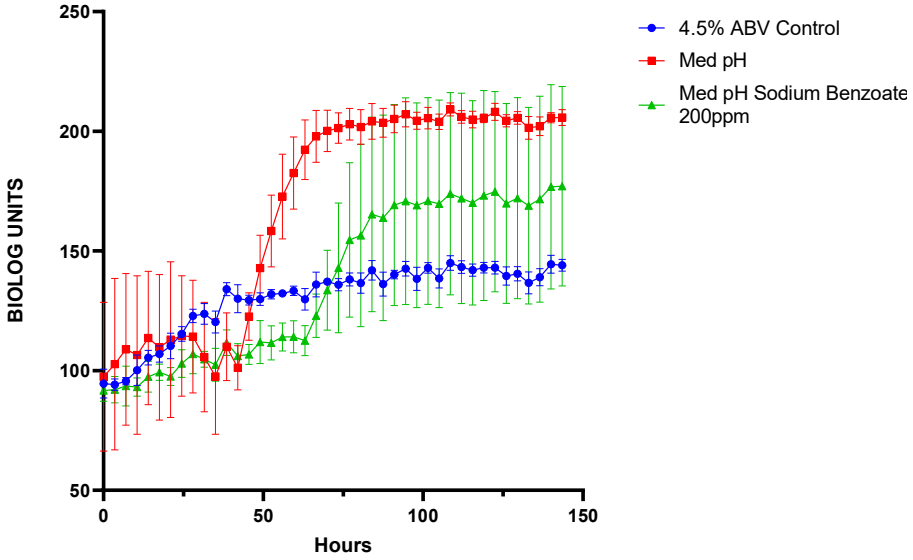

Figure 45: Growth of P. membranifaciens in Brand 1 4.5% and Brand 1 0.5% Med pH with and without 200ppm sodium benzoate. Results are means of three replicates, error bars are ± standard deviation

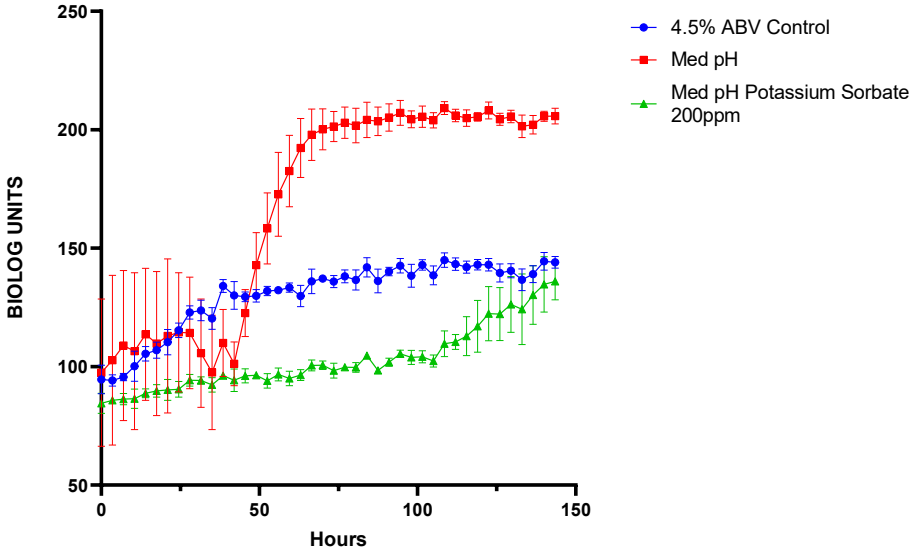

Figure 46: Growth of P. membranifaciens in Brand 1 4.5% and Brand 1 0.5% Med pH with and without 200ppm potassium sorbate. Results are means of three replicates, error bars are ± standard deviation

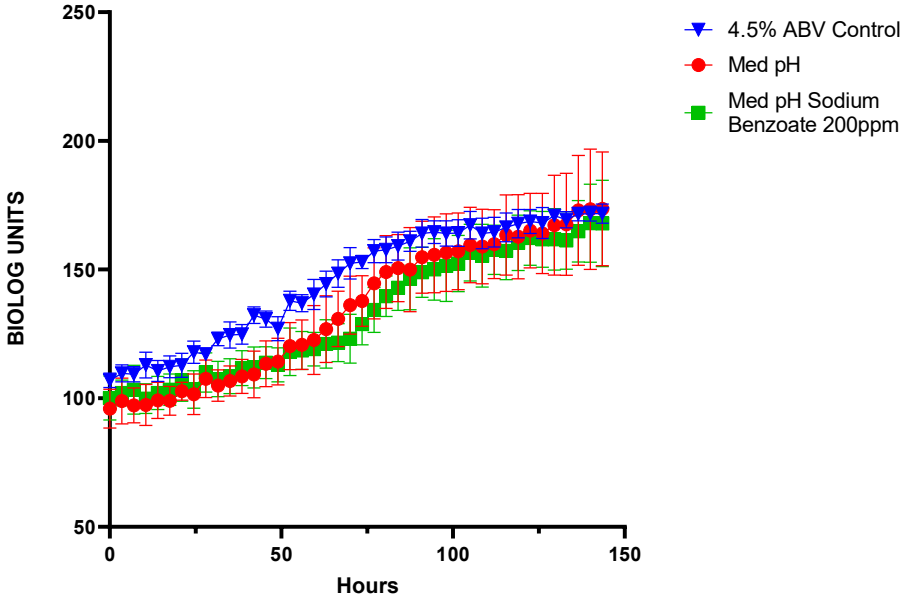

Figure 47: Growth of L. brevis in Brand 1 4.5% and Brand 1 0.5% Med pH with and without 200ppm sodium benzoate. Results are means of three replicates, error bars are ± standard deviation

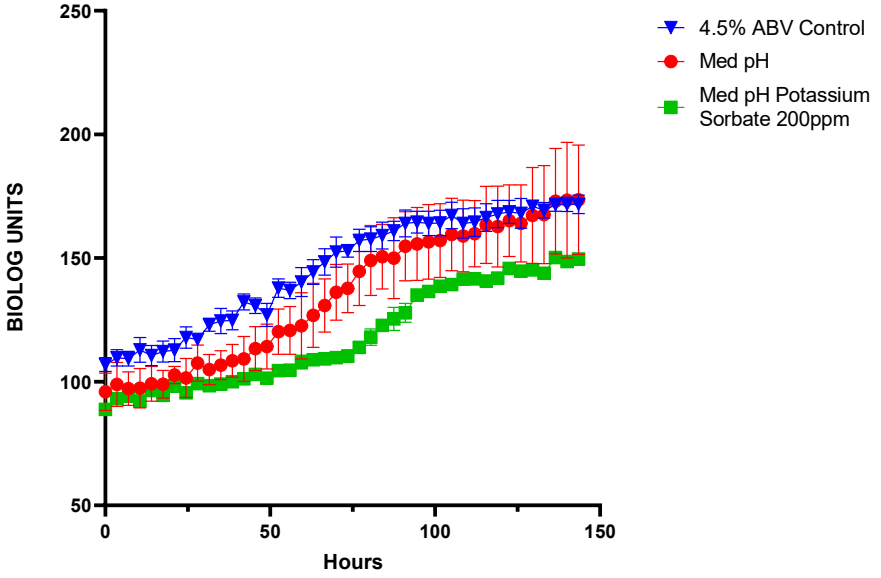

Figure 48: Growth of L. brevis in Brand 1 4.5% and Brand 1 0.5% Med pH with and without 200ppm potassium sorbate. Results are means of three replicates, error bars are ± standard deviation

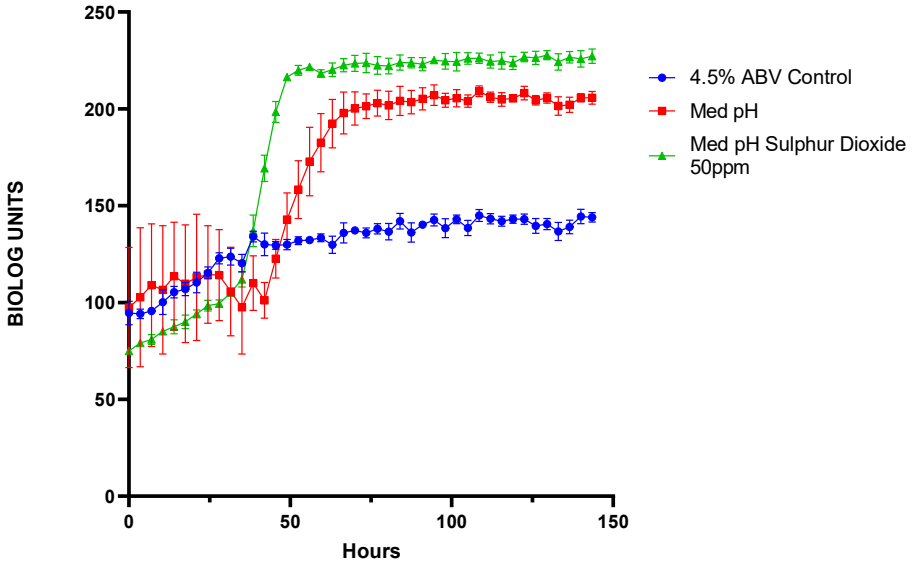

Figure 49: Growth of P. membranifaciens in Brand 1 4.5% and Brand 1 0.5% Med pH with and without 50ppm sulphur dioxide. Results are means of three replicates, error bars are ± standard deviation

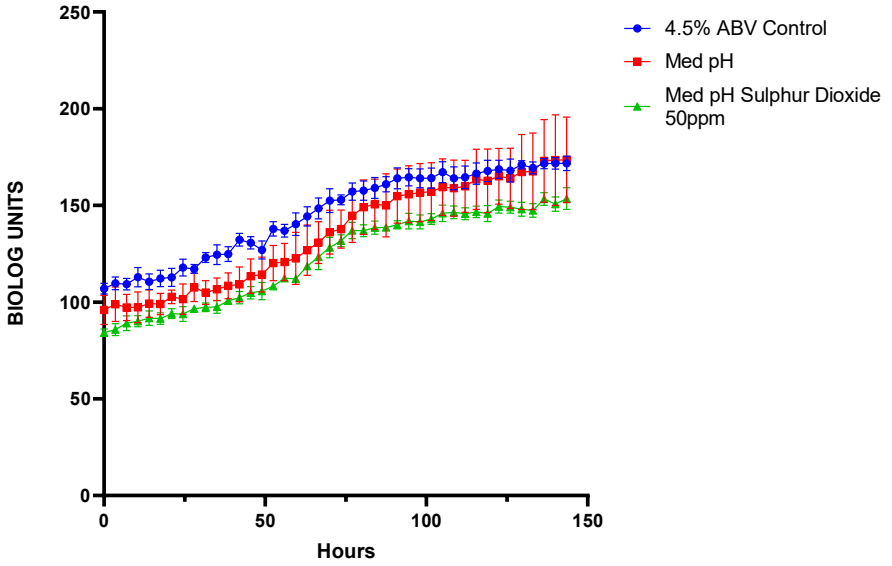

Figure 50: Growth of L. brevis in Brand 1 4.5% and Brand 1 0.5% Med pH with and without 50ppm sulphur dioxide. Results are means of three replicates, error bars are ± standard deviation

4.3 Omnilog® Spoilage Trials to Assess Preservative Dose Response at Three pH Levels

4.3.1 Introduction

Although all the preservatives tested here are some of the most widely used and affordable, they can still add a significant cost to production. Most preservatives also have an assigned acceptable daily intake (ADI) which given higher doses and high consumption could be exceeded (WHO, 2021), so if a lower dose could be used to achieve a similar result that would be advantageous. As previously mentioned in Sections 1.7.1, 1.7.2 and 1.7.3, pH can affect the level of undissociated acid present in solution and so impact the preservatives effectiveness. A lower pH within a reasonable range for beer could be shown to allow a lower dose of preservative to be similarly effective to a higher dose at a higher pH, which could allow pH adjustment to be used for greater stability or lower preservative use. In addition, the presence of sulphites must be labelled on all food and drink products if over 10ppm. If doses under 10ppm were able to achieve a similar effect to higher doses at a higher pH, then sulphites wouldn't have to be declared as an allergen, allowing for a 'clean' label.

In this section three samples of Brand 1 0.5% were used at Low (3.8), Med (4.15) and High (4.55) pH were used in combination with 200ppm, 100ppm and 50ppm potassium sorbate, 200ppm, 100ppm and 50ppm sodium benzoate or 50ppm, 20ppm and 10ppm sulphur dioxide (Section 2.20). *P. membranifaciens* and *L. brevis* were dosed into each variable as described in Section 2.20 and incubated for 6 days at 20°C. Results are trials in triplicate with ± standard deviation, presented as Biolog Units every 3.5 hours.

4.3.2 Results

Here Brand 1 0.5% was dosed with 200ppm, 100ppm, 50ppm or 0ppm sodium benzoate or potassium sorbate or 50ppm, 20ppm, 10ppm or 0ppm sulphur dioxide at Low, Med and High pH levels. The samples were inoculated with *P. membranifaciens* or *L. brevis* as described in Section 2.20.

Shown in Figure 51 as the dose of sodium benzoate increases from 0ppm to 200ppm the level of growth of *P. membranifaciens* decreases and the lag phase increases. Although there may be similar trends for Med pH and High pH samples the errors of the 200ppm sodium benzoate sample for Figure 52 and 200ppm and 50ppm sodium benzoate as well as the control for high pH (Figure 53) are too large to infer any trend. As shown in Figure 54 as the potassium sorbate dose increases from 0ppm to 200ppm the level of growth of *P. membranifaciens* decreases and the lag phase increases. Although 50ppm and 100ppm doses seem to be effective at restricting the growth of *P. membranifaciens* in Brand 1 0.5% the 200ppm dose was by far the most effective. A similar dose response relationship was seen with the Med pH sample (Figure 55). However, in the High pH sample (Figure 56) the 50ppm and 100ppm samples aren't clearly lower than the High pH control. This suggests that the high pH may be affecting the ability of potassium sorbate to inhibit *P. membranifaciens* but more so at these lower doses, as the 200ppm dose was still very effective. The results in Figure 57 and Figure 58 show an indication that 20ppm and 50ppm doses of sulphur dioxide reduced the lag phase of growth for *P. membranifaciens*. This effect was smaller in the High pH sample (Figure 59).

In Figures 60, 61, 62 there were no clear growth differences of *L. brevis* between any of the sodium benzoate concentrations at any pH. In Figures 63, 64, 65 there were no clear differences in growth between the lowest (50ppm potassium sorbate) and highest (200ppm potassium sorbate) doses at any pH. However, at the low pH all doses of

potassium sorbate appeared to reduce growth as compared to the control (Figure 63). As seen in Figures 66, 67, 68 there were no clear growth differences between any of the sulphur dioxide concentrations at any pH.

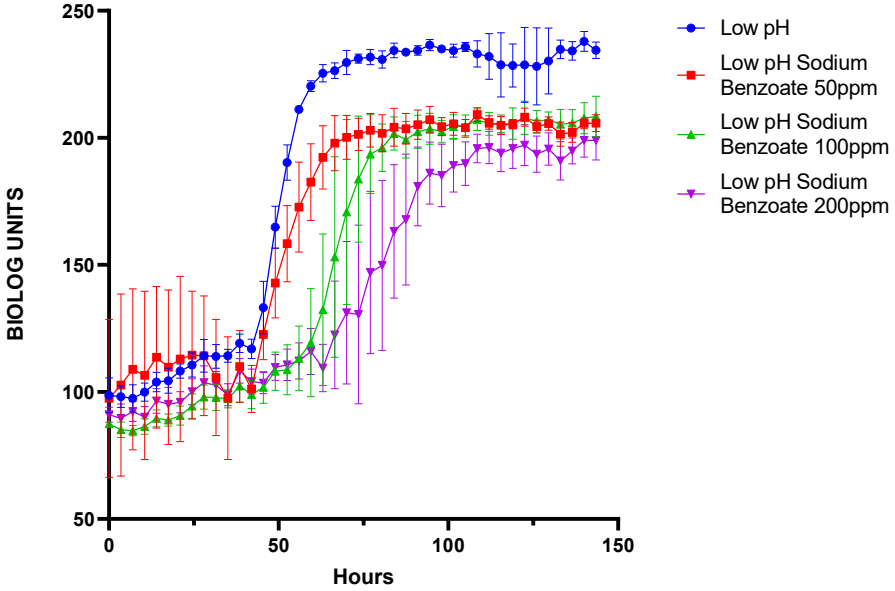

Figure 51: Growth of P. membranifaciens in Brand 1 0.5% Low pH with 50ppm, 100ppm or 200ppm sodium benzoate. Results are means of three replicates, error bars are ± standard deviation

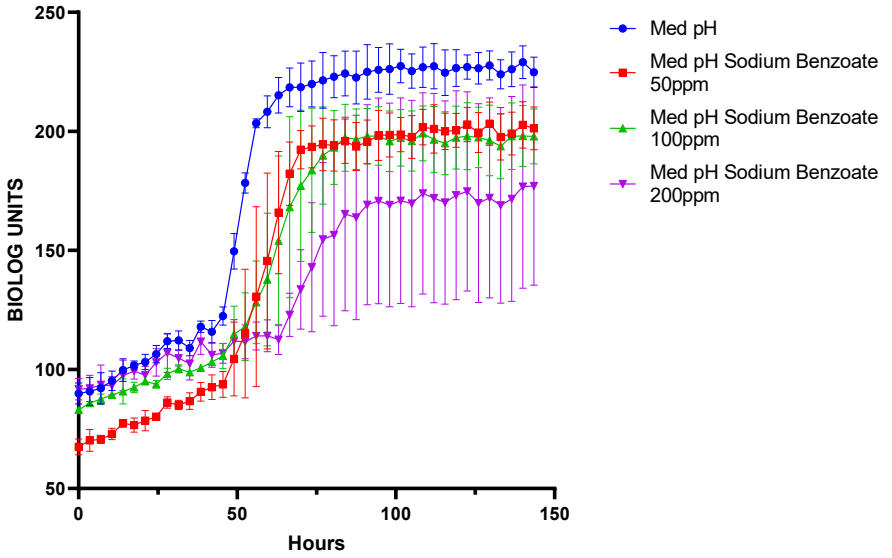

Figure 52: Growth of P. membranifaciens in Brand 1 0.5% Med pH with 50ppm, 100ppm or 200ppm sodium benzoate. Results are means of three replicates, error bars are ± standard deviation

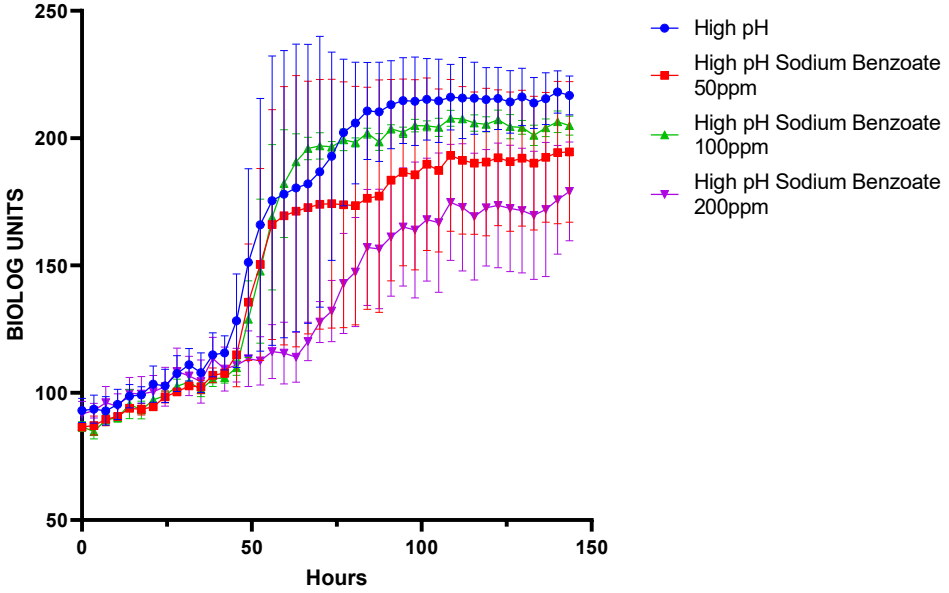

Figure 53: Growth of P. membranifaciens in Brand 1 0.5% High pH with 50ppm, 100ppm or 200ppm sodium benzoate. Results are means of three replicates, error bars are ± standard deviation

