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The impact of composition, environment and hygiene on the microbiome and spoilability of draught beer

by

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Abbreviations

4-ethylguaiacol	4-EG
4-ethylphenol	4-EP
4-vinylguaiacol	4-VG
4-vinylphenol	4-VP
Acetic acid bacteria	AAB
Adenosine diphosphate	ADP
Adenosine trisphosphate	ATP
Alcohol by volume	ABV
Autoinducer peptide	AIP
British beer & pub association	BBPA
Carbon dioxide	CO_2
Clean-in-Place	CiP
Exopolysaccharides	EPS
Foam on Beer	FOB
Fusarium head blight	FHB
General practitioner	GP
Gram	g
Hectolitre	hL
High performance liquid chromatography	HPLC
Horizontal gene transfer	HGT
Internal transcribed spacer region	ITS
Lactic acid bacteria	LAB
Limit of detection	LOD
Limit of quantification	LOQ
Lin’s copper media agar	LCMA
Liquid chromatography mass spectrometry	LC-MS
Litre	L
Medium density polyethylene	MDP
Microlitre	µL
Micromolar	µM
Milligram	mg

Millilitre	mL
Millimolar	mM
N-acyl homoserine lactones	ATP
Nanomolar	nM
National health service	NHS
Next-generation sequencing	NGS
No and low alcoholic beverages	NABLAB
Optical density	OD
Oxford nanopore technologies	ONT
Parts per million	ppm
Polyethylene terephthalate	PET
Polymerase chain reaction	PCR
Polystyrene	PS
Polyvinyl chloride	PVC
Pasteurisation units	PU
Quality index	QI
Raka-ray	RR
Reverse osmosis	RO
Tricarboxylic acid cycle	TCA
United Kingdom	UK
Viable but non culturable	VBNC
WLD	WL differential agar
WLN	WL nutrient agar
Yeast, peptone, dextrose	YPD

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Abstract

Since the mid-1970s draught beer sales have been in decline. The on-trade has been impacted by changing consumer preferences, increased taxation, a competitive off-trade market and more recently the COVID-19 pandemic. Off-trade beer served in bottle/can is in the condition set by the brewer and will be 'in-spec'. Comparatively beer served at the on-trade is subject to variable beer-line hygiene and therefore quality is variable from account to account. Assuring beer quality at the on-trade is vital to a brewer's reputation and economic sustainability. But in light of changing dispense parameters and increased complexity, variable hygiene regimes and a declining market – there remain significant challenges ahead.

Draught beer microbiology has been a largely ignored area of research in the brewing industry since the early to mid-20th Century. Largely, our knowledge of draught beer spoilage microorganisms has been driven from historical works – with little to no work conducted on the microbiome of draught beer. Moreover, irrespective of the style of beer, beer spoilage microorganisms are presumed consistent irrespective of the distinct environmental differences between styles. Therefore, our primary work aimed to identify the 'culturable' microflora of draught beer across a range of styles. It was revealed in this work *Brettanomyces* sp. and *Acetobacter* sp. were ubiquitous spoilers of draught beer irrespective of style, and beer style did influence the microflora abundance. However, it was recognised in this work the culturable microbiome was not reflective of the true microbiome due to the phenomenon of 'non-culturability'. This work was later repeated using Oxford Nanopore Technologies MinION Next-generation sequencing (NGS) tool. For the first time NGS was conducted on draught

beer samples and revealed a number of new microorganisms from draught beer – but further demonstrated differences between beer styles and the impact of poor hygiene.

Biofilms in the brewing process are well-documented and although recognised in the on-trade are not discussed with equal importance. Biofilms can harbour numerous microorganisms and act as a source of fresh contamination after cleaning. Current cleaning practices at the on-trade do not utilise the four elements of 'Sinner's circle' (mechanical action or temperature) and would be classed as an ineffective CiP regime in the brewing industry and elsewhere. This project aimed to firstly develop a method capable of replicating and quantifying biofilms in dispense systems, which was both affordable, reproducible, simple, and high throughput. After the development of this method, it was proceeded by initial work on introducing the missing elements of the 'Sinner's circle', which was revealed to improve cleaning effectiveness using the assay.

Finally, it was noted throughout these works beers were spoiling at different rates despite being of the same style. In this work beers were subject to a range of analyses such as LC-MS for tracking tricarboxylic acids during forcing, high performance anion-exchange chromatography for fermentable quantification, free amino nitrogen (FAN), and pyruvic acid quantification. Moreover, the impact of supplementing a range of beers with sugars of varying complexity was analysed. This work revealed maltose to be a poorly used sugar irrespective of style or source inoculum, FAN and pyruvic acid to be potential spoilage indicators, and revealed beer spoilability began to assess the predictability of beers spoilability.