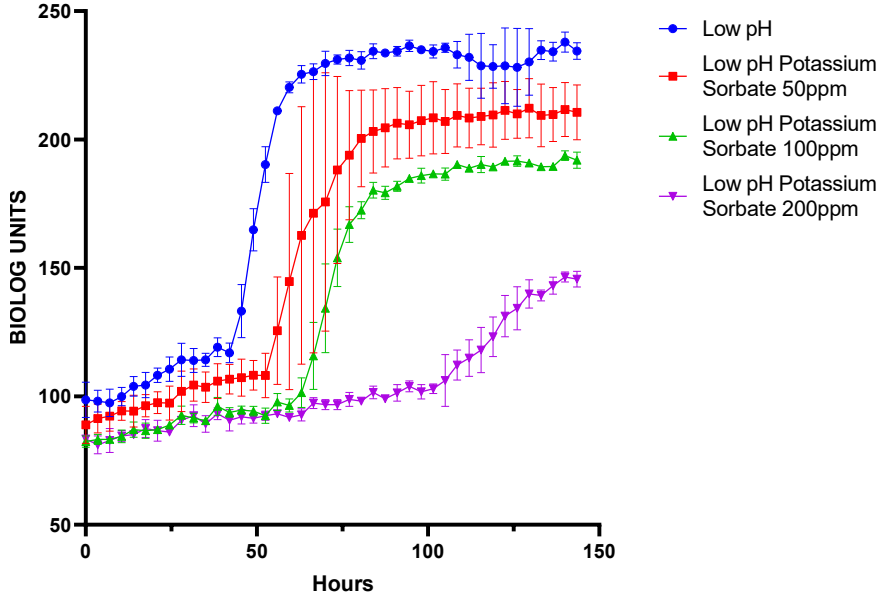

Figure 54: Growth of P. membranifaciens in Brand 1 0.5% Low pH with 50ppm, 100ppm or 200ppm potassium sorbate. Results are means of three replicates, error bars are ± standard deviation

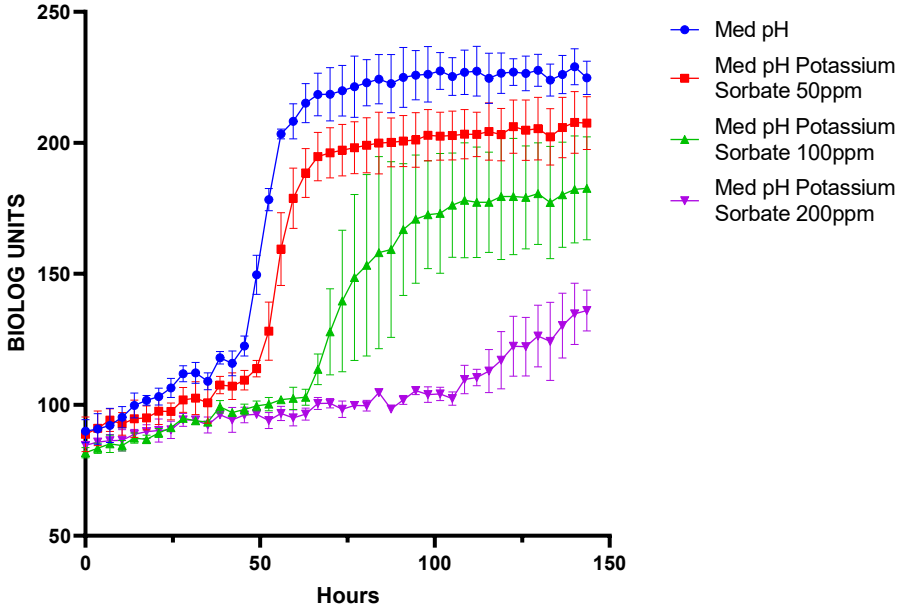

Figure 55: Growth of P. membranifaciens in Brand 1 0.5% Med pH with 50ppm, 100ppm or 200ppm potassium sorbate. Results are means of three replicates, error bars are ± standard deviation

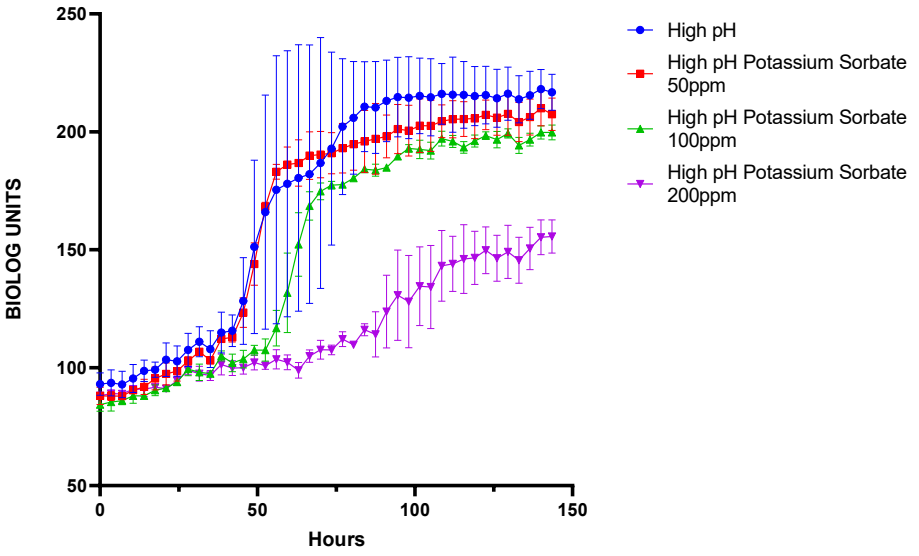

Figure 56: Growth of P. membranifaciens in Brand 1 0.5% High pH with 50ppm, 100ppm or 200ppm potassium sorbate. Results are means of three replicates, error bars are ± standard deviation

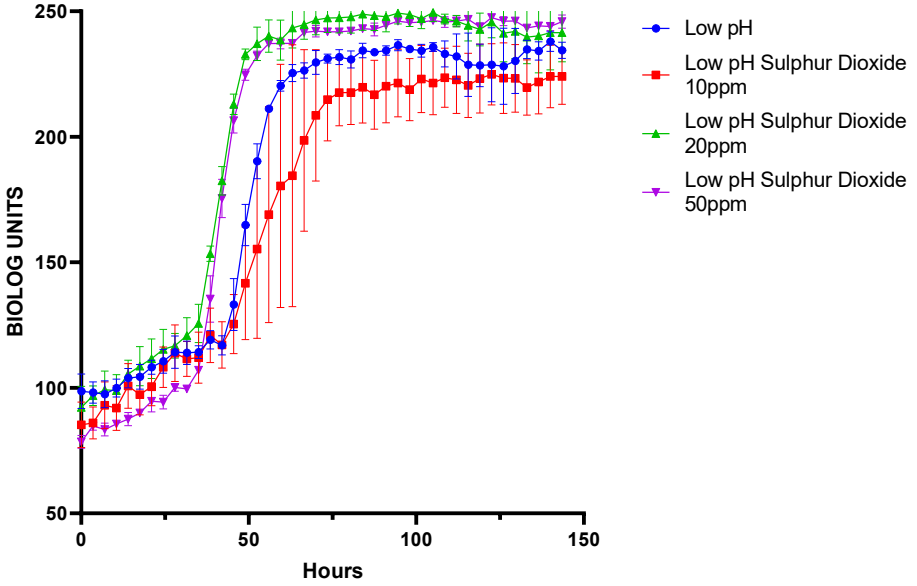

Figure 57: Growth of P. membranifaciens in Brand 1 0.5% Low pH with 10ppm, 20ppm or 50ppm sulphur dioxide. Results are means of three replicates, error bars are ± standard deviation

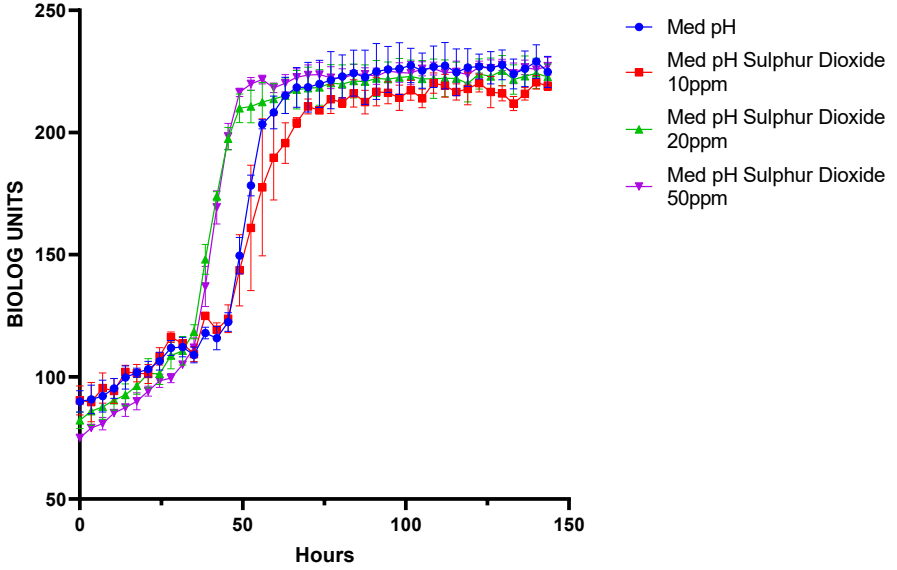

Figure 58: Growth of P. membranifaciens in Brand 1 0.5% Med pH with 10ppm, 20ppm or 50ppm sulphur dioxide. Results are means of three replicates, error bars are ± standard deviation

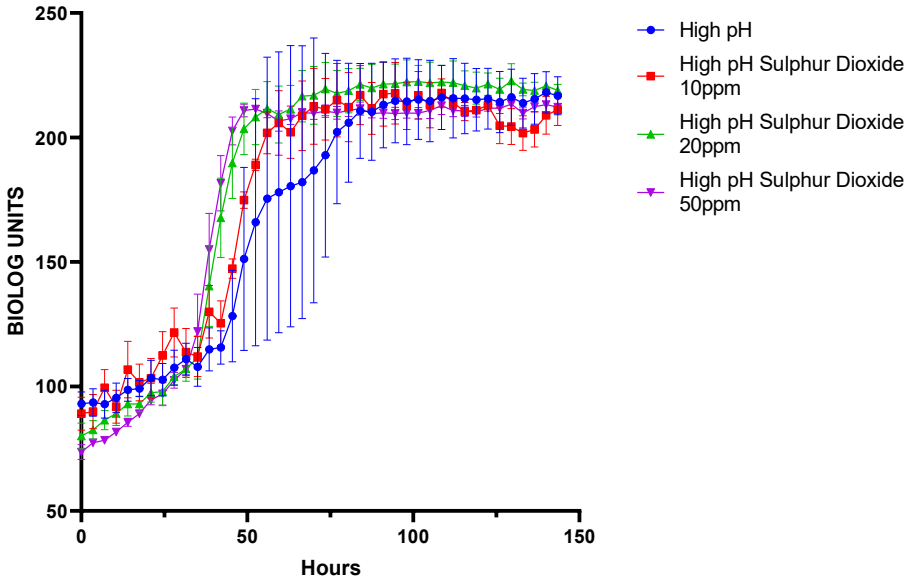

Figure 59: Growth of P. membranifaciens in Brand 1 0.5% High pH with 10ppm, 20ppm or 50ppm sulphur dioxide. Results are means of three replicates, error bars are ± standard deviation

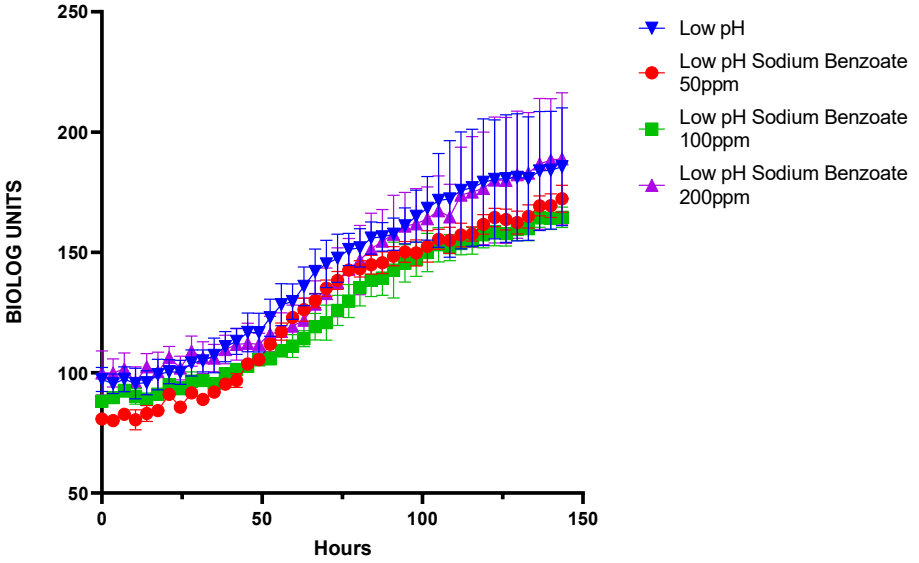

Figure 60: Growth of L. brevis in Brand 1 0.5% Low pH with 50ppm, 100ppm or 200ppm sodium benzoate. Results are means of three replicates, error bars are ± standard deviation

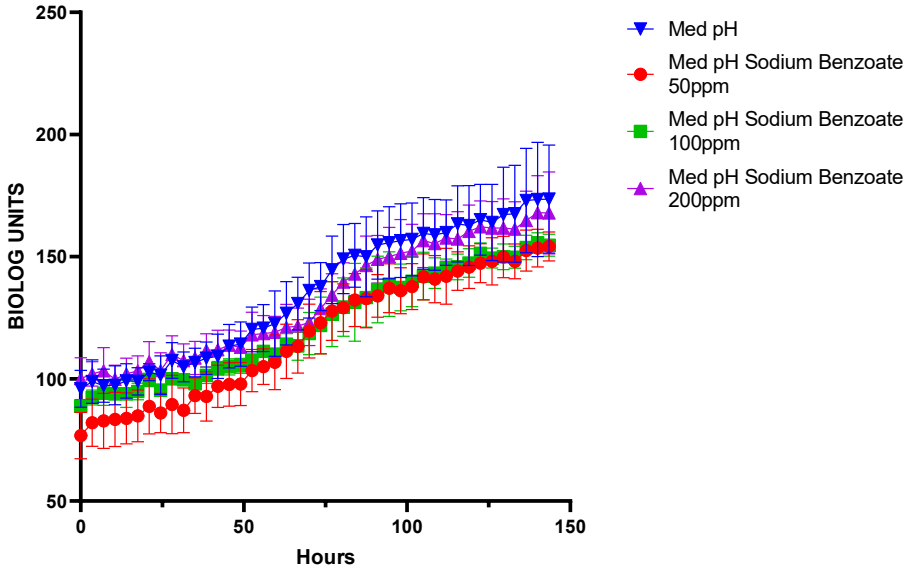

Figure 61: Growth of L. brevis in Brand 1 0.5% Med pH with 50ppm, 100ppm or 200ppm sodium benzoate. Results are means of three replicates, error bars are ± standard deviation

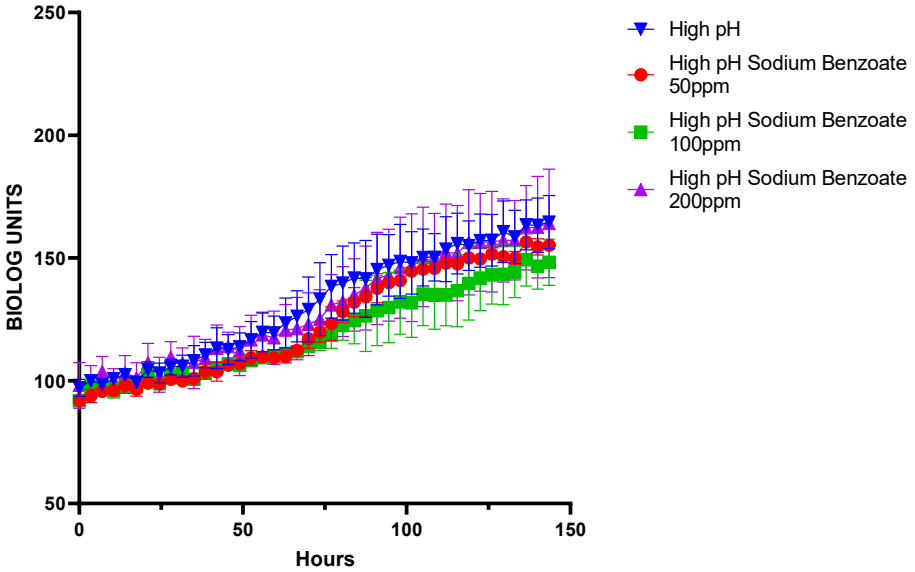

Figure 62: Growth of L. brevis in Brand 1 0.5% High pH with 50ppm, 100ppm or 200ppm sodium benzoate. Results are means of three replicates, error bars are ± standard deviation

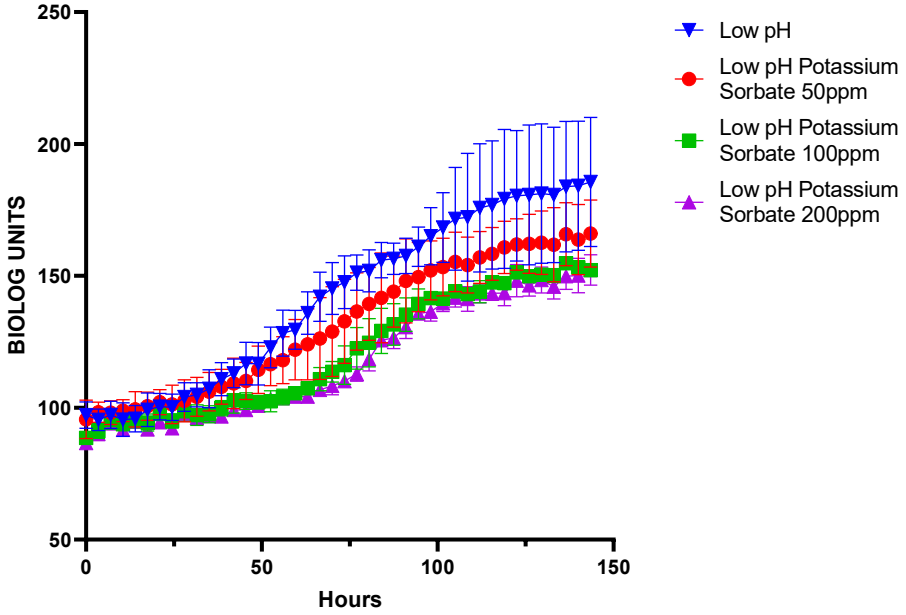

Figure 63: Growth of L. brevis in Brand 1 0.5% Low pH with 50ppm, 100ppm or 200ppm potassium sorbate. Results are means of three replicates, error bars are ± standard deviation

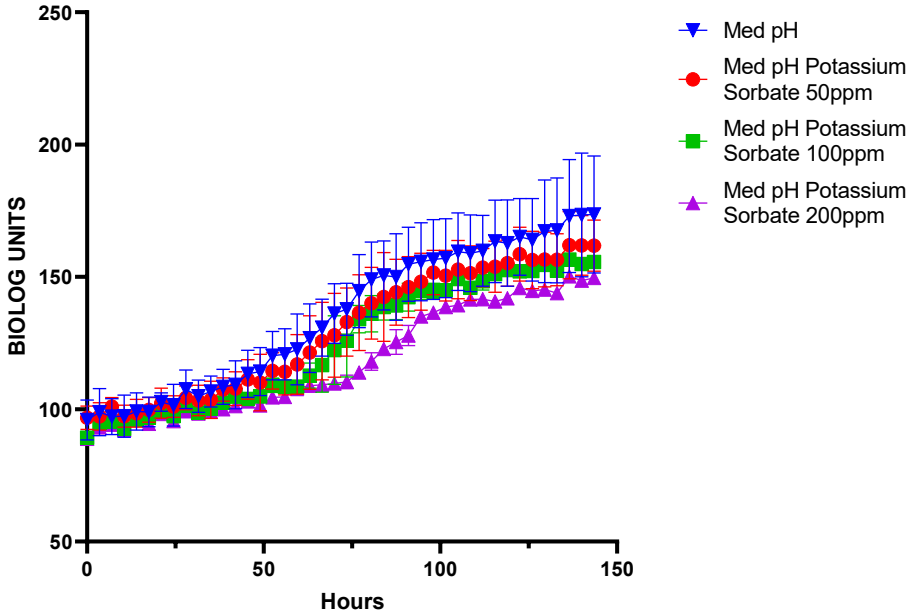

Figure 64: Growth of L. brevis in Brand 1 0.5% Med pH with 50ppm, 100ppm or 200ppm potassium sorbate. Results are means of three replicates, error bars are ± standard deviation

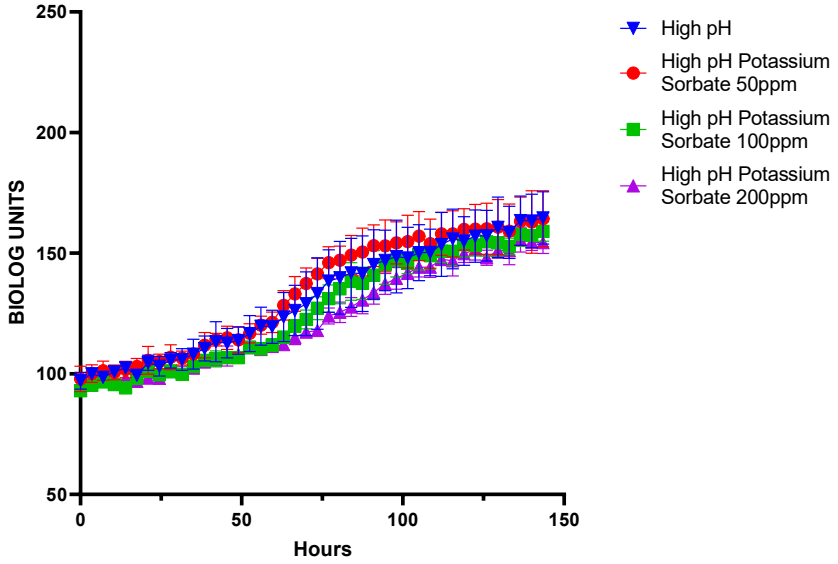

Figure 65: Growth of L. brevis in Brand 1 0.5% High pH with 50ppm, 100ppm or 200ppm potassium sorbate. Results are means of three replicates, error bars are ± standard deviation

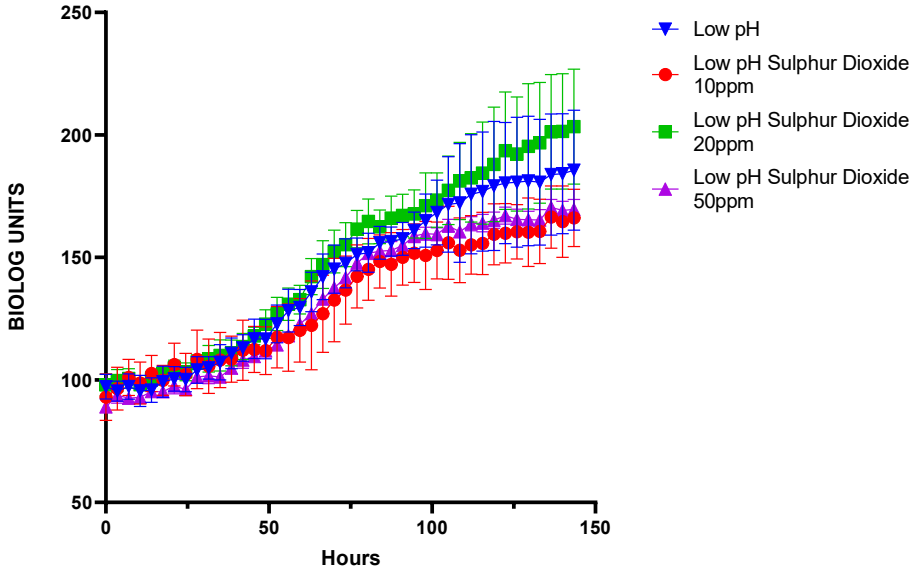

Figure 66: Growth of L. brevis in Brand 1 0.5% Low pH with 10ppm, 20ppm or 50ppm sulphur dioxide. Results are means of three replicates, error bars are ± standard deviation

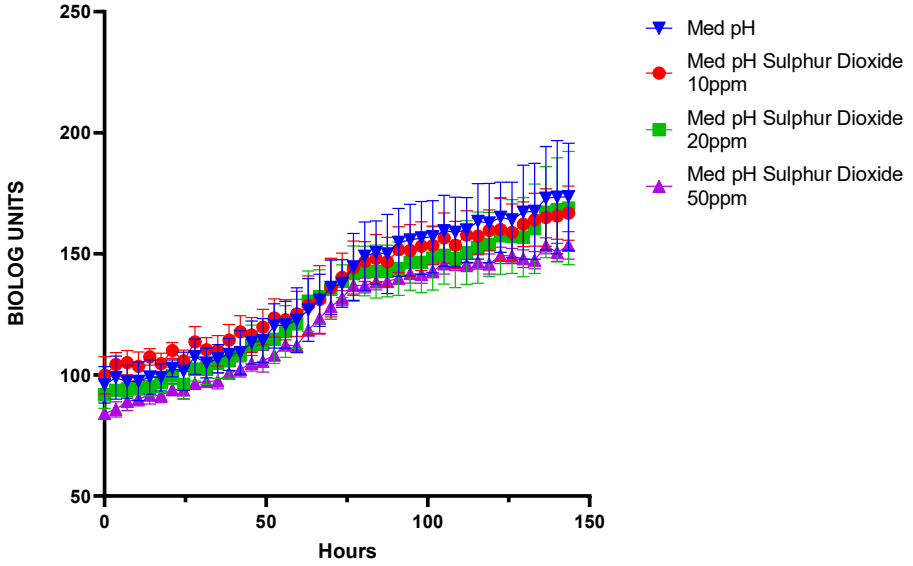

Figure 67: Growth of L. brevis in Brand 1 0.5% Med pH with 10ppm, 20ppm or 50ppm sulphur dioxide. Results are means of three replicates, error bars are ± standard deviation

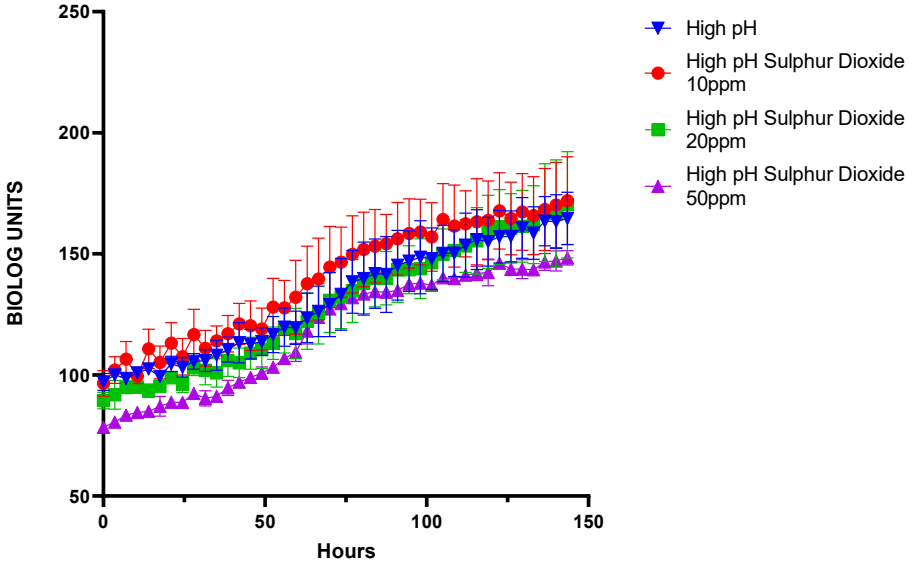

Figure 68: Growth of L. brevis in Brand 1 0.5% High pH with 10ppm, 20ppm or 50ppm sulphur dioxide. Results are means of three replicates, error bars are ± standard deviation

4.4 Omnilog® Spoilage Trials to Assess Preservative Combinations at Three pH Levels

4.4.1 Introduction

Preservatives are often not used alone, combinations can be more effective (Ceylan *et al.,* 2004; Stanojevic *et al.,* 2009). If one preservative is able to inhibit the growth of yeast and moulds, but has little effect against bacteria, it could be advantageous to add another preservative that is known to inhibit bacteria effectively. In addition, due to the differing mechanisms of antimicrobial action a combination can sometimes yield synergistic effects where the combination of the two produce greater antimicrobial action than the sum of their parts (Ceylan *et al.,* 2004).

In this section Brand 1 0.5% was used at Low (3.8), Med (4.15) and High (4.55) pH with preservative combinations:

- 50ppm sodium benzoate and 50ppm potassium sorbate (Lower dose combination to assess for synergism compared to single 200ppm dose)
- 100ppm sodium benzoate and 100ppm potassium sorbate (Lower dose combination to assess for synergism compared to single 200ppm dose)
- 200ppm sodium benzoate and 20ppm sulphur dioxide (highest currently permitted dose of both preservatives in NABLAB keg)
- 200ppm potassium sorbate and 20ppm sulphur dioxide (Potassium sorbate dose comparison to currently highest permitted preservative dose in NABLAB keg)

These were compared to the Low, Med and High pH controls as well as the 200ppm sodium benzoate and 200ppm potassium sorbate samples. *P. membranifaciens* and *L. brevis* were dosed into each variable as described in Section 2.20 and incubated for 6 days at 20°C. Results are trials in triplicate with ± standard deviation, presented as Biolog Units every 3.5 hours.

4.4.2 Results

Shown in Figures 69 and 70 both combinations showed less growth inhibition than the individual 200ppm doses of sodium benzoate or potassium sorbate with 200ppm potassium sorbate being by far the most effective at inhibiting *P. membranifaciens.* This suggests that there are not synergistic effects between sodium benzoate and potassium sorbate. Sodium benzoate 200ppm Med pH results were omitted from (Figure 70) due to excessive standard deviation, for the same reason the 50ppm sodium benzoate 50ppm potassium sorbate combination and 200ppm sodium benzoate results with High pH Brand 1 0.5% were also omitted.

The results seen in Figures 72, 73, 74 show no clear differences in growth of *L. brevis* between any of the preservative concentrations at any pH. This suggests that the pH of the solution was not affecting the antimicrobial activity of sodium benzoate or potassium sorbate at the doses used.

Shown in Figure 75 the addition of 20ppm sulphur dioxide with 200ppm sodium benzoate had greater growth from *P. membranifaciens* than sodium benzoate alone, this effect seemed to be even greater in Figure 76 where there appeared to be no difference between the control and the 200ppm sodium benzoate and 20ppm sulphur dioxide combination. However, the opposite was seen in Figure 77 where the addition of sulphur dioxide seemed to improve outcomes beyond an additive effect, as sulphur dioxide on its own showed little to no growth prevention for *P. membranifaciens.* The results in Figure 76 are likely due to error in dosing as it would be expected for the 200ppm sodium benzoate and 20ppm sulphur dioxide combination to have at least some effect. In addition Figure 77 shows this combination performing in a very similar way to the potassium sorbate and sulphur dioxide combination in Figure 83 also suggesting a dosing error. These trials would have to repeated to confirm the results shown here.

As shown in Figures 78, 79, 80 show no clear differences in growth of *L. brevis* between any of the preservative concentrations at any pH. This suggests that there is synergistic action between sodium benzoate and sulphur dioxide at the concentrations tested.

As shown in Figure 81 both 200ppm potassium sorbate and the 200ppm potassium sorbate and 20ppm sulphur dioxide combination were effective at inhibiting *P. membranifaciens,* with the sulphur dioxide addition possibly negatively affecting potassium sorbates antimicrobial effects. This was also seen in Figure 82 and Figure 83 indicating that 20ppm sulphur dioxide may be aiding in the growth of *P. membranifaciens* in these conditions or interacting with potassium sorbate and reducing its effectiveness.

As shown in Figure 84 200ppm potassium sorbate and the 200ppm potassium sorbate 20ppm sulphur dioxide combination show some inhibition on the growth of *L. brevis* as also seen in Figure 63. However, there was no difference between the two variables. The same can be said for Figure 85, but not for Figure 86 where no differences were seen, suggesting there were no synergistic effects between potassium sorbate and sulphur dioxide at the concentrations tested.

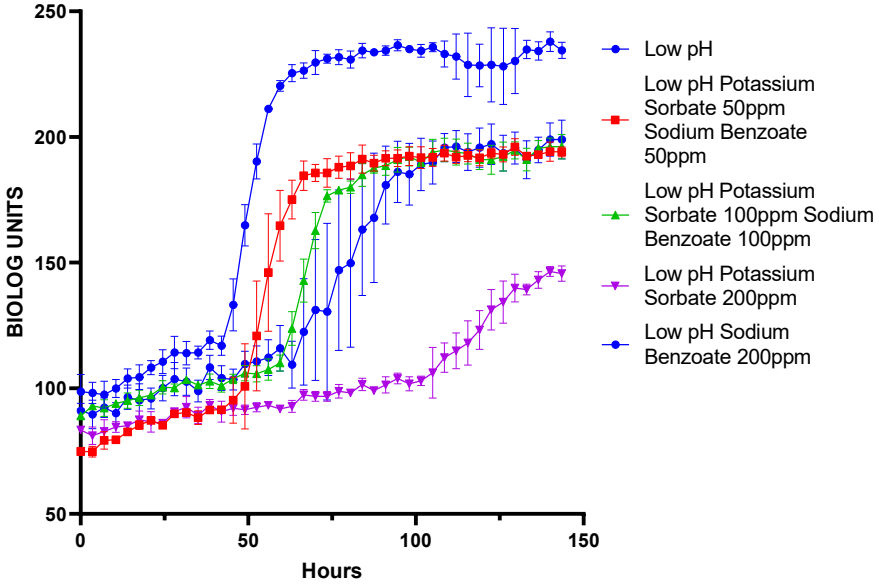

Figure 69: Growth of P. membranifaciens in Brand 1 0.5% Low pH with 50ppm sodium benzoate and potassium sorbate, 100ppm sodium benzoate and potassium sorbate, 200ppm sodium benzoate or 200ppm potassium sorbate. Results are means of three replicates, error bars are ± standard deviation

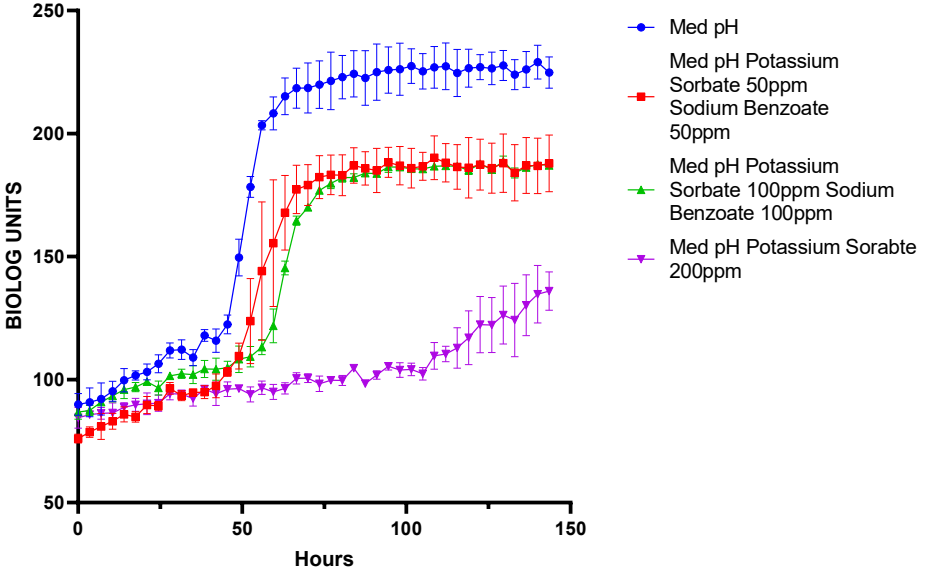

Figure 70: Growth of P. membranifaciens in Brand 1 0.5% Med pH with 50ppm sodium benzoate and potassium sorbate, 100ppm sodium benzoate and potassium sorbate, and 200ppm potassium sorbate. Results are means of three replicates, error bars are ± standard deviation

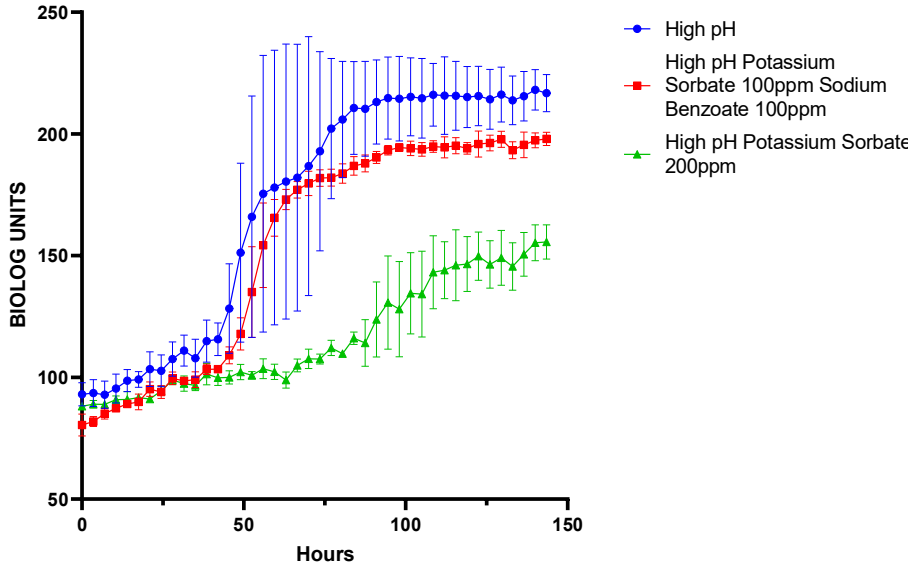

Figure 71: Growth of P. membranifaciens in Brand 1 0.5% High pH with 100ppm sodium benzoate and potassium sorbate, or 200ppm potassium sorbate. Results are means of three replicates, error bars are ± standard deviation

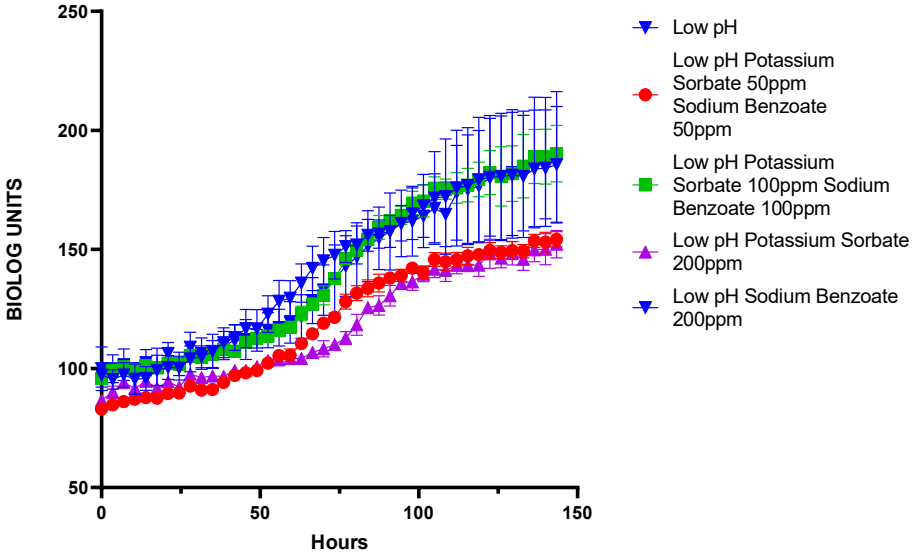

Figure 72: Growth of L. brevis in Brand 1 0.5% Low pH with 50ppm sodium benzoate and potassium sorbate, 100ppm sodium benzoate and potassium sorbate, 200ppm sodium benzoate or 200ppm potassium sorbate. Results are means of three replicates, error bars are ± standard deviation