Chapter I: Introduction

1.1 A brief overview of draught beer

Alcoholic beverages have been a part of human society for more than 5,000 years; with evidence of brewing during the ancient Egyptian era. Hence, beer is arguably the oldest recipe in the world and its continuation in popular culture is testament to its significance in society. Beer continued to develop over the proceeding centuries, with hops first on record as being used in the 9th Century, the move to the mass-production of beer in the 14th and 15th Centuries by monasteries etc., and the significant impact of the industrial revolution enabled better control of fermentation coupled with the invention of the thermometer and hydrometer (Meussdoerffer, 2009). Consequently, over time brewing processes have improved, giving rise to the plethora of beer brands and styles seen today, with diverse flavours, colours, haze, and aromas. Similarly, the methods of packaging beer and beer stability improved significantly from the late 19th Century, beginning with Louis Pasteur. Pasteurisation was first detailed in the 'Studies on Beer' work published by Louis Pasteur in 1876. This work discusses the requirements to pasteurise beer to decrease the risk of infection. Initial methods used cabinets of steam and water, requiring high amounts of energy. Hence, during the early 20th Century methods to recapture the steam and hot water were developed (Wray, 2015). It was around the same period where draught beer began being served from 'pressurised containers', with artificial carbonation being introduced in 1936, driven by the consequence of pasteurisation inactivating live yeast *in situ* (Wray, 2015). By the 1970's draught beer had become synonymous with draught kegged beer, rather than traditional cask ales.

Bottled beer can be sourced back to over 400 years ago (Poelmans and Swinnen, 2011). Original bottled ale would undergo secondary fermentation *in situ*, carbonating

the beverage which is comparable to traditional wooden cask ales. Original glass bottles could not take the strain of the carbon dioxide; thus, bottles were required to be kept cool. However, as discussed earlier, the adoption of pasteurisation enabled the stabilisation of bottled beer. It was not until post the First World War that bottled beers popularity was rapidly increasing. As such, it was predicted in the 1950s bottled beer would take a market share majority, which would later be proven true.

Since the early 1970s, UK draught beer sales have been in almost linear decline. There are several reasons for this, key contributors include socioeconomic and political pressures. Since the mid-1970s draught beer sales have been in decline. The on-trade has been impacted by changing consumer preferences, increased taxation, a competitive off-trade market and more recently the COVID-19 pandemic. Off-trade beer served in bottle/can is in the condition set by the brewer and will be 'in-spec'. Comparatively beer served at the on-trade is subject to variable beer-line hygiene and therefore quality is variable from account to account. Assuring beer quality at the on-trade is vital to a brewer's reputation and economic sustainability.

Arguably for the dispense of draught beer, there is little-to-no control or consensus on line management and hygiene, resulting in variable beer quality. The lack of consistency of draught beer quality in the on-trade compared to bottled/canned beer has contributed to the increasing consumer shift to 'small pack' cans and bottles from supermarkets where consumers experience a consistently high-quality product. Furthermore, the growing disparity between price and quality where draught beer can exceed £5.00 per pint, a similar price to four cans of beer from a supermarket.

A common misconception is beer is immune to spoilage due to its antimicrobial components. Typically, lager will consist of ethanol (3-5% v/v), hop bitter iso-α-acids, low dissolved oxygen, depleted nutrients, and high CO_2. These conditions collectively are inhibitory to most microorganisms. However, some microorganisms are capable of surviving and proliferating in beer. Consequently, those adapted to a beers environment will – through growth and metabolism damage flavour, haze, and the aroma of beer.

1.2 Economic challenges facing the brewing industry

The UK brewing industry is currently going through its toughest period in modern history, simultaneously changing consumer preferences, political pressures, climate mandates and healthier living are continually increasing production costs and reducing turnover. With the on-going COVID-19 pandemic, the hospitality sector has been one of the worst affected industries through forced national closures.

1.2.1 Market trend of draught beer sales in the UK

In the UK draught beer sales have been in continuous decline since the mid-1970s. The British Beer and Pub Association (BBPA) statistical handbook reports a further 1.5% reduction of draught beer sales between 2017 and 2018, with sales responsible for just 41.9% of total UK beer sales. To put this into perspective, in 1980 draught beer sales accounted for 78.8% of total UK beer sales (BBPA, 2018).

Alcohol is always either purchased through the ‘on-trade’ (pubs, restaurants, hotels) or the ‘off-trade’ (shops, supermarkets, etc.). Historically drinking alcohol is a staple of socialising amongst most adults in the UK, therefore sales heavily favoured the on-

trade retailer. Increasingly, consumers are favouring packaged beer and in 2015 packaged beer sales were greater than sales of draught beer.

1.2.2 Healthier living and dietary requirements

Western culture often promotes an over-indulgence of luxury, whether it be travelling, food, or drink. Consequently, the UK has an ongoing obesity issue, which comes with its own problems regarding over reliance on NHS resources, demands on GPs and long-term disease, putting increasing strain on the NHS.

Brewers are conscious of the changing attitudes of consumers and political pressures, and in the past 10 years low and no alcohol beer sales have risen annually. Producing low and no alcohol beers is a clear example of brewers adapting to consumer demands. In April 2020, the BBPA reported no and low alcohol beer sales have increased 33% annually (BBPA, 2018).