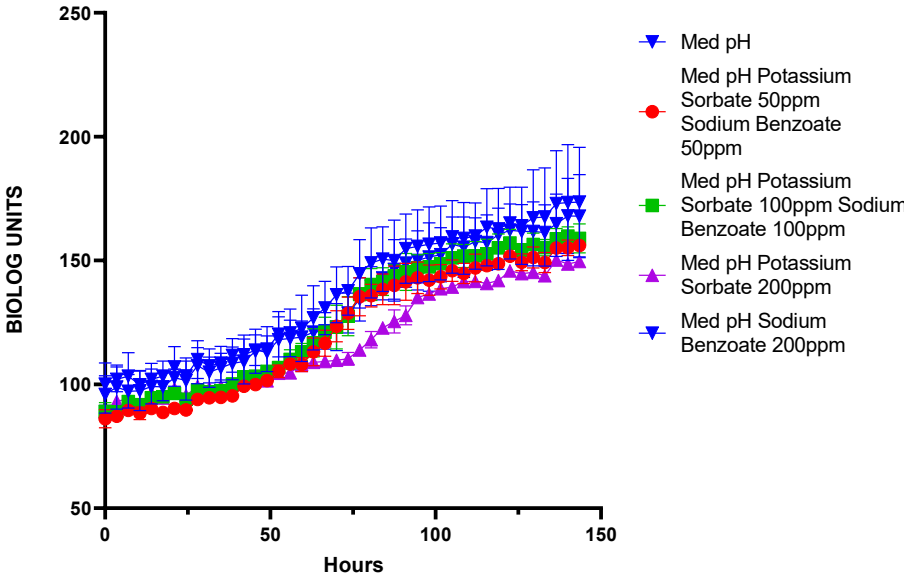

Figure 73: Growth of L. brevis in Brand 1 0.5% Med pH with 50ppm sodium benzoate and potassium sorbate, 100ppm sodium benzoate and potassium sorbate, 200ppm sodium benzoate or 200ppm potassium sorbate. Results are means of three replicates, error bars are ± standard deviation

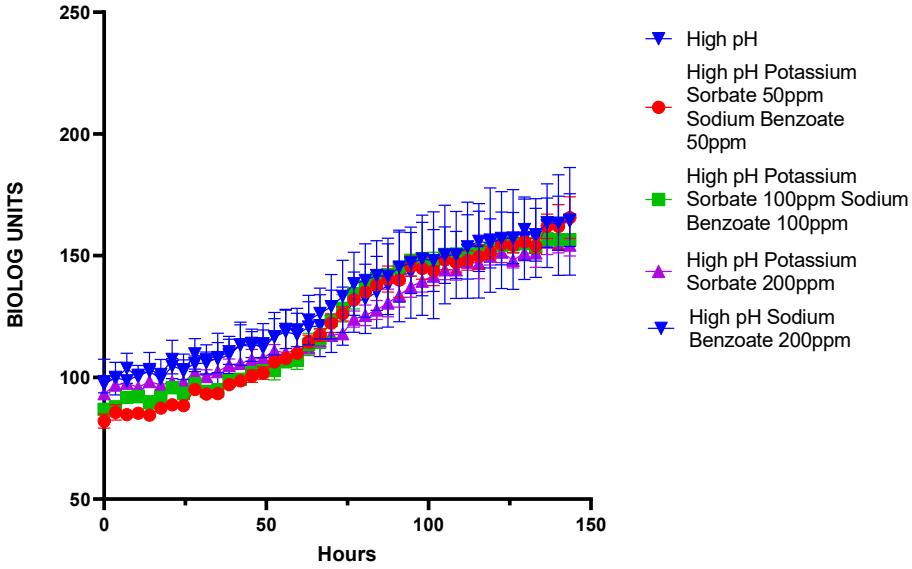

Figure 74: Growth of L. brevis in Brand 1 0.5% High pH with 50ppm sodium benzoate and potassium sorbate, 100ppm sodium benzoate and potassium sorbate, 200ppm sodium benzoate or 200ppm potassium sorbate. Results are means of three replicates, error bars are ± standard deviation

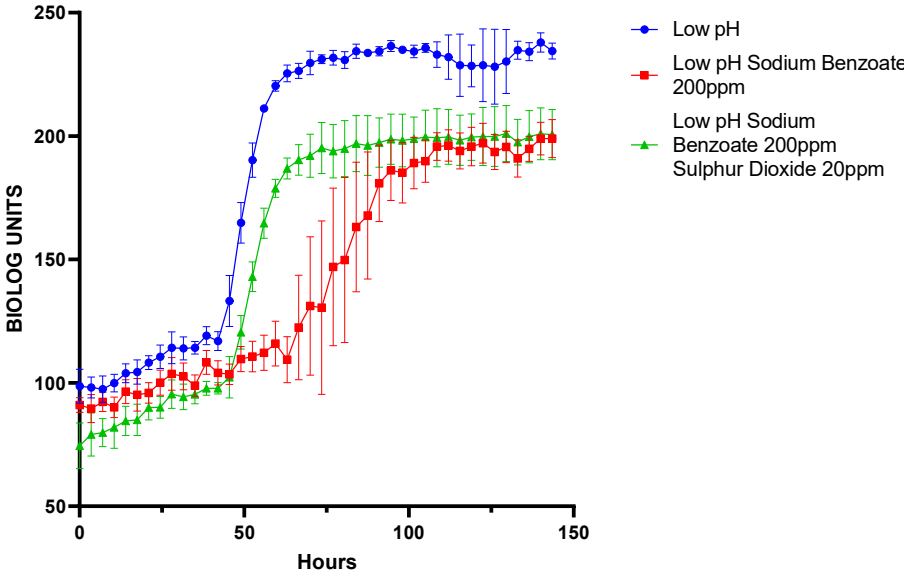

Figure 75: Growth of P. membranifaciens in Brand 1 0.5% Low pH with 200ppm sodium benzoate or 200ppm sodium benzoate and 20ppm sulphur dioxide. Results are means of three replicates, error bars are ± standard deviation

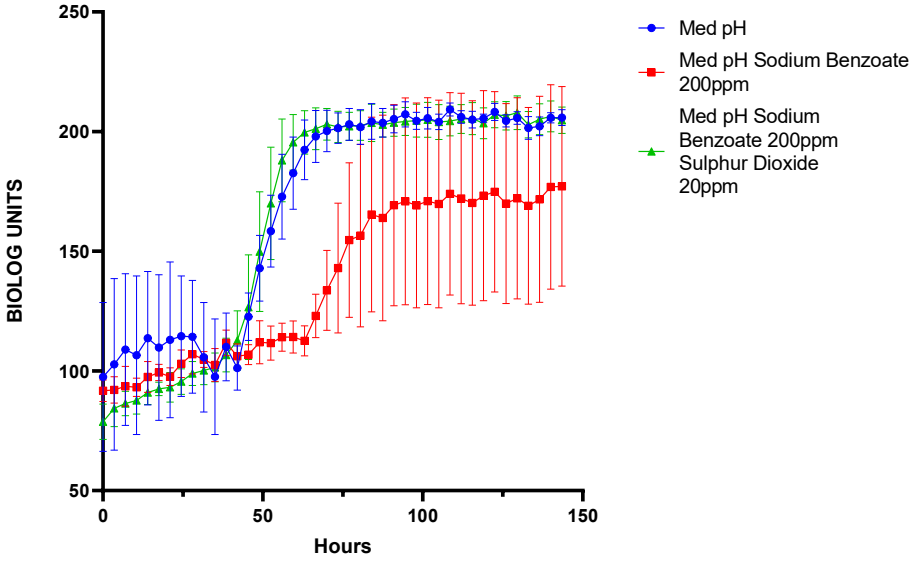

Figure 76: Growth of P. membranifaciens in Brand 1 0.5% Med pH with 200ppm sodium benzoate or 200ppm sodium benzoate and 20ppm sulphur dioxide. Results are means of three replicates, error bars are ± standard deviation

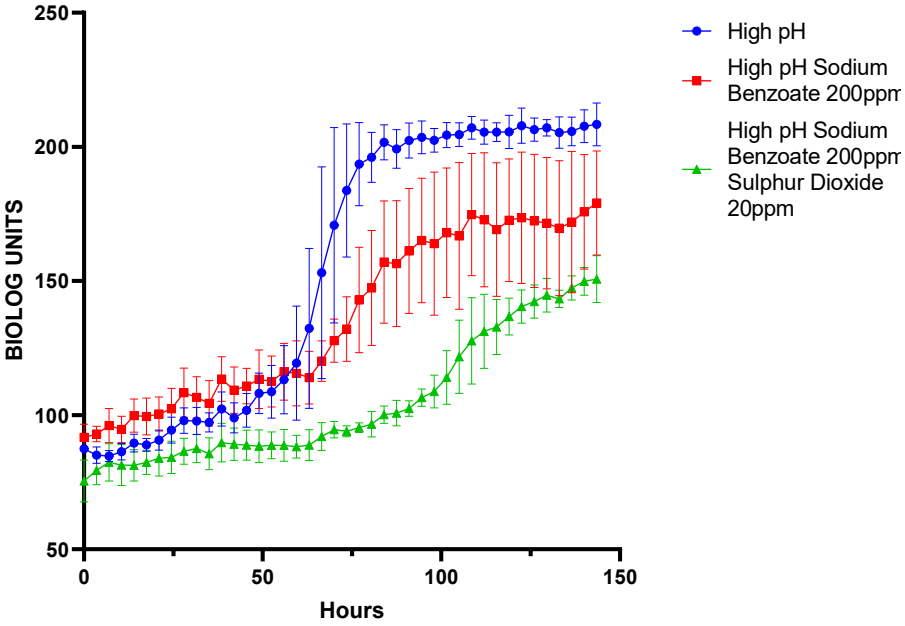

Figure 77: Growth of P. membranifaciens in Brand 1 0.5% High pH with 200ppm sodium benzoate or 200ppm sodium benzoate and 20ppm sulphur dioxide. Results are means of three replicates, error bars are ± standard deviation

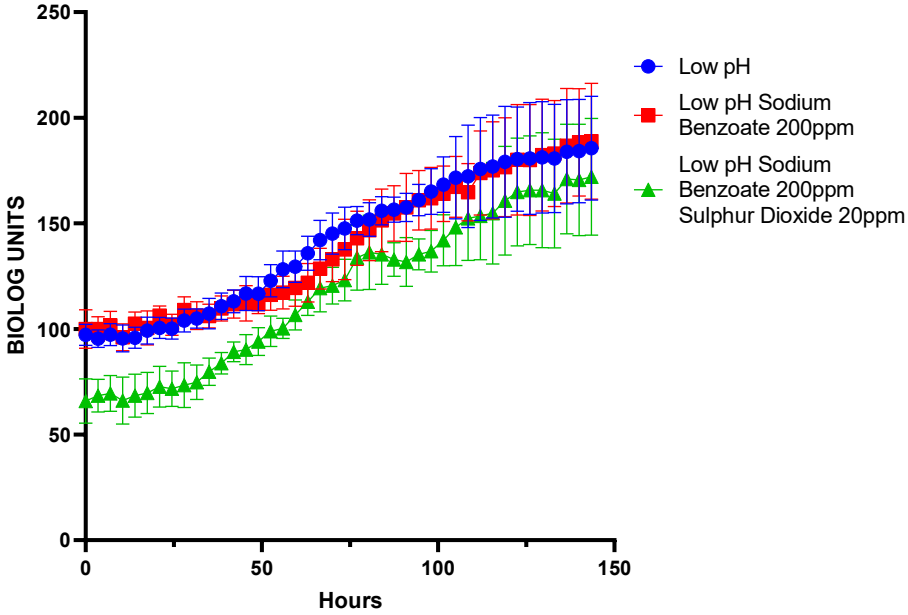

Figure 78: Growth of L. brevis in Brand 1 0.5% Low pH with 200ppm sodium benzoate or 200ppm sodium benzoate and 20ppm sulphur dioxide. Results are means of three replicates, error bars are ± standard deviation

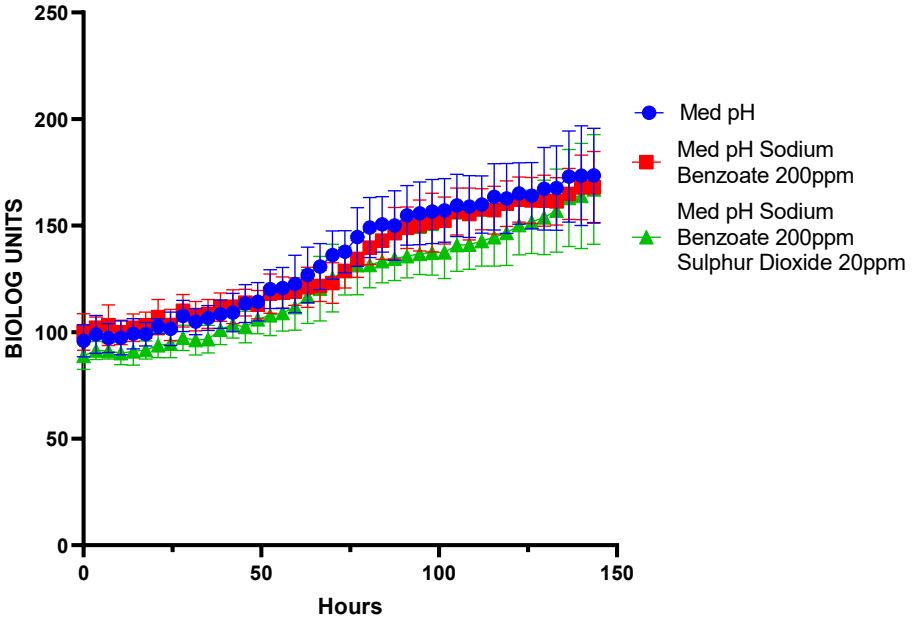

Figure 79: Growth of L. brevis in Brand 1 0.5% Med pH with 200ppm sodium benzoate or 200ppm sodium benzoate and 20ppm sulphur dioxide. Results are means of three replicates, error bars are ± standard deviation

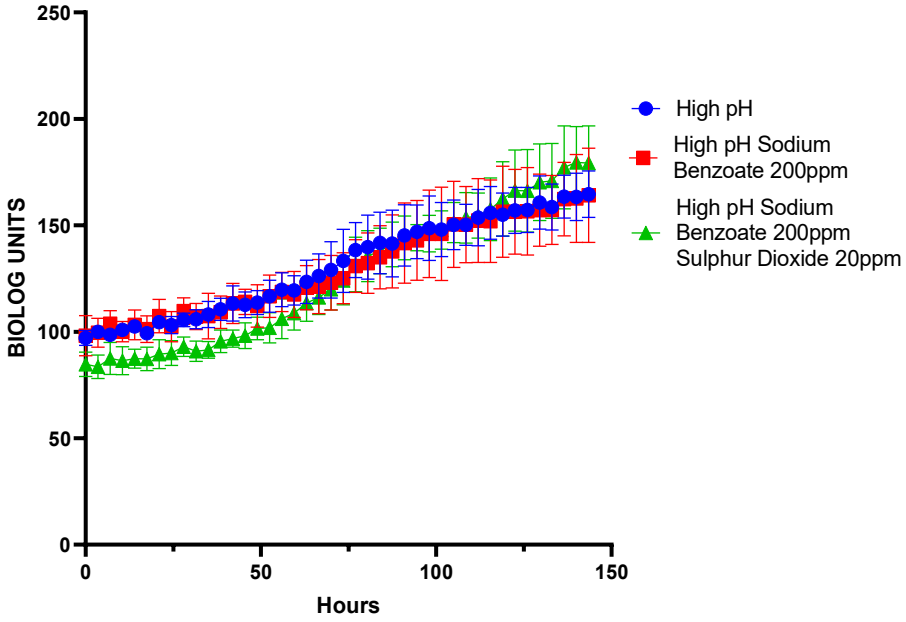

Figure 80: Growth of L. brevis in Brand 1 0.5% High pH with 200ppm sodium benzoate or 200ppm sodium benzoate and 20ppm sulphur dioxide. Results are means of three replicates, error bars are ± standard deviation

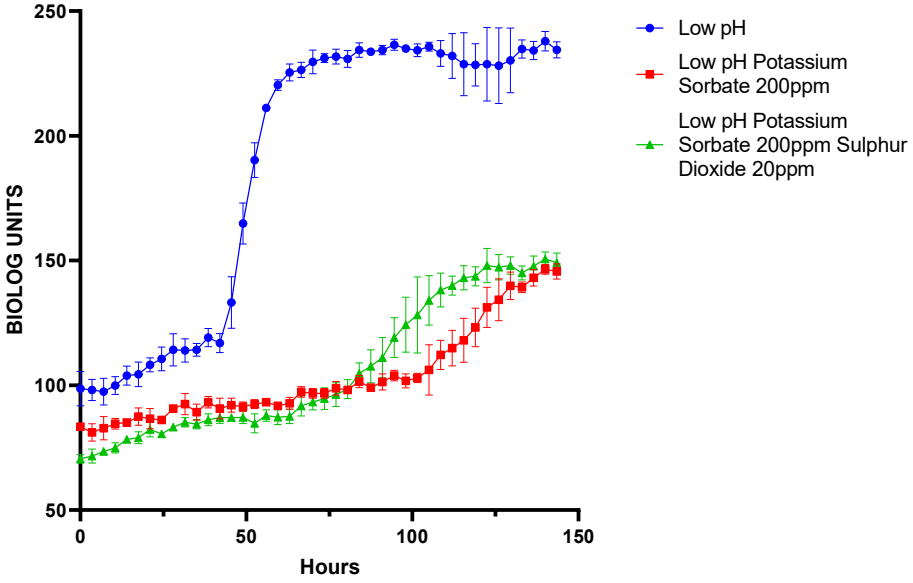

Figure 81: Growth of P. membranifaciens in Brand 1 0.5% Low pH with 200ppm potassium sorbate or 200ppm potassium sorbate and 20ppm sulphur dioxide. Results are means of three replicates, error bars are ± standard deviation

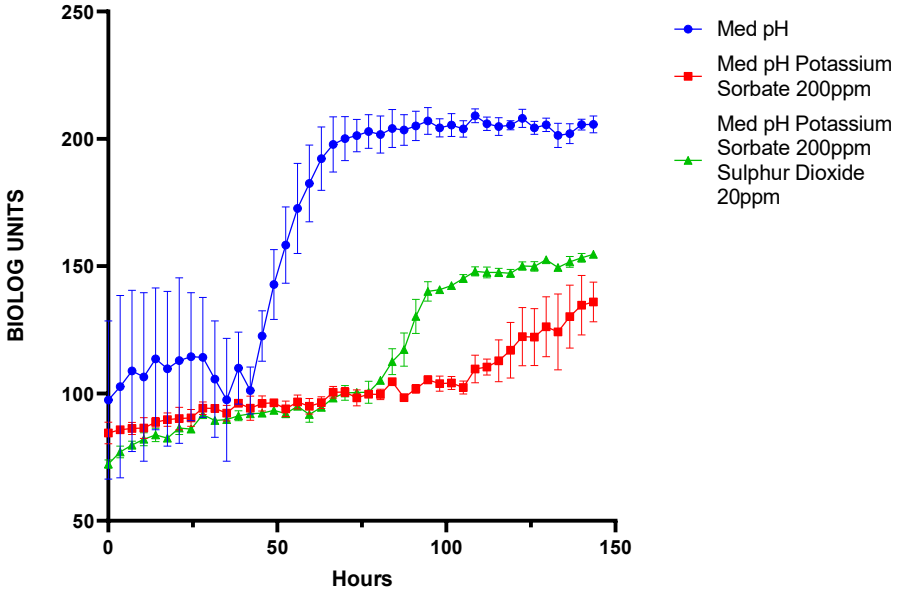

Figure 82: Growth of P. membranifaciens in Brand 1 0.5% Med pH with 200ppm potassium sorbate or 200ppm potassium sorbate and 20ppm sulphur dioxide. Results are means of three replicates, error bars are ± standard deviation

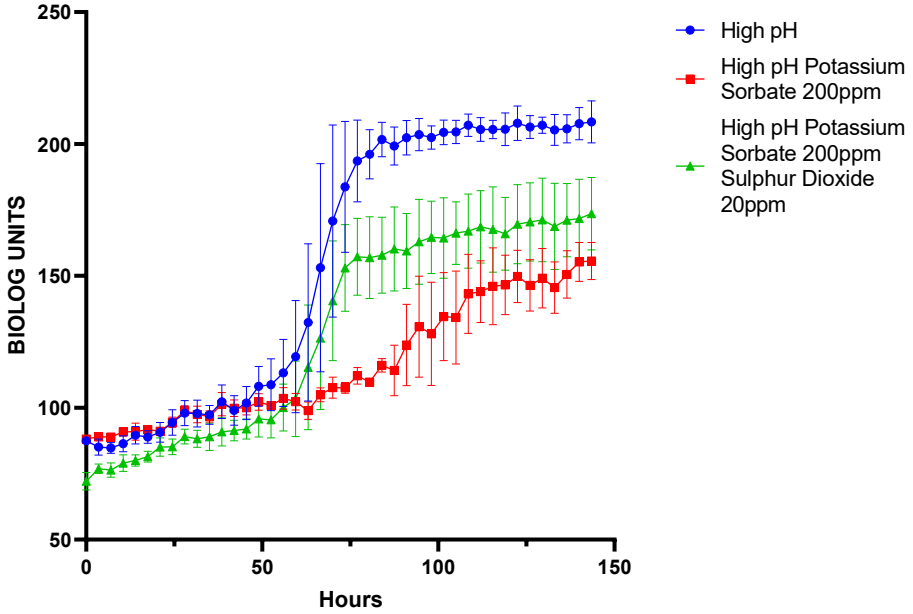

Figure 83: Growth of P. membranifaciens in Brand 1 0.5% High pH with 200ppm potassium sorbate or 200ppm potassium sorbate and 20ppm sulphur dioxide. Results are means of three replicates, error bars are ± standard deviation

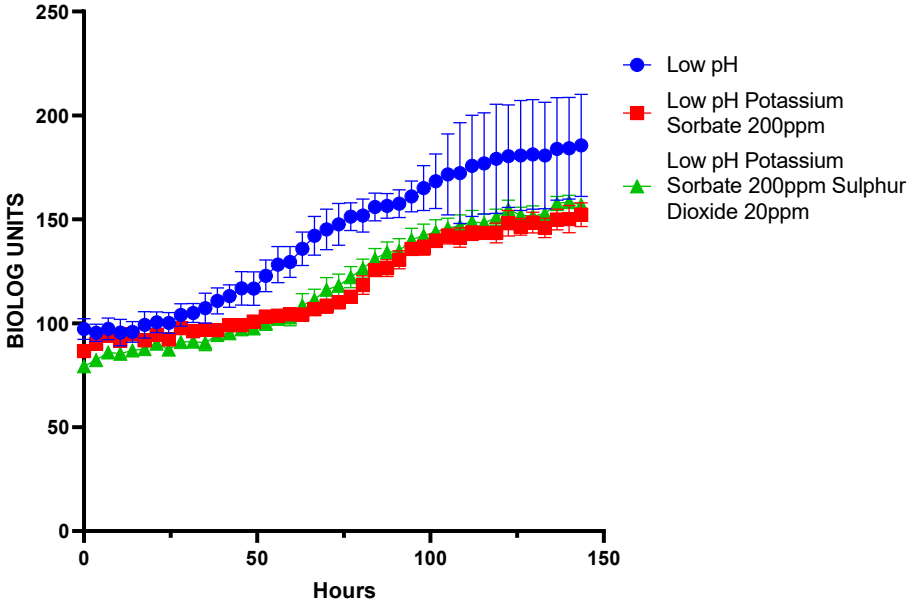

Figure 84: Growth of L. brevis in Brand 1 0.5% Low pH with 200ppm potassium sorbate or 200ppm potassium sorbate and 20ppm sulphur dioxide. Results are means of three replicates, error bars are ± standard deviation

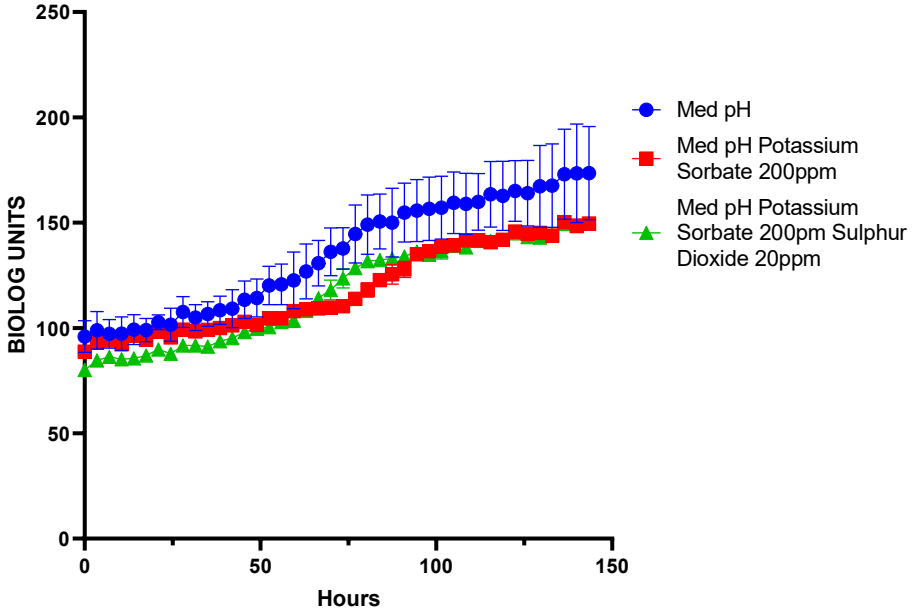

Figure 85: Growth of L. brevis in Brand 1 0.5% Med pH with 200ppm potassium sorbate or 200ppm potassium sorbate and 20ppm sulphur dioxide. Results are means of three replicates, error bars are ± standard deviation

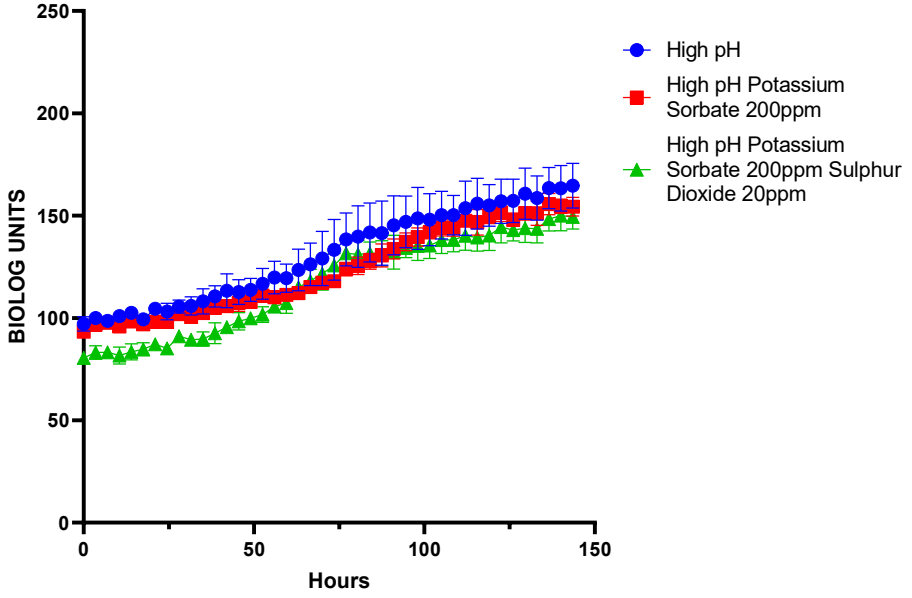

Figure 86: Growth of L. brevis in Brand 1 0.5% High pH with 200ppm potassium sorbate or 200ppm potassium sorbate and 20ppm sulphur dioxide. Results are means of three replicates, error bars are ± standard deviation

4.5 Discussion of Omnilog® Results

Throughout this work it has been noted numerous times that the antimicrobial activity of sodium benzoate, potassium sorbate and sulphur dioxide is dependent on the pH of the solution they are in Section 1.7.1, 1.7.2 and 1.7.3. Additionally, each preservative possesses a different range in which they are most effective. This sections trials on the Omnilog® were designed to ascertain whether the difference in the amount of undissociated acid, within reasonable upper and lower bounds for the pH of a beer, would translate to a measurable change in the preservative's antimicrobial effects against *P. membranifaciens* and *L. brevis*. Additionally, it was designed to assess different doses and combinations of preservatives for possible synergistic effects.

A clear effect of each preservative on growth of *P. membranifaciens* or *L. brevis* was not seen between the different pH values. There are a number of reasons why this could be. The dose used could have not been high enough to see any significant changes, as in Section 4.3 (Figure 54) the 50ppm and 100ppm doses of potassium sorbate were much less effective at inhibiting the growth of *P. membranifaciens* than the 200ppm dose. The undissociated portion of the preservative is the anti-microbially effective dose. For example, a 200ppm dose of potassium sorbate at a pH of 4.40 would have 140ppm undissociated acid, at a pH of 4.0 it would have 172ppm of undissociated acid, a 32ppm increase. At 100ppm this would be halved to just a 16ppm difference between 4.0 and 4.4 pH (Sofos and Busta, 1981). Sodium benzoate has a steeper dissociation curve (Figure 8) so the increase in undissociated acid with a similar drop in pH should be greater, leading to a stronger anti-microbial activity. However, this was not observed. This could be due to the microorganisms tested showing resistance to a certain level of the effective undissociated acid dose present, especially as some *Lactobacillus* have shown the ability to metabolise and resist potassium sorbate (Catherine *et al.,* 2014). If the microorganism can withstand 100ppm of undissociated acid, no matter how low the pH is, a 100ppm of the salt form will

not be enough of a dose to have the desired inhibitory effect. Whereas in this example a 200ppm dose would just have to be greater than 50% undissociated acid to start to become effective, this would have the benefit of also being within a reasonable pH for beer. The dissociation constant is not only affected by pH, but it can also be affected by temperature and possibly other compounds that are found in beers (Liewen and Marth, 1985). This suggests that the dissociation of these preservatives may need to be measured directly in beer, or the testing dose increased to further understand how pH and other parameters may affect these preservatives effectiveness in beer.

Three dose levels for sodium benzoate, potassium sorbate and sulphur dioxide were also tested to determine the dose response relationship. Although the addition of preservatives to beer has a low cost there is little sense in using more than required. This is especially true for sulphur dioxide, as it must be declared at over 10ppm as an allergen. If the same antimicrobial effect could be exhibited at below 10ppm then it could be used as such and so would no longer have to be labelled as an allergen, possibly increasing the number of customers who can consume it and leaving it with a 'clean' label. There was a clear dose response relationship for potassium sorbate and sodium benzoate, with 200ppm of potassium sorbate being much more effective than 50ppm or 100ppm (Section 4.3.2). Sodium benzoate showed an incremental improvement as the dose increased. Sulphur dioxide showed no clear differences between the 10ppm, 20ppm and 50ppm doses. The doses for sulphur dioxide were chosen based on 10ppm being the allergen declaration limit, 20ppm being legal limit for addition into NABLAB keg beer and 50ppm being the legal limit permitted in cask beer (EU Regulation 1129, 2011). None of these doses had any noticeable effect on the growth of *P. membranifaciens* or *L. brevis.*

Combinations of the three preservatives were also tested to assess for any synergistic effects between the preservatives, and if there were, whether a lower dose combination

could be a viable alternative to a single higher dose preservative. Synergistic effects could take place due to the preservatives utilising different mechanisms of action in inhibiting microorganisms (Section 1.7.1; 1.7.2; 1.7.3). The spoilage trials in Section 3.3.4 showed that potassium sorbate was more effective at reducing the growth of *P. membranifaciens* whereas sodium benzoate was more effective at inhibiting *L. brevis.* This suggests that perhaps a combination of the two would be advantageous especially as in a real draught dispense system there would be many different spoilage organisms (Quain, 2015). However, none of the combinations tested were better than the 200ppm dose of potassium sorbate against *P. membranifaciens* or *L. brevis.* There may be other beer spoilage organisms that could show particular sensitivity to one preservative, as was shown in Section 3.3.6 (Figure 28) where in Brand 1 0.5% *Rahnella spp.* was completely inhibited by the addition of just 20ppm sulphur dioxide, where in the same conditions 200ppm of sodium benzoate or potassium sorbate could only reduce its growth slightly. The spoilage trials showed a better inhibition of *L. brevis* by sodium benzoate compared to potassium sorbate; this was not observed in the Omnilog® trials. This may be due to poor growth of *L. brevis* in these trials.

The growth of *L. brevis* in these trials was poorer than expected, despite the use of Brand 1 0.5% as the test medium which allowed for the greatest growth of *L. brevis* in the spoilage trials (Figure 17). The lack of growth reduces the potential differences in growth between the different variables. This is most likely due to excess dissolved oxygen in the beer samples. The spoilage trials (Section 2.17) were set up in a way to emulate the conditions of a draught dispense line, specifically at the nozzle where the beer is open to the air and contamination (Quain, 2016). The CO_2 in solution would decrease after dosing and dissolved oxygen would increase over the course of incubation. However, due to limitations with setting up 96-well plates and pipetting small volumes, the beer samples for the Omnilog® trials had to be degassed. This also meant that their dissolved oxygen content

was likely higher than in the spoilage trials. The *L. brevis* used was clearly somewhat tolerant to dissolved oxygen as evidenced by its growth in all the tested beers in the spoilage trials (Figure 14). However, the mechanism by which *L. brevis* can resist the negative effects of radical oxygen species is energy intensive (Archibald and Fridovich, 1981). The Omnilog® trials were possibly too high in dissolved oxygen for *L. brevis* to achieve comparable growth to the spoilage trials. However, if *L. brevis* was struggling to grow in these conditions it would have been thought that the addition of preservatives would have prevented its growth completely by adding an extra microbiological hurdle into an environment it was already struggling to grow in. This trial should be re-assessed with *L. brevis* under anaerobic conditions to increase its growth and aid in differentiating all the variables tested.

One of the main shortfalls of the Omnilog® method, and likely source of some of the large errors seen in the data, is the inoculum to test solution ratio. The wells are only 100µl in size. The trial was set-up to have a 10µl inoculum which is 10% of the total capacity of the well, this means that carryover of latent nutrients from the inoculum and most importantly the low pH of the inoculum would have likely significantly affected the parameters of the test solution. In future, the organisms should be grown, pelleted by centrifuge, and then resuspended in the test solution itself. This would eliminate carryover of nutrients and the pH lowering effect of the original inoculum. In addition, it was advised that dosing at an OD of 0.5 would be best for achieving clear results with the Omnilog®**,** this is not consistent with the dose rate of 10^3 CFU/ml for the other trials. With an OD of 0.5 it would be likely that the dose would far exceed this with the 10% inoculum rate. Ideally the preservatives and pH differences should be tested against a number of spoilage microorganisms that the products are likely to be exposed to. In the future, the dose rate should be determined as it was in the spoilage trials. However, If these hurdles could be overcome the Omnilog® could be a good tool for assessing the effects of varying beer composition and especially

preservative concentrations, as well as any synergistic effects of these preservatives on beer spoilage microorganisms.

Chapter 5: Conclusions and Future Work

5.1 Conclusions

The demand for no or low alcohol alternatives to alcoholic beverages has increased and is growing more rapidly than the alcoholic beverage sector (IWSR, 2022). The majority of this growth is coming specifically from no-alcohol and low-alcohol beers (NABLABs). A newly expanding area is the serving of NABLABs in keg on draught dispense systems (IWSR, 2022). However, this environment is susceptible to microbial contamination, and the removal or restriction of ethanol in NABLABs can increase their susceptibility to spoilage (Quain, 2021). This reduction in ethanol can also allow foodborne pathogens such as *E. coli* 0157:H7 and *S.* Typhimurium to grow (Menz *et al.,* 2011; L'Anthoën and Ingledew, 1996). The main aim of this thesis is to help determine a consistent, economical and practical way for breweries producing NABLABs for the on-trade market to serve a safe, stable and quality product to consumers, as well as developing a method by which producers can test their products susceptibility to spoilage and pathogen growth. This was achieved by further investigating whether NABLABs are more susceptible to microbial spoilage than their full alcohol counterparts and the effects of preservatives and pH on microbial growth, in addition to identifying whether the NABLABs selected could provide an environment conducive to pathogen growth, and whether preservatives could be used to inhibit their growth. The composition of beers tested was also assessed to identify factors that may increase spoilage of a NABLAB other than reduction in ethanol content.

In Chapter 3, three NABLABs, Brand 1 0.5%, Brand 2 0.5%, Brand 3 0.5% and one full alcohol beer, Brand 1 4.5%, were challenge tested with *P. membranifaciens*, *L. brevis* or

Rahnella spp. To determine if there were significant differences in the growth of these organisms between the different NABLABs as compared to the full alcohol beer. All the beers tested have differing concentrations of fermentable sugars (Section 3.1). The beer spoilage yeast *P. membranifaciens* was able to grow more in all the NABLABs than the full alcohol beer. It seems that ethanol was the main inhibiting factor in this growth as there were no significant differences between the NABLABs growth despite them having large differences in sugar composition, pH and elemental composition. Additionally, *P. membranifaciens* is a non-fermentative yeast, and so does not typically produce ethanol, which could mean that it has a greater sensitivity to its anti-microbial effects. It also cannot utilise maltose and only poorly utilises glucose (Kurtzman, 2010)**.** Contrary to *P. membranifaciens*, the ability for *L. brevis* to grow and spoil NABLABs seems to be tied to the fermentable sugar content, specifically glucose. The beers with the highest glucose exhibited the greatest growth of *L. brevis,* this was regardless of ABV. Brand 1 0.5% had the highest glucose concentration and highest growth, Brand 2 0.5% had a similar level of growth to Brand 1 4.5%, and lowest in glucose Brand 3 0.5% showed the lowest growth. *Rahnella spp.* is a Gram-negative bacteria found in the brewery environment but is often not considered a beer spoiler due to its ethanol sensitivity (Van Vuuren and Priest, 2003). It was not able to grow in Brand 1 4.5%. It was able however, to grow in Brand 1 0.5% and Brand 2 0.5%, although not to the degree that *P. membranifaciens* and *L. brevis* could.

These results show that there is more to the susceptibility of a beer to microbial growth than its ethanol content. The parameters which allow successful growth differs between microorganisms (Van Vuuren and Priest, 2003; Priest, 2003; Campbell, 2003). A NABLAB will not always spoil more than a full alcohol beer, its spoilability is determined by the action of all the microbiological hurdles in combination. Although aerobic non-fermentative wild yeasts such as *P. membranifaciens* may show less growth in full alcohol beers, this may not be the case for fermentative yeasts like *Saccharomyces* species. These yeasts are more

tolerant to ethanol, low pH and can ferment maltose easily. So, beers high in maltose such as Brand 1 0.5% and Brand 3 0.5% would likely spoil to a greater degree than Brand 2 0.5% (Briggs *et al.,* 2004). Brand 2 0.5% is produced by restricted fermentation (with a low original gravity) with an ale yeast. This is what allows it to have such a low maltose concentration, as regular yeasts ferment maltose readily (Briggs *et al.,* 2004). Conversely, many other brewers who use restricted fermentation use 'lazy yeasts' which cannot, or have a reduced ability to ferment maltose. This would lead them to have a higher maltose concentration and so possibly be more vulnerable to spoilage by fermentative yeasts (Capece *et al.,* 2021; Muller *et al.,* 2020). For unpasteurised products or NABLABs served on-trade through draught dispense, large amounts of residual fermentable sugars would have the added negative effect of allowing alcoholic fermentation. This could lead to an increase in ABV, potentially above the legally declared limit.

The reduction in glucose and fructose remaining in NABLABs, and all other beers for that matter, is likely to slow the growth of *L. brevis,* and thus reduce its spoilage impact (Kim *et al.,* 2009; Behr *et al.,* 2006; Geissler *et al.,* 2016) (Section 3.2.4). Changing the formulation, or production method of a beer, to reduce the concentration of these simple sugars should be one of the main goals of any brewer looking to reduce risk of *L. brevis* related spoilage. Additionally, this would be likely to have a positive effect on reducing the growth of other beer spoilage organisms, as many microorganisms can utilise these substrates (Kim *et al.,* 2009; Behr *et al.,* 2006; Geissler *et al.,* 2016).

The growth of the non-beer spoilage organism *Rahnella spp.* in NABLABs supports the idea that these beers need to be treated as a different product to regular full alcohol beer (Section 3.2.4). Existing beer spoilage microbes may spoil NABLABs to a greater degree, but there is also a whole host of microorganisms that cannot or struggle to grow in full strength beer, that we now must consider as spoilage risks. The strict anaerobes *Megasphaera* and

Pectinatus are two of them. Although they have both been implicated in beer spoilage incidences, which seem to have been increasing over the years, they are still not a common occurrence (Suzuki, 2011, Vriesekoop *et al.,* 2012). This increase in incidence is mostly being attributed to the improvement in dissolved oxygen control throughout the brewing process, as the growth of both of these organisms is inhibited by oxygen (Juvonen, 2015). *Megasphaera* is sensitive to ethanol concentrations as low as 2.8% ABV and often not considered a spoilage threat to beers over 4% ABV. However, a recent study found *Megasphaera cerevisiae* NSB1 can grow in 5% ABV beer (Bergsveinson *et al.,* 2016). *Pectinatus* is slightly more resistant being able to grow in 4% ABV beers but not >5% ABV. They are both also sensitive to pH levels below 4.2. NABLABs that are subject to higher-than-average beer pH, due to their production method, may make them even more susceptible to these strict anaerobes (Juvonen, 2015).

Other organisms that might need to be considered are those which can spoil soft drinks, such as *Alicyclobacillus* which has been isolated from beer previously (Munford *et al.*, 2017). Contamination with *Alicyclobacillus* can cause serious organoleptic spoilage of soft drinks without producing a significant amount of turbidity, making it harder to detect visually. Additionally, it is a spore-forming bacteria and so can sometimes survive pasteurisation (Smit *et al.,* 2011; Sourri *et al.,* 2022). It typically requires hot conditions for growth, but as NABLABs are increasing in popularity worldwide, they will likely also be served in markets where these temperatures are more realistic (Smit *et al.,* 2011; Sourri *et al.,* 2022). Luckily however, it is a Gram-positive bacteria and so is sensitive to iso- α acids, with some suggesting that iso- α acids should be used in soft drinks as an antimicrobial agent to prevent the growth of *Alicyclobacillus* (Maca *et al.,* 2007). However, this does not rule out the possibility of it acquiring the *horA* and/or *horC* genes and becoming more resistant to iso- α acids, thus allowing it to survive and possibly grow in NABLABs (Munford *et al.*, 2017; Umegatani *et al.*, 2022). *Rahnella spp.* itself did not grow as readily as *L. brevis*

or *P. membranifaciens,* but as with *Alicyclobacillus* this does not prevent it from producing off-flavours and aromas, which for *Rahnella spp.* can result in large amounts of DMS and diacetyl (Van Vuuren and Priest, 2003).

The addition of preservatives, sodium benzoate, potassium sorbate and sulphur dioxide were then tested to see if they could be used to introduce a new hurdle to NABLABs, to replace the missing ethanol and compensate for the other hurdle changes. When dosed at 200ppm, sodium benzoate and potassium sorbate were found to both be effective at inhibiting the growth of *P. membranifaciens* in NABLABs in the spoilage trials and the Omnilog® trials (Sections 3.3.2, 3.3.4 and 4.2.2). They also reduced growth to a level that was comparable or better than the full alcohol beer (Section 3.4.2). This alone shows that use of these preservatives would be beneficial when serving NABLABs through draught dispense systems. In addition, both of these preservatives were also seen to inhibit *L. brevis* in the spoilage trials as well reducing its growth to comparable or better than the full alcohol counterpart (Sections 3.3.2, 3.3.4 and 3.4.2), further adding to the conclusion that addition of these preservatives would be beneficial to preventing spoilage. Although both sodium benzoate and potassium sorbate showed an effect against *Rahnella spp.* It was not as positive as the effect that just 20ppm of sulphur dioxide showed against *Rahnella spp.* in Brand 1 0.5%, where no growth was observed (Section 3.3.6). There was however, growth observed in 20ppm sulphur dioxide dosed into Brand 2 0.5%. This could be due to the pH being higher, as this is a major factor in the effectiveness of sulphur dioxide as an anti-microbial. But it could also be due to the fact that Brand 2 0.5% was produced by restricted fermentation. The lack of fermentation from this production method leaves the beer with a high concentration of aldehydes, which give the beer a 'Worty' aroma often associated with NABLABs (Blanco *et al.,* 2016; Muller *et al.,* 2020). These aldehydes can bind sulphur dioxide and essentially negate the antimicrobial action they could have provided and so effectively reduce the dose available (Lisanti *et al.,* 2019). As the beers produced by reverse

osmosis and vacuum distillation undergo a full fermentation, they do not tend to suffer from elevated aldehyde levels to the same extent (Blanco *et al.,* 2016; Muller *et al.,* 2020). This could explain why sulphur dioxide was effective in Brand 1 0.5%. In addition to the inhibition of *Rahnella spp.* and possibly other similar bacteria, sulphur dioxide helps to prevent oxidation. This causes 'staling' of beer and can reduce quality and in-turn the shelf-life. However, the addition of sulphur dioxide did seem to encourage the formation of pellicle by *P. membranifaciens,* this prevented accurate measurement of growth due to pellicle increasing turbidity, but not necessarily because of cell growth. This phenomenom is likely due to the antioxidant effect of sulphur dioxide, as pellicle forming yeasts are hypothesised to do so when there is not sufficient dissolved oxygen in solution, as there is a higher concentration of oxygen at the air liquid interface. So, sulphur dioxide could possibly worsen pellicle formation in areas such as the dispense nozzle, which should be considered if used. If pellicle forms on the dispense nozzle the next customer could get an unpleasant experience, which obviously needs to be avoided. So, if the anti-oxidant and limited anti-microbial properties outweigh the possible increase in pellicle formation, it seems that the combination of 20ppm sulphur dioxide and 200ppm sodium benzoate or 200ppm potassium sorbate (if permitted) could be a viable strategy to slow microbial growth and allow the serving of NABLABs on draught dispense systems. This would negate the need for specialist equipment or any major cleaning procedure changes within premises wishing to serve NABLABs on draught.

In Chapter 4 Brand 1 0.5% was challenge tested with *P. membranifaciens* and *L. brevis* to determine the effect of pH on preservative effectiveness, varying doses of preservatives, and whether combinations of preservatives could lead to synergistic effects. There was no clear indication that the pH of the sample impacted the antimicrobial effectiveness of sodium benzoate, potassium sorbate or sulphur dioxide against *P. membranifaciens* or *L. brevis.* The effects could have been too small to see with the low number of replicates, and

so repetition of these trials would be needed. In addition, the test spoilage organisms were grown in Brand 1 0.5% and then dosed straight into the well of the Omnilog® plate. The growth of these organisms could reduce the pH of the inoculum, especially *L. brevis*. This would have brought the pH of the test sample down as the inoculum was 10% of the final volume. If it was reduced significantly enough the differences between the percentage undissociated acid available would be small (Sofos and Busta, 1981; Baird and Parker, 1980) (Figures 7 and 8). Sodium benzoate and potassium sorbate showed a clear dose response relationship on the inhibition of *P. membranifaciens*. However, the lower doses (50ppm and 100ppm) did not appear to show enough inhibition for them to be used instead of the full 200ppm dose. No combinations of preservatives tested showed any signs of anti-microbial synergistic effects. The growth of *L. brevis* in these trials was low and did not show a normal binomial distribution, this affected the quality of all the results from the Omnilog® where *L. brevis* was tested. This could have been due to an elevated dissolved oxygen content in the samples due to the way the trials were set-up and run, this was further discussed in Section 4.5.

It is not only spoilage organisms that we should be concerned about, the reduction in ethanol concentration can also allow the growth of food borne pathogens such as *E. coli* 0157:H7 and *S.* Typhimurium. None of the beers tested (as received) saw growth of *E. coli* 0157:H7. Brand 1 0.5% and Brand 3 0.5% did not see growth of *S.* Typhimurium however Brand 2 0.5% did after 7 days. This was likely enabled by the high pH of Brand 2 0.5% but was still surprising considering the lack of glucose, as it is *S.* Typhimurium's preferred carbon source (Dandekar *et al.,* 2012). Brand 1 0.5% didn't allow the growth of *S.* Typhimurium, so it was adjusted with a small addition of malt extract and a pH adjustment. This kept the modified Brand 1 0.5%'s parameters within what is reasonable for other NABLABs that have been previously analysed (Appendix 1). The increase in pH and the introduction of additional sugars allowed *S.* Typhimurium to grow within 72 hours in a beer

it was previously unable to grow in Figure 41. This allowed the assessment of preservatives in preventing the growth of *S.* Typhimurium in a NABLAB. Sodium benzoate and potassium sorbate at 200ppm and sulphur dioxide at 20ppm, just as in the spoilage trials, were tested and all found to be effective agents at preventing *S.* Typhimurium growth. This means that the suggested combination of 200ppm sodium benzoate or potassium sorbate with the addition of 20ppm sulphur dioxide would also be effective at inhibiting *S.* Typhimurium as well as reducing the growth of spoilage organisms. *E. coli* 0157:H7 was not able to grow in the modified Brand 1 0.5%, so its sensitivity to sodium benzoate, potassium sorbate and sulphur dioxide was tested in un-modified Brand 1 0.5%. The preservatives had no effect on the survival of *E. coli* 0157:H7 compared to the control, this does not mean however that they would not be effective at preventing their growth (Mohammadzadeh-Aghdash *et al.,* 2019). But the ability of these preservatives to inhibit *E. coli* 0157:H7 in NABLABs needs to be tested in the near future. Two other NABLABs and a lactose dosed Brand 1 0.5% were briefly challenge tested with *E. coli* 0157:H7, they were all found to be a viable medium for the growth of *E. coli* 0157:H7. However, the other NABLABs were not readily available in the quantities required and variation of pH and PG were large between bottles. The Lactose modified Brand 1 0.5% was not pursued further due to the tendency of lactose to break down into glucose and galactose under heating (Berg and Boekel, 1994). The lactose solution was sterilised by autoclaving, this may have caused this break down. When it was used in a lactose modified Brand 1 0.5% and challenge tested with *E. coli* 0157:H7 it showed growth after 5 days, for *S.* Typhimurium it showed growth after only 3 days. As the majority of *Salmonella* species cannot utilise lactose, it was expected that this growth was due to break down of lactose (McDonough *et al.,* 2000). The level to which this would happen between batches would be difficult to control and so the malt extract dosed Brand 1 0.5% was used instead to aid in consistency. The effects of lactose addition on the growth of *E. coli* 0157:H7 still needs to be assessed as *E. coli* 0157:H7 is known to be able to

ferment lactose (Rahal *et al.,* 2012) which is a common addition to NABLABs to add body to beer (Big Drop, 2022; Club Soda, 2022).

To be able to restrict the growth of pathogens without the need for preservatives NABLABs should have a pH below 4.2, but ideally as low as possible, this can be achieved by addition of acids such as lactic acid. However, the addition of lactic acid can affect organoleptic properties of the beer causing a sour taste if too much is used (Siebert, 1999). The use of a mineral acid such as phosphoric acid rather than an organic one may be a better option in this case, as mineral acids are less sour at the same pH than organic acids (Siebert, 1999). This could possibly allow the pH to be reduced to below 4.0 while still being palatable, which would even further reduce the risk of pathogen growth and may aid in preservative effectiveness. However, mineral acids are not permitted as food additives in beer, although they are permitted and commonly used in soft drinks (EU Regulation 1129, 2011). A pH as low as 4.0 may even be effective at inhibiting food-borne pathogens in a NABLAB which has high fermentables, as Menz *et al.* showed that a pH of 4.0 inhibited *E. coli* 0157:H7 and *S.* Typhimurium in wort (Menz *et al.,* 2010). The reduction in pH should also be used in combination with the reduction of glucose and fructose to reduce the available carbon source for any food-borne pathogens. Additionally, the IBU of any NABLAB should not be less than 5 IBU to prevent the growth of *L. monocytogenes* and any other Gram-positive pathogens (Menz *et al.,* 2010). The variation in the production methods and styles of NABLAB may mean that some of these recommendations are not possible, and so in this case the product would ideally be challenge tested to ensure no growth of *E. coli* 0157:H7 and *S.* Typhimurium.

5.2 Future Work

The increase in demand for NABLABs served in keg through draught dispense systems brings an extra complexity to ensuring that the customer receives a safe product. Now, despite the abilities of breweries to provide a commercially sterile product, the growth of food borne pathogens in NABLABs is feasible (Section 3.5.3) (Menz *et al.,* 2011; L'Anthoën and Ingledew, 1996). Even if a NABLAB satisfies all the criteria to reduce the risk of growth, low pH, low fermentable sugars, >5 IBU and the permitted preservative additions, it is not clear that this will always be enough to prevent pathogen growth. Thus, further research into this area is required, to provide more well-defined recommendations for a NABLAB composition which will not allow the growth of pathogens and reduce their susceptibility to spoilage. This could be achieved with the use of the Omnilog® as was originally intended for this thesis. NABLABs with varying concentrations of fermentable sugars and pH levels could all be run in tandem, to determine a safe pH level for each level of fermentables. This would give brewers a guideline to base their high pH specification limit for their NABLABs based on fermentable sugar analysis. In addition, preservatives could be tested as they were against the spoilage organisms in Chapter 4. More methods should be developed for the investigation of microbial growth in NABLABs and regular beers with varying physical and chemical parameters. With their main purpose being to improve understanding of how beer composition and production methods effect their microbiological stability, in an environment similar to that of a brewery, or draught dispense system.

This work has highlighted the differences in spoilage of NABLABs by different microorganisms; it may be advantageous to further investigate which microorganisms are most likely to spoil NABLABs preferentially. This could be achieved by collecting samples of draught served NABLABs from on-trade premises, forcing those samples as performed in (Quain, 2021) and then assessing the microbial population with quantitative PCR. This

would provide the quantity of each microbe in the sample, the microbes that are most often found to make up the majority of the population will be those that are the best at spoiling NABLABs. These microorganisms can then be targeted and individually assessed to ascertain their growth requirements and sensitivity to preservatives. With Next-Gen sequencing the microflora of NABLABs and regular beers could be assessed rapidly, with the ability to sample a draught dispense system daily to assess the microflora change over the serving life of a beer. This could also be utilised to identify microorganisms which persist even after line cleaning, possibly through the formation of biofilms. Collecting the full genome sequences of beer spoilers found in trade could help us to understand the niche adaptation to beer (Geissler *et.al,* 2016).

In addition to looking at the microbiome currently found in draught NABLABs, new potential spoilage organisms should also be assessed. Although not entirely new to the brewing environment, *Megasphaera* and *Pectinatus* are two beer spoilers that could become a major problem for NABLABs. The removal of ethanol makes NABLABs a much more hospitable environment for these ethanol sensitive strict anaerobes (Juvonen, 2015). So their ability to grow in a variety of NABLABs with varying parameters and production methods needs to be determined, as well as the effects of sodium benzoate, potassium sorbate and sulphur dioxide on their growth. This work also showed, with the growth of *Rahnella spp.* in two of the three NABLABs tested, that non-beer spoilage organisms also need to be considered when investigating the microbiological stability of NABLABs. This is a new niche and so it is inevitable that new microorganisms will find and thrive in it 'Everything is everywhere but the environment selects' (Becking, 1934).

Many microorganisms can rapidly adapt to new environments and can also acquire genes, such as *horA* and *horC* which confer hop resistance, via horizontal gene transfer. A strain of *B. cereus* was isolated from a full alcohol beer and appears to be hop resistant (Wang *et al.,*

2017). This could also possibly happen with other food-borne pathogens such as *L. monocytogenes*. In addition, this can also occur in spoilage organisms and so we must always be aware of the risk of newly emerging beer spoilers or pathogens. Some species of *Lactobacillus* can metabolise potassium sorbate, so if we were to use it regularly to stabilise NABLABs, sorbic acid metabolising organisms may become the dominant spoilage organism. Through natural selection this could lead them to becoming better at metabolising potassium sorbate, essentially negating its effectiveness, and then allowing other microorganisms without the means to metabolise it to grow again. Additionally, if the genes responsible for this metabolization are plasmid bound, they may be transferrable to other bacteria.

As the preservatives tested were deemed to be a viable solution to reducing the growth of the beer spoilage organisms, the effects that the preservatives themselves have on beer quality need to be assessed. Potassium sorbate and Sodium benzoate are used in many soft drinks already due to their high taste thresholds, although sodium benzoate is known to cause 'oral-prickling' in higher doses (Catherine *et al.,* 2014; Lück and Jager, 1997; Otero-losada, 2003). However, their organoleptic effects have not been tested directly in beer. They may react with other beer compounds or as described previously be metabolised by microorganisms found in beer which in turn produces off-flavours. In addition, they could have effects on other physical and chemical parameters of beer such as foam stability and formation, colour or perceived bitterness.

So perhaps it may be better to develop a draught dispense system that is more resistant to contamination than relying on preservatives which may end up encouraging preservatives resistance in spoilage organisms. Some newer technologies being considered for use in the brewery environment include, silver impregnation of lines, especially draught dispense lines to produce an anti-microbial effect and to aid in the prevention of biofilm formation

(Mohanta *et al.,* 2020). Another is pulsed electric fields (PEF) which use short pulses of electricity for an anti-microbial effect (Puligundla *et al.,* 2018). This is being posed as an alternative to thermal-pasteurisation as it avoids heating the product and so doesn't affect its organoleptic properties (Bamforth, 2011). There is a possibility that these could be used separately or in combination within the dispense line to inactivate any microbes present and prevent them from growing and spoiling the beer.

5.3 Key Conclusions

- NABLABs can be more susceptible to spoilage but this is not always the case and will vary depending on the composition of the NABLAB and the microorganisms it is exposed to.
- NABLABs are an entirely new niche for microorganisms and so may be susceptible to spoilage by microorganisms that brewers have previously not had to contend with.
- The food-borne pathogens *E. coli* 0157:H7 and *S.* Typhimurium can grow in NABLABs, but not always, similarly to the spoilage organisms this depends on the composition of the NABLAB.
- A NABLAB with a high fermentable sugar content (glucose, fructose and maltose) and high pH (>4.4) is more likely to be susceptible to the growth of food-borne pathogens and spoilage organisms.
- When dosed at 200ppm sodium benzoate and potassium sorbate are effective at inhibiting *S.* Typhimurium*, P. membranifaciens*, *L. brevis* and *Rahnella spp*. A 20ppm dose of sulphur dioxide is effective at inhibiting the growth of *S.* Typhimurium and *Rahnella spp*.
- If NABLABs are to be served through standard draught dispense systems they should be stabilised with preservatives, to reduce their level of susceptibility to microbial spoilage within a range that would be expected for a full-alcohol beer, and to further reduce the risk of any food-borne pathogen growth.

Chapter 6: References and Appendix

6.1 References

Ameer, M. A., Wasey, A., Salen P., (2021) *Escherichia Coli* (*E Coli* 0157 H7) [Updated 2021 Dec 29]. In: StatPearls, Treasure Island (FL): StatPearls Publishing; 2022 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK507845/

Anderson, P., Kokole, D., & Llopis, E. J., (2021) *Production, Consumption, and Potential Public Health Impact of Low-and No-Alcohol Products: Results of a Scoping Review*. *Nutrients*, 13 (9). MDPI https://doi.org/10.3390/nu13093153

Aneja, K. R., Dhiman, R., Aggarwal, N. K., & Aneja, A., (2014) *Emerging Preservation Techniques for Controlling Spoilage and Pathogenic Microorganisms in Fruit Juices. International Journal of Microbiology*, 2014. Hindawi Publishing Corporation http://dx.doi.org/10.1155/2014/758942

Archibald, F. S., & Fridovich, I., (1981) *Manganese and Defenses Against Oxygen Toxicity in Lactobacillus plantarum*. *Journal of Bacteriology*, 145 (1), 442-451

Arici, M., & Daglioglu, O., (2002) B*oza: A Lactic Acid Fermented Cereal Beverage as a Traditional Turkish Food*. *Food Reviews International,* 18 (1), 39–48

Ashurst, J.V., Truong, J., & Woodbury, B., (2021) *Salmonella Typhi*. [Updated 2021 Aug 12]. In: StatPearls. Treasure Island (FL): StatPearls Publishing; 2022 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK519002/

Ashurst, P. R., (2016) *Chemistry and Technology of Soft Drinks and Fruit Juices 3rd Edition*, John Wiley & Sons

Azeredo, D. R. P., Alvarenga, V., Sant'Ana, A. S., & Sabaa Srur, A. U. O., (2016) *An Overview of Microorganisms and Factors Contributing for the Microbial Stability of Carbonated Soft Drinks*. *Food Research International*, 82, 136–144 Elsevier Ltd https://doi.org/10.1016/j.foodres.2016.01.024

Becking, L.G.M.B., (1934) *Geobiologie of Inleiding tot de Milieukunde* (No. 18-19). WP Van Stockum & Zoon

Baik. H. S., Bearson. S., Dunbar. S., Foster. J. W., (1996) *The Acid Tolerance Response of Salmonella Typhimurium Provides Protection Against Organic Acids*. *Microbiology*, 142, 3195-3200

Baird-Parker, A. C., (1980) *Organic Acids*. *Microbial Ecology of Foods*, 1, 126

Bamforth, C. W., (2011) *125th Anniversary Review: The Non-Biological Instability of Beer*. *Journal of the Institute of Brewing*, 117(4), 488–497

Bamforth, C. W., (Ed.)., (2006) *Brewing: New Technologies*. Elsevier Science & Technology

Banks, J. G., & Board, R. G., (1982) *Sulfite-Inhibition of Enterobacteriaceae Including Salmonella in British Fresh Sausage and in Culture Systems*. *Journal of Food Protection*, 45(14), 1292–1297 https://doi.org/10.4315/0362-028x-45.14.1292

Barker, C., & Park, S. F., (2001) *Sensitization of Listeria monocytogenes to Low pH, Organic Acids, and Osmotic Stress by Ethanol*. *Applied and Environmental Microbiology*, 67(4), 1594–1600

Basaran-Akgul, N., Churey, J. J., Basaran, P., & Worobo, R. W., (2009) *Inactivation of Different Strains of Escherichia coli O157:H7 in Various Apple Ciders Treated with Dimethyl Dicarbonate (DMDC) and Sulfur Dioxide (SO_2) as an Alternative Method. Food Microbiology*, 26(1), 8–15

BBPA briefing 1, (2018) *Hygienic Management of Cellars and Cleaning of Beer Dispense Lines 1*. General Cellar Management http://beerandpub.com/wp-content/uploads/2017/Briefings/Line-Cleaning-Guidance.pdf

Behr, J., Gänzle, M. G., & Vogel, R. F., (2006) *Characterization of a Highly Hop-Resistant Lactobacillus brevis Strain Lacking Hop Transport*. *Applied and environmental microbiology*, 72(10), 6483–6492 https://doi.org/10.1128/AEM.00668-06

Bellut, K., & Arendt, E. K., (2019) *Chance and Challenge: Non-Saccharomyces Yeasts in Nonalcoholic and Low Alcohol Beer Brewing–A Review*. *Journal of the American Society of Brewing Chemists*, 77(2), 77–91

Berg, H. E., & Van Boekel, M. A. J. S., (1994) *Degradation of Lactose During Heating of Milk.* Reaction pathways. *Netherlands Milk and Dairy Journal*, 48, 157-175

Bergsveinson, J., Baecker, N., Pittet, V., & Ziola, B., (2015) *Role of Plasmids in Lactobacillus brevis BSO 464 Hop Tolerance and Beer Spoilage*. *Applied and environmental microbiology*, 81(4), 1234–1241 https://doi.org/10.1128/AEM.02870-14

Big Drop, (2022) Accessed: 29/09/2022 https://shop.bigdropbrew.com/pages/faqs

Bintsis, T., (2017) *Foodborne Pathogens*. *AIMS microbiology*, *3*(3), 529–563 https://doi.org/10.3934/microbiol.2017.3.529

Bisson. L. F., (1999) *Stuck and Sluggish Fermentations*. *The American Journal of Enology and Viticulture*, 50, 107–119

Blanco, C. A., Andrés-Iglesias, C., & Montero, O., (2016) *Low-Alcohol Beers: Flavor Compounds, Defects, and Improvement Strategies*. *Critical Reviews in Food Science and Nutrition*, 56(8), 1379–1388 https://doi.org/10.1080/10408398.2012.733979

Bokulich, N. A., & Bamforth, C. W., (2013) *The Microbiology of Malting and Brewing*. *Microbiology and Molecular Biology Reviews*, 77(2), 157–172

Booth, I. R., (1985) *Regulation of Cytoplasmic pH in Bacteria*. *Microbiological reviews,* 49 (4)

Bose, N., Auvil, D.P., Moore, E.L., & Moore, S.D., (2021) Microbial Communities in Retail Draft Beers and the Biofilms They Produce. *Microbiology Spectrum*, 9

Boulton, C., & Quain, D., (2001) Brewing yeast and fermentation. Blackwell Science

Brányik, T., Silva, D. P., Baszczyňski, M., Lehnert, R., & Almeida E Silva, J. B., (2012) *A Review of Methods of Low Alcohol and Alcohol-Free Beer Production. Journal of Food Engineering*, 108 (4), 493–506

Briggs, D. E., Boulton, C. A., Brookes, P. A., & Stevens, R., (2004) *Brewing Science and Practice*. Woodhead Publishing

Bunker, H. J., (1955) *The Survival of Pathogenic Bacteria in Beer*. Proceedings of the European Brewery Convention, 5, 330-341

Butzke. C. E., (2010) *Winemaking Problems Solved*, 1st Edition, Woodhead

Caballero. B., Finglas. P. M., & Toldrá. F., (2016) *Encyclopedia of Food and Health 1st Edition*, Elsevier

Campbell, I., & Msongo, H. S., (1991) *Growth of Aerobic Wild Yeasts*. *Journal of the Institute of Brewing*, 97(4), 279–282 https://doi.org/10.1002/j.2050-0416.1991.tb01069.x

Campbell, I., (2003) *Wild Yeasts in Brewing and Distilling*, *Brewing microbiology* (Third ed.), 247-266, Springer

Capece, A., de Fusco, D., Pietrafesa, R., Siesto, G., & Romano, P., (2021) *Performance of Wild Non-Conventional Yeasts in Fermentation of Wort Based on Different Malt Extracts to Select Novel Starters for Low-Alcohol Beers*. *Applied Sciences*, 11(2), 1–17 https://doi.org/10.3390/app11020801

Cath, T. Y., Childress, A. E., & Elimelech, M., (2006) *Forward osmosis: Principles, Applications, and Recent Developments*. *Journal of Membrane Science*, 281, (1–2), 70–87

Catherine, L. D., George, T. F. Jr., Madelyn S. B., Alison, R. & Behling, H. E. C., (2014) *Sorbic Acid*. *Encyclopedia of chemical technology*, 24, (1), 92

Centers for Disease Control and Prevention (2022) National Center for Emerging and Zoonotic Infectious Diseases (NCEZID) Last updated 16th June 2022 https://www.cdc.gov/salmonella/index.html#:~:text=CDC%20estimates%20Salmonella%20bacteria%20cause,the%20United%20States%20every%20year.

Ceylan, E., Fung, D. Y. C., & Sabah, J. R., (2004) *Antimicrobial Activity and Synergistic Effect of Cinnamon with Sodium Benzoate or Potassium Sorbate in Controlling Escherichia coli O157:H7 in Apple Juice*. *Journal of Food Science*, 69, (4), 102-106

Chisholm, M. G., & Samuels, J. M., (1992) *Determination of the Impact of the Metabolites of Sorbic Acid on the Odor of a Spoiled Red Wine*, *Journal of Agricultural and Food Chemistry*, 40, 630-633

Ciosek, A., Fulara, K., Hrabia, O., Satora, P., & Poreda, A., (2020) *Chemical Composition of Sour Beer Resulting from Supplementation the Fermentation Medium with Magnesium and Zinc Ions*. *Biomolecules*, 10(12), 1–14 https://doi.org/10.3390/biom10121599

Club Soda, (2022) Accessed: 29/09/2022 https://joinclubsoda.com/an-introduction-to-non-alcoholic-beer/

Code of Federal Regulations, (2022) Title 21, Volume 3, (Accessed 08/09/2022) https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/cfrsearch.cfm?fr=184.1733

Code of Federal Regulations, (2022) Title 27, Part 7, Section 7.71, (Accessed 15/5/2023) https://www.govinfo.gov/app/details/CFR-2021-title27-vol1/CFR-2021-title27-vol1-sec7-71/summary

Cojocaru, G. A., & Antoce, A. O., (2012) *Chemical and Biochemical Mechanisms of Preservatives used in Wine: A Review Knowing the Particularities of Different Varieties of Grapes*, *Scientific Papers*, Series B, Horticulture, LVI

Cruz, C. D., Esteve, P., & Tammela, P., (2021) *Evaluation and Validation of Biolog OmniLog® System for Antibacterial Activity Assays*. *Letters in Applied Microbiology*, *72*(5), 589–595 https://doi.org/10.1111/lam.13450

Dandekar, T., Fieselmann, A., Popp, J., & Hensel, M., (2012) *Salmonella enterica: A Surprisingly Well-Adapted Intracellular Lifestyle*. *Frontiers in Microbiology*, 3, https://doi.org/10.3389/fmicb.2012.00164

Daniels, J. A., Krishnamurthi, R., & Rizvi, S. S. H., (1984) *A Review of Effects of Carbon Dioxide on Microbial Growth and Food Quality*, *Journal of Food Protection*, 48 (6), 532-537

de las Heras. J., Aldamiz-Echevarria. L., Martínez-Chantar. M., Delgado. T., (2016) *An Update on the use of Benzoate, Phenylacetate and Phenylbutyrate Ammonia Scavengers for Interrogating and Modifying Liver Nitrogen Metabolism and its Implications in Urea Cycle Disorders and Liver Disease*. *Expert opinion on drug metabolism & toxicology*, 13 (4), 439-448

De Nederlandse Recalme Code, (2022) *De Nederlandse Recalme Code met Informatie Over de Stichting Reclame Code en de Werkwijze van de Reclame Code Commissie en het College van Beroep & Jaarcijfers*, Edited 2022

del Olmo, A., Calzada, J., & Nuñez, M., (2017) *Benzoic acid and its Derivatives as Naturally Occurring Compounds in Foods and as Additives: Uses, Exposure, and Controversy*. *Critical Reviews in Food Science and Nutrition*, 57(14), 3084–3103

Department of Health and Social Care, (2018) Low Alcohol Descriptors Guidance https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/763840/low-alcohol-descriptors-guidance.pdf

Dong, M., Masuyer, G., & Stenmark, P., (2019) *Botulinum and Tetanus Neurotoxins*. *Annual Review of Biochemistry*, 88, 811–837

Doyle, A. A., & Stephens, J. C., (2019) *A Review of Cinnamaldehyde and its Derivatives as Antibacterial Agents*. *Fitoterapia*, 139 https://doi.org/10.1016/j.fitote.2019.104405

Drinks International, (2021) https://drinksint.com/news/fullstory.php/aid/9561/Research_exposes_high_demand_for_draft_alcohol-free_beer.html

Du, Q., Liu, Y., Song, Y., & Qin, Y., (2020) *Creation of a Low-Alcohol-Production Yeast by a Mutated SPT15 Transcription Regulator Triggers Transcriptional and Metabolic Changes During Wine Fermentation*. *Frontiers in Microbiology*, *11*. https://doi.org/10.3389/fmicb.2020.597828

EBC Technology and Engineering Forum, (1995) *Beer Pasteurisation: Manual of Good Practice*, Getränke-Fachverlag Hans Carl, Nürnberg

Enikova, R., Kozereva, M., Ivanova, T., Yang'ozova, Z., (1985) *Sanitary Microbiological Analysis of Boza*. *Khranitelnopromishlena Nauka,* 1985, 1, 73–81

Eßlinger, H., (2009) *Handbook of Brewing* (1. Aufl. ed.). Wiley-VCH

EU Regulation, (2011) Commission Regulation (EU) No 1129/2011 of 11 November 2011 amending Annex II to Regulation (EC) No 1333/2008 of the European Parliament and of the Council by establishing a Union list of food additives Text with EEA relevance http://data.europa.eu/eli/reg/2011/1129/oj

EU Regulation, (2011) Commission Regulation (EU) No 1169/2011 of 25 October 2011 amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=celex%3A32011R1169

Feldmann, H., & Branduardi, P., (2012) *Yeast: Molecular and Cell Biology (2nd completely rev. and greatly enl. ed.).* Weinheim: Wiley-Blackwell

Fernandez. J.L. & Simpson, J., (1995) *Measurement and prediction of the susceptibility of lager beer to spoilage by lactic acid bacteria*. Journal of Applied Bacteriology, 78, 419-425

Firmino, D. F., Cavalcante, T. T. A., Gomes, G. A., Firmino, N. C. S., Rosa, L. D., de Carvalho, M. G., & Catunda, F. E. A., (2018) *Antibacterial and Antibiofilm Activities of Cinnamomum Sp. Essential Oil and Cinnamaldehyde: Antimicrobial Activities. Scientific World Journal*, 2018 https://doi.org/10.1155/2018/7405736

Foster, J. W., (2004) *Escherichia coli Acid Resistance: Tales of an Amateur Acidophile*. *Nature Reviews Microbiology*, 2 (11), 898–907

Friedman, D. E., Patten, K. A., Rose, J. B., Barney, M. C., & Wisconsin, M., (1997) *The Potential For Cryptosporidium Parvum Oocyst Survival In Beverages Associated With Contaminated Tap Water*. *Journal of Food Safety,* 17, 125-132

Garcia-Fuentes, A., Wirtz, S., Vos, E., & Verhagen, H., (2015) *Short Review of Sulphites as Food Additives*. *European Journal of Nutrition & Food Safety*, 5(2), 113–120 https://doi.org/10.9734/ejnfs/2015/11557

Gardner L. K., Lawrence G. D., (1993) *Benzene Production from Decarboxylation of Benzoic Acid in the Presence of Ascorbic Acid and a Transition-Metal Catalyst*. *Journal of Agricultural and Food Chemistry*, 41, 693-695

Geissler, A. J., Behr, J., & Vogel, R. F., (2016) *Multiple Genome Sequences of the Important Beer-Spoiling Species Lactobacillus backii.* Genome announcements, 4(4) https://doi.org/10.1128/genomeA.00826-16

Gernat, D. C., Brouwer, E., & Ottens, M., (2020) *Aldehydes as Wort Off-Flavours in Alcohol-Free Beers—Origin and Control*. *Food and Bioprocess Technology*, *13*(2), 195–216 https://doi.org/10.1007/s11947-019-02374-z

Graziano, M. U., Graziano, K. U., Pinto, F. M. G., Bruna, C. Q. de M., de Souza, R. Q., & Lascala, C. A., (2013) *Effectiveness of Disinfection with Alcohol 70% (w/v) of Contaminated Surfaces not Previously Cleaned*. *Revista Latino-Americana de Enfermagem*, 21(2), 618–623

Guido, L. F., (2016) *Sulfites in beer: Reviewing Regulation, Analysis and Role*. *Scientia Agricola*, 73 (2), 189–197) https://doi.org/10.1590/0103-9016-2015-0290

Gutnick, D., Calvo, J. M., Klopotowski, T., & Ames, B. N., (1969) *Compounds which serve as the Sole Source of Carbon or Nitrogen for Salmonella Typhimurium LT-2*. *Journal of bacteriology*, 100(1), 215–219 https://doi.org/10.1128/jb.100.1.215-219.1969

Heineken, (2021) Accessed: 20/9/2022, https://www.heineken.co.uk/heineken-uk-strengthens-online-presence-for-blade/

Hill, A. E., (2015) *13 -Traditional Methods of Detection and Identification of Brewery Spoilage Organisms. Brewing Microbiology: Managing Microbes, Ensuring Quality and Valorising Waste*. *Food Science, Technology, and Nutrition*, 289, 271-286 Woodhead Publishing

Hornsey, I., (2013) *Brewing 2nd Edition*, RSC Publishing

Hutkins, R. W., & Nannen, N. L., (1993) *pH Homeostasis in Lactic Acid Bacteria. Journal of Dairy Science*, 76 (8), 2354-2365

Independent, (2021) Accessed: 17/09/2022 https://www.independent.ie/irish-news/draught-alcohol-free-guinness-set-to-land-in-pubs-next-monthin-keg-so-small-its-a-can-40524041.html

IWSR, (2022) *No- and Low-Alcohol Strategic Study*, https://www.theiwsr.com/wp-content/uploads/IWSR-2022-No-and-Low-Alcohol-Press-Release.pdf

IWSR, (2022) https://www.theiwsr.com/no-and-low-alcohol-category-value-surpasses-11bn-in-2022/

Jaskula, B., Kafarski, P., Aerts, G., & de Cooman, L., (2008) *A Kinetic Study on the Isomerization of Hop α-acids*. *Journal of Agricultural and Food Chemistry*, 56(15), 6408–6415

Jeffery, I.A., Karim, S., (2022) *Botulism*. [Updated 2022 May 12]. In: StatPearls. Treasure Island (FL): StatPearls Publishing; 2022 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK459273

Jeon, S. H., Kim, N. H., Shim, M. B., Jeon, Y. W., Ahn, J. H., Lee, S. H., Hwang, I. G., & Rhee, M. S., (2015) *Microbiological Diversity and Prevalence of Spoilage and Pathogenic Bacteria in Commercial Fermented Alcoholic Beverages (Beer, Fruit Wine, Refined Rice Wine, and Yakju*). *Journal of Food Protection*, 78(4), 812–818

Jevons, A. L., & Quain, D. E., (2021) *Draught Beer Hygiene: Use of Microplates to Assess Biofilm Formation, Growth and Removal*. *Journal of the Institute of Brewing*, 127(2), 176–188

Jin, L. Z., Ho, Y. W., Abdullah, N., & Jalaludin, S., (1998) *Growth Performance, Intestinal Microbial Populations, and Serum Cholesterol of Broilers Fed Diets Containing Lactobacillus Cultures*. *Poultry science*, 77(9), 1259–1265 https://doi.org/10.1093/ps/77.9.1259

Juvonen, R., (2015) *9 - Strictly Anaerobic Beer-Spoilage Bacteria Brewing microbiology : Managing Microbes, Ensuring Quality and Valorising Waste. Food Science, Technology, and Nutrition*, 289, 195-218 Woodhead Publishing

Karabín, M., Hudcová, T., Jelínek, L., & Dostálek, P., (2016) *Biologically Active Compounds from Hops and Prospects for Their Use*. *Comprehensive Reviews in Food Science and Food Safety*, 15(3), 542–567

Kenyon, W. J., Thomas, S. M., Johnson, E., Pallen, M. J., & Spector, M. P., (2005) *Shifts From Glucose to Certain Secondary Carbon-Sources Result in Activation of the Extracytoplasmic Function Sigma Factor σE in Salmonella enterica Serovar Typhimurium*. *Microbiology*, 151(7), 2373–2383 https://doi.org/10.1099/mic.0.27649-0

Kim, J. H., Shoemaker, S. P., & Mills, D. A., (2009) *Relaxed Control of Sugar Utilization in Lactobacillus brevis*. *Microbiology*, 155(4), 1351–1359 https://doi.org/10.1099/mic.0.024653-0

Kim, S. A., Park, H. J., Lee, S. H., Hwang, I. G., & Rhee, M. S., (2014) *Short Communication: Fate of Major Foodborne Pathogens and Bacillus cereus Spores in Sterilized and Non-Sterilized Korean Turbid Rice Wine (Makgeolli). Food Control*, 39(1), 139–145

Kornberg, H., & Lourenco, C., (2006) *A Route for Fructose Utilization by Escherichia coli Involving the Fucose Regulon*. *Proceedings of the National Academy of Sciences,* 103(51), 19496-19499. www.pnas.orgcgidoi10.1073pnas.0609429103

Krofta, K., & Mikyška, A., (2014) *Hop Beta Acids: Properties, Significance and Utilization. Kvasny Prumysl*, 60(4), 96–105. https://doi.org/10.18832/kp2014010

Krogerus, K., & Gibson, B., (2020) *A Re-evaluation of Diastatic Saccharomyces cerevisiae Strains and their Role in Brewing*. *Applied Microbiology and Biotechnology*, 104(9), 3745–3756. Springer. https://doi.org/10.1007/s00253-020-10531-0

Krogerus, K., Eerikäinen, R., Aisala, H., Gibson, B., (2021) *Repurposing Brewery Contaminant Yeast as Production Strains for Low-alcohol Beer Fermentation*, *Yeast*, 39(1-2), 156-169

Kurtzman. C. P., Fell. J. W., Boekhout T., (2010) *The Yeasts: A Taxonomic Study*, 5th Edition, Elsevier

Kyselová L., Bránayik T., (2015) *20 - Quality Improvement and Fermentation Control in Beer. Advances in Fermented Foods and Beverages*, *Food Science, Technology and Nutrition*, 477-500 Woodhead Publishing

L' Anthoën, N.C., and Ingledew, W.M., (1996) *Heat Resistance of Bacteria in Alcohol Free Beer*, *Journal of the American Society of Brewing Chemists*. 54(1), 32 –36

Lachenmeier, D. W., Reusch, H., Sproll, C., Schoeberl, K., & Kuballa, T., (2008) *Occurrence of Benzene as a Heat-induced Contaminant of Carrot Juice for Babies in a General Survey of Beverages*. *Food Additives and Contaminants*, 25(10), 1216–1224. https://doi.org/10.1080/02652030802036230

Lewis, M. J., & Young T. W., (2002) *Brewing 2nd Edition*. Springer Science & Business

Liett, O. R., (1995) *Aspects of the Analysis, Role, and Fate of Sulphur Dioxide in Beer · A Review*, *MBAA Technical Quarterly*, 32(4), 213-221

Liewen, M. B., & Marth, E. H., (1985) *Growth and Inhibition of Microorganisms in the Presence of Sorbic Acid: A Review*. *Journal of Food Protection* 48(4), 364-375

Lisanti, M. T., Blaiotta, G., Nioi, C., & Moio, L., (2019) *Alternative Methods to SO_2 for Microbiological Stabilization of Wine*. *Comprehensive Reviews in Food Science and Food Safety*, 18(2), 455–479. Blackwell Publishing Inc https://doi.org/10.1111/1541-4337.12422

López, M. G., Roche, E., Rodríguez, E., (2020) *Contaminant Microbiota in Craft Beers.* Journal of Microbiology, *Biotechnology and Food Sciences*, 9(6), 1181–1186 https://doi.org/10.15414/JMBFS.2020.9.6.1181-1186

Londesborough, J., (2001) *Fermentation of Maltotriose by Brewer's and Baker's Yeasts. Biotechnology Letters*, 23, 1995-2000

Loutfy, M. R., Austin, J. W., Blanchfield, B., & Fong, I. W., (2003) *An Outbreak of Foodborne Botulism in Ontario*. *Canadian Journal of Infectious Diseases and Medical Microbiology*, 14(4), 206-209

Lück, E., & Jager, M., (1997). *Sorbic Acid*. *Antimicrobial Food Additives: Characteristics, Uses, Effects*, 2nd Revised and Enlarged Edition, 152–167, Springer

Mallett, J. R., Stuart, M. S., & Quain, D. E., (2018) *Draught Beer Hygiene: A Forcing Test to Assess Quality*. *Journal of the Institute of Brewing*, 124(1), 31–37

Maca, H. W., Barney, M. C., & Ryder D. S., (2007) European Patent No. 1 799 054 B1, European Patent Office

Matoulková, D., Kosař, K., Slabý, M., & Sigler K., (2012) *Occurrence and Species Distribution of Strictly Anaerobic Bacterium Pectinatus in Brewery Bottling Halls*, *Journal of the American Society of Brewing Chemists*, 70(4), 262-267, https://doi.org/10.1094/ASBCJ-2012-0910-01

Martin, J. D., Werner, B. G., & Hotchkiss, J. H., (2003) *Effects of Carbon Dioxide on Bacterial Growth Parameters in Milk as Measured by Conductivity*. *Journal of Dairy Science*, 86(6), 1932–1940

McDonough, P. L., Shin, S. J., & Lein, D. H., (2000) *Diagnostic and Public Health Dilemma of Lactose-Fermenting Salmonella enterica Serotype Typhimurium in Cattle in the North-eastern United States*. *Journal of clinical microbiology*, 38(3), 1221–1226. https://doi.org/10.1128/JCM.38.3.1221-1226.2000

McDowell R.H., Sands E.M., Friedman H., (2021) *Bacillus Cereus*. [Updated 2021 Sep 16]. In: StatPearls. Treasure Island (FL): StatPearls Publishing; 2022 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK459121/

McDevitt, C. A., Ogunniyi, A. D., Valkov, E., Lawrence, M. C., Kobe, B., McEwan, A. G., & Paton, J. C., (2011) *A Molecular Mechanism for Bacterial Susceptibility to Zinc*. *PLoS Pathogens*, *7*(11) https://doi.org/10.1371/journal.ppat.1002357

Meier-Dörnberg, T., Kory, O. I., Jacob, F., Michel, M., & Hutzler, M., (2018) *Saccharomyces cerevisiae Variety diastaticus Friend or Foe? - Spoilage Potential and Brewing Ability of Different Saccharomyces cerevisiae Variety diastaticus Yeast Isolates by Genetic, Phenotypic and Physiological Characterization*. *FEMS Yeast Research*, 18(4)

Membré, J. M., Leporq, B., Vialette, M., Mettler, E., Perrier, L., Thuault, D., & Zwietering, M., (2005) *Temperature Effect on Bacterial Growth Rate: Quantitative Microbiology Approach Including Cardinal Values and Variability Estimates to Perform Growth Simulations on/in Food*. *International Journal of Food Microbiology*, *100*(1–3), 179–186 https://doi.org/10.1016/j.ijfoodmicro.2004.10.015

Menz, G., Aldred, P., & Vriesekoop, F., (2008) *Pathogens in Beer. Beer in Health and Disease Prevention*, 2008, 403–413

Menz, G., Aldred, P., & Vriesekoop, F., (2011) *Growth and Survival of Foodborne Pathogens in Beer*. *Journal of Food Protection*, 74(10), 1670–1675

Menz, G., Vriesekoop, F., Zarei, M., Zhu, B., & Aldred, P., (2010) *The Growth and Survival of Food-borne Pathogens in Sweet and Fermenting Brewers' Wort*. *International Journal of Food Microbiology*, 140(1), 19–25

Mitropoulou, G., Nikolaou, A., Santarmaki, V., Sgouros, G., & Kourkoutas, Y., (2020) *Citrus medica and Cinnamomum zeylanicum Essential Oils as Potential Biopreservatives Against Spoilage in Low Alcohol Wine Products. Foods*, 9(577)

Mohammadzadeh-Aghdash, H., Akbari, N., Esazadeh, K., & Dolatabadi, J. E. N., (2019) *Molecular and Technical Aspects on the Interaction of Serum Albumin with Multifunctional Food Preservatives*. *Food chemistry*, 293, 491-498

Mohanta, Y. K., Biswas, K., Jena, S. K., Hashem, A., Abd_Allah, E. F., & Mohanta, T. K., (2020) *Anti-biofilm and Antibacterial Activities of Silver Nanoparticles Synthesized by the Reducing Activity of Phytoconstituents Present in the Indian Medicinal Plants*. *Frontiers in Microbiology*, *11* https://doi.org/10.3389/fmicb.2020.01143

Msagati, T., (2012) *The Chemistry of Food Additives and Preservatives* (1. Aufl. ed.). Hoboken: Wiley-Blackwell

Muller, C., Neves, L. E., Gomes, L., Guimarães, M., & Ghesti, G., (2020) *Processes for Alcohol-free Beer Production: A Review*. *Food Science and Technology*, 40(2), 273–281

Munford, A. R. G., Alvarenga, V. O., Prado-Silva, L. do, Crucello, A., Campagnollo, F. B., Chaves, R. D., Oteiza, J. M., & Sant'Ana, A. S., (2017) *Sporeforming Bacteria in Beer: Occurrence, Diversity, Presence of Hop Resistance Genes and Fate in Alcohol-free and Lager Beers*. *Food Control*, *81*, 126–136 https://doi.org/10.1016/j.foodcont.2017.06.003

National Center for Biotechnology Information, (2022) PubChem Compound Summary for CID 23676745, Potassium sorbate. Retrieved August 24, 2022 from https://pubchem.ncbi.nlm.nih.gov/compound/Potassium-sorbate

Murphy & Son, (Accessed 14/09/2022) https://www.murphyandson.co.uk/wp-content/uploads/2021/04/Cedarex-Light-std-Issue-3-Muntons-Rev-10.04.2018.pdf

National Center for Biotechnology Information, (2022) PubChem Compound Summary for CID 62371, Magnesium dibenzoate. Retrieved August 24, 2022 from https://pubchem.ncbi.nlm.nih.gov/compound/Magnesium-dibenzoate

Nsogning, S. D., Fischer, S., & Becker, T., (2018) *Investigating on the Fermentation Behavior of Six Lactic Acid Bacteria Strains in Barley Malt Wort Reveals Limitation in Key Amino Acids and Buffer Capacity*. *Food microbiology*, 73, 245–253 https://doi.org/10.1016/j.fm.2018.01.010

Otero-Losada, M. E., (2003) *Differential Changes in Taste Perception Induced by Benzoic Acid Prickling*. *Physiology and Behavior*, 78(3), 415–425

Paradh, A. D., Mitchell, W. J., & Hill, A. E., (2011) *Occurrence of Pectinatus and Megasphaera in the Major UK Breweries*. *Journal of the Institute of Brewing*, *117*(4), 498–506 https://doi.org/10.1002/j.2050-0416.2011.tb00497.x

Paradh, A. D., (2015) *Gram-negative Spoilage Bacteria in Brewing. Brewing Microbiology,* 175-194. Woodhead Publishing

Pennington, T. H., (2014) *E. coli O157 Outbreaks in the United Kingdom: Past, Present, and Future*. *Infection and Drug Resistance,* 7, 211–222. Dove Medical Press Ltd

Petrova, O. E., & Sauer, K., (2016) *Escaping the Biofilm in More than one way: Desorption, Detachment or Dispersion*. *Current Opinion in Microbiology,* 30, 67–78

Powell, C. D., & Diacetis, A. N., (2007) *Long Term Serial Repitching and the Genetic and Phenotypic Stability of Brewer's Yeast. Journal of the Institute of Brewing*, 113(1), 67–74

Priest, F., (2003) *Gram-Positive Brewery Bacteria*, *Brewing microbiology* (Third ed.), 188-218, Springer

Puligundla, P., Pyun, Y. R., & Mok, C., (2018) *Pulsed Electric Field (PEF) Technology for Microbial Inactivation in Low-alcohol Red Wine*. *Food Science and Biotechnology*, *27*(6), 1691–1696 https://doi.org/10.1007/s10068-018-0422-1

Purwasasmita, M., Kurnia, D., Mandias, F. C., Khoiruddin, & Wenten, I. G., (2015) *Beer Dealcoholization using Non-porous Membrane Distillation*. *Food and Bioproducts Processing*, 94, 180–186

Quain, D. E., (2015) *16 -Assuring the Microbiological Quality of Draught Beer. Brewing Microbiology: Managing Microbes, Ensuring Quality and Valorising Waste*. *Food Science, Technology, and Nutrition*, 289, 271-286, Woodhead Publishing

Quain, D. E., (2016) *Draught Beer Hygiene: Cleaning of Dispense Tap Nozzles*. *Journal of the Institute of Brewing*, 122(3), 388–396

Quain, D. E., (2021) *The Enhanced Susceptibility of Alcohol-free and Low Alcohol Beers to Microbiological Spoilage: Implications for Draught Dispense*. *Journal of the Institute of Brewing*, 127(4), 406–416

Quirós. C., Herrero. M., García. L., Díaz. M., (2012) *Effects of SO_2 on Lactic Acid Bacteria Physiology when used as Preservative Compound in Malolactic Fermentation. Journal of the Institute of Brewing*. 118(1), 89-96

Rachon, G., Raleigh, C. P., & Pawlowsky, K., (2021) *Heat Resistance of Yeast Ascospores and their Utilisation for the Validation of Pasteurisation Processes for Beers*. *Journal of the Institute of Brewing*, 127(2), 149–159

Rachon, G., Rice, C. J., Pawlowsky, K., & Raleigh, C. P., (2018) *Challenging the Assumptions Around the Pasteurisation Requirements of Beer Spoilage Bacteria. Journal of the Institute of Brewing*, 124(4), 443–449

Rahal, E. A., Kazzi, N., Nassar, F. J., & Matar, G. M., (2012) *Escherichia coli O157:H7-Clinical Aspects and Novel Treatment Approaches*. *Frontiers in cellular and infection microbiology*, 2(138) https://doi.org/10.3389/fcimb.2012.00138

Rainbow, C., (1971) *Spoilage organisms in breweries*. *Process Biochem*, 31, 15–17.

Rogalla, D., Bomar, P. A., (2021) *Listeria Monocytogenes*. [Updated 2021 Jul 10]. In: StatPearls Treasure Island (FL): StatPearls Publishing; 2022 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK534838

Ruiz, P., Celada, L., Seseña, S., & Palop, M. L., (2018) *Leuconostoc mesenteroides in the Brewing Process: A Controversial Role*. *Food Control*, 90, 415–421 https://doi.org/10.1016/j.foodcont.2018.03.019

Ryu, J. H., Kim, H., Frank, J. F., & Beuchat, L. R., (2004) *Attachment and Biofilm Formation on Stainless Steel by Escherichia coli O157:H7 as Affected by Curli Production. Letters in applied microbiology*, 39(4), 359–362

Saez, J. S., Lopes, C. A., Kirs, V. E., & Sangorrín, M., (2011) *Production of Volatile Phenols by Pichia manshurica and Pichia membranifaciens Isolated from Spoiled Wines and Cellar Environment in Patagonia*. *Food Microbiology*, 28(3), 503–509 https://doi.org/10.1016/j.fm.2010.10.019

Sakamoto, K., & Konings, W. N., (2003) *Beer Spoilage Bacteria and Hop Resistance*. *International Journal of Food Microbiology*, 89(2-3), 105–124

Salanță, L. C., Coldea, T. E., Ignat, M. V., Pop, C. R., Tofană, M., Mudura, E., Borșa, A., Pasqualone, A., & Zhao, H., (2020) *Non-alcoholic and Craft Beer Production and Challenges*. *Processes*, 8(11), 1–22 https://doi.org/10.3390/pr8111382

Sheth, N. K., Wisniewski, T. R., & Franson, T. R., (1988) *Survival of Enteric Pathogens in Common Beverages: An in Vitro Study*. *American Journal of Gastroenterology*, 83(6), 658-660

Shimwell, J. L., (1935) *The Resistance of Beer Towards Saccharobacillus pastorianus*. *Journal of the Institute of Brewing*, 41(3), 245–258

Siebert, K. J., (1999) *Modeling the Flavor Thresholds of Organic Acids in Beer as a Function of their Molecular Properties*. *Food Quality and Preference,* 10, 129-137

Simpson, W. J., Hammond. J. R. M., (1991) *Antibacterial Action of Hop Resin Materials*. *EBC Congress,* 185-192

Smart, K., (2002) *Brewing Yeast Fermentation Performance*. John Wiley & Sons, Incorporated.

Smit, Y., Cameron, M., Venter, P., & Witthuhn, R. C., (2011) *Alicyclobacillus Spoilage and Isolation - A Review*. *Food Microbiology*, 28(3), 331–349

Sofos, J. N., & Busta, F. F., (1981) *Antimicrobial Activity of Sorbate*. *Journal of Food Protection*, 44(8), 614-622

Sourri, P., Tassou, C. C., Nychas, G. J. E., & Panagou, E. Z., (2022) *Fruit Juice Spoilage by Alicyclobacillus: Detection and Control Methods—A Comprehensive Review*. *Foods*, 11(5) https://doi.org/10.3390/foods11050747

Stanojevic, D., Comic, L., Stefanovic, O., Solujic-Sukdolak. S., (2009) *Antimicrobial Effects of Sodium Benzoate, Sodium Nitrite and Potassium Sorbate and their Synergistic Action in Vitro*. *Bulgarian Journal of Agricultural Science*, 15(4), 307-311

Steele, M., (2012) *IPA: Brewing Techniques, Recipes, and the Evolution of India Pale Ale*. Boulder, Colo.: Brewers Publications

Stewart, P. S., (2003) *Diffusion in Biofilms*, *Journal of Bacteriology*, 185(5), 1485-1491

Sun, Y., Fukamachi, T., Saito, H., & Kobayashi, H., (2011) *ATP Requirement for Acidic Resistance in Escherichia coli*. *Journal of Bacteriology*, 193(12), 3072–3077 https://doi.org/10.1128/JB.00091-11

Suzuki, K., (2011) *125th Anniversary Review: Microbiological Instability of Beer Caused by Spoilage Bacteria*. *Journal of the Institute of Brewing*, 117(2), 131–155

Suzuki, K., (2015) *7 - Gram-positive Spoilage Bacteria in Brewing. Brewing Microbiology: Managing Microbes, Ensuring Quality and Valorising Waste*. *Food Science, Technology, and Nutrition,* 289, 141-173, Woodhead Publishing

Suzuki, K., Iijima, K., Sakamoto, K., Saihi, M., & Yamashita, H., (2006) *A Review of Hop Resistance in Beer Spoilage Lactic Acid Bacteria. Journal of the Institute of Brewing*, 112(2), 173–191

Van Vuuren, H. J. J., Priest, F., (2003) *Gram-Negative Brewery Bacteria*, *Brewing microbiology* (Third ed.), 219-246, Springer

Vriesekoop, F., Krahl, M., Hucker, B., & Menz, G., (2012) *125th Anniversary Review: Bacteria in Brewing: The Good, the Bad and the Ugly*. *Journal of the Institute of Brewing*, 118(4), 335–345

Wang, C., Cui, Y., & Qu, X., (2018) *Mechanisms and Improvement of Acid Resistance in Lactic Acid Bacteria. Archives of Microbiology*, 200(2), 195–201

Wang, W., Liu, Y., Sun, Z., Du, G., & Li, X., (2017) *Hop Resistance and Beer-spoilage Features of Foodborne Bacillus cereus Newly Isolated from Filtration-sterilized Draft Beer. Annals of Microbiology*, 67(1), 17–23 https://doi.org/10.1007/s13213-016-1232-4

Wang, X., Wang, Z., Feng, T., (2022) *Screening of Yeast in Various Vineyard Soil and Study on Its Flavor Compounds from Brewing Grape Wine*. *Molecules*. 2022; 27(2), 512 https://doi.org/10.3390/molecules27020512

Wareing, P., & Davenport, R. R., (2007) *Microbiology of Soft Drinks and Fruit Juices. Chemistry and Technology of Soft Drinks and Fruit Juices: Second Edition* (279–299). Blackwell Publishing Ltd

Warth, A. D., (1988) *Effect of Benzoic Acid on Growth Yield of Yeasts Differing in Their Resistance to Preservatives*. *Applied and Environmental Microbiology*, 54(8), 2091-2095

World Health Organisation, (2021) Accessed: 20/9/2022 https://apps.who.int/food-additives-contaminants-jecfa-database/Home/Chemical/4530

Willaert, R., (2006) *Section V. Beverages: The Beer Brewing Process: Wort Production and Beer Fermentation*. *Handbook of Food Products Manufacturing*, 1, 443–506. John Wiley & Sons, Inc

Wray, E., (2018) Antiformin S Pipe Cleaning Detergent, Murphy and Son LTD, https://www.murphyandson.co.uk/wp-content/uploads/2018/09/Antiformin-S-PCD-TDS-2018.pdf

Xiong, Z. R., Chen, A., Jiang, G. Z., Lewis, A. G., Sislak, C. D., Cobo, M., Worobo, R. W., & Gibney, P. A., (2021) *Evaluation of Foodborne Pathogen Die-off in Back-sweetened Wine and Apple Cider Models*. *Journal of Food Protection*, 84(6), 1023–1032 https://doi.org/10.4315/JFP-20-351

Zhao, T., Doyle, M. P., & Besser, R. E., (1993) *Fate of Enterohemorrhagic Escherichia coli 0157:H7 in Apple Cider with and Without Preservatives*. *Applied and Environmental Microbiology*, 59(8), 2526-2530

6.2 APPENDIX

Appendix 1: Analysis of beers from L'Anthoën and Ingledew 1996, Quain 2021 and Section 3.1.2. Table is sorted from lowest to highest level of fermentable sugars

	Beer	ABV	pH	Fermentables g/100ml (Glucose, fructose, and maltose)	Glucose g/100ml	Fructose g/100ml	Maltose g/100ml	Maltotriose g/100ml
Bartlett	Brand 2 0.5%	0.42	4.46	0.039	0.005	0.007	0.027	0.051
L'Anthoën	Regular	3.84	4.26	0.18	0	0	0.18	0.32
Bartlett	Brand 1 4.5%	4.42	4.41	0.219	0.014	0.098	0.107	0.124
Bartlett	Brand 1 0.5%	0.46	4.26	0.328	0.052	0.102	0.174	0.229
Bartlett	Brand 3 0.5%	0.51	4.19	0.329	0.003	0.067	0.259	0.223
Quain	PL8	4.59	3.98	0.43				
Quain	PL9	4.38	4.3	0.68				
L'Anthoën	Falcon Light	0.38	4.04	1.16	0.72	0	0.44	0.39
L'Anthoën	O'Doul's	0.38	4.86	1.18	0.78	0.05	0.35	0
Quain	AFB5	0.03	4.44	2.1				
Bartlett	MB1 0.5%	0.37	4.44	2.543	0.352	0.117	2.074	0.654
Quain	AFB3	0	3.9	2.73				
L'Anthoën	Molson Exel	0.46	3.64	2.84	0.68	0.12	2.04	0.55
Quain	AFB6	0	4.38	3.05				
L'Anthoën	Labatt 0.5	0.34	4.72	3.27	0.74	0.17	2.36	0.72
L'Anthoën	Miller Sharps	0.53	4.01	3.44	1.13	0.13	2.18	0.61
L'Anthoën	Tourtel	0.48	5.25	3.76	0.7	0.14	2.92	0.72
Quain	LAB2	0.38	4.61	3.9				
Quain	AFB4	0.02	4.04	4.1				
Quain	LAB1	0.4	4.15	4.2				
Quain	AFB2	0.03	4.28	4.25				
Quain	AFB1	0.01	4.06	4.37				