1.2.3 Travel, legislation, and cost of driving

Society is becoming ever more reliant on vehicles, whether it be for work, sightseeing, meeting family etc. Almost any occasion is made simple by the availability of a car. Most families now possess at least one car, and it is more common for a family to own a car than not. With this, there has inevitably been an increase in road collisions and fatalities, which can often be linked to the presence of drugs and alcohol. Subsequently, increased legislation, has deterred people from drink driving.

Often, when we look back in history at generations gone by behaviours and lack of governance (e.g., smoking) it is accompanied by both shock and bemusement, “how did they not know better?”. However, in the case of drink driving, the UK seems to

have been ahead of the curve. In 1872 it was deemed an offence to be drunk whilst manning carriages, horses, cattle, and steam engines. For the proceeding 80-90 years, many updates to the law were introduced to include 'mechanically propelled' vehicles, however it was not until 1967 that a legal driving limit was set. A blood alcohol limit of 80mg of alcohol per 100mL of blood was set, which still stands today.

In 1991 'causing death by driving while under the influence of alcohol or drugs' carried a compulsory custodial sentence, a maximum of 5 years. Now, the same law carries a compulsory sentence, a maximum of 14 years, unlimited fine, a driving ban for at least 2 years and the requirement to resit an 'extended driving test'.

1.2.4 Choice

Oddly, the sheer amount of choice available is a possible factor driving draught beer sales down. There are over 3,000 breweries in the UK and the number of diverse beers available to the consumer is overwhelming (BBPA, 2020). In the on-trade there are only so many draught beer taps (fonts) available and these are often prioritised for national 'big brand' beers and ciders (e.g., Carling, Fosters, Heineken, Strongbow, and Guinness). For those willing to expand beyond these brands there may be no option but to opt for a canned/bottled product. From my own experience, the millennial generation is preferring a fruitier beverage; the popularity amongst young drinkers of fruit ciders (Strongbow Dark Fruit, Kopperberg etc.) is growing, with only Strongbow Dark Fruit commonly available on draught. Fruit ciders in 2015 held a 25% market share of cider, in 2019 this had risen to 40% (Carling Partnership). Hence for those preferring beer over cider opt for 'craft' beers from breweries such as Beavertown, Tiny Rebel or Neon Raptor. These craft beverages are often packed with fruity flavours in contrast to a standard lager.

1.2.5 Alcohol advertisement and consumption restrictions

A central question around alcohol advertisement is whether total consumption is increased by advertisement or only brand choice is influenced for the consumer (Saffer and Dave, 2002). Limiting advertising restricts the ability of a brewer to maximise the potential of their brands and therefore profits. Strict limits on advertising were first introduced by France in 1993, which was soon expanded to the European Union (Saffer and Dave, 2002). Any alcoholic beverages or those classed as foodstuff over 0.5% ABV are subject to advertising regulations in the United Kingdom. Although there are no restrictions on location, time, media-channel, type, or advertiser on alcohol. One of the major restrictions is there must be no appeal to anyone <18 years of age, hence adverts in places targeting children, such as children's TV are not permitted.

1.2.6 Socioeconomic influences

The consumer shift to packaged beer is an unfortunate reality, where beer at the on-trade is increasingly becoming a luxury due to rising costs. Due to the rising political pressures to control the UK binge culture by rising taxes on alcohol sales, it has forced on-trade retailers to adapt. Increased prices, staff redundancies, reduced cellar management and increased reliance on technology are just some examples of how the on-trade is responding. Reduced staff eventually results in poorer service for the consumer, increasing frustration and further less time is input into managing dispense lines appropriately will inevitably impact beer quality. Poor draught beer, or 'off-beer', is an unpleasant experience and often results in customers complaining, leaving, or changing brand.

UK binge culture is a term popularised by the media and has subsequently directed pressure towards the government to begin tackling the issue. Further, other downstream issues with alcohol in regard to addiction, domestic abuse, and poor health provide extra credence to reducing alcohol consumption. In response, the UK government has increased VAT (up 5% since 2015) and excise duty on beer has risen by 60% since 2000.

As the minimum wage lags behind the rate of inflation, the amount of disposable income per household is shrinking, whilst alcohol prices rise. Further rental prices are astronomical, particularly in London, UK, and is further driving prices up. In London, UK, prices average at over £5.50 per pint, the UK average currently sits at £4.07 (2022) a 7% increase on 2021. Therefore, it is becoming increasingly popular for people to purchase packaged beer from off-licenses and drink at home rather than at the local pub/bar.

1.2.7 2020 COVID-19 pandemic

The COVID-19 pandemic closed all on-trade accounts for much of the year and when allowed to open operations were under economically difficult restrictions:

- 22:00pm national curfew
- 2 metre social distancing
- 6-person limit indoors per group
- Table service only

Under government instruction, food and drink establishments were required to ask all customers to sanitise their hands before entry and advised to check temperatures.

Further, front of house hygiene was at the forefront as one case of COVID-19 could result in temporary closure. Coupled with staff redundancies during the pandemic, many hygienic practices away from the public eye were arguably infrequent or forgotten. For example, time consuming line cleaning may have been 'parked' as there was no longer enough staff to manage both table service and dispense hygiene and insufficient turnover to hire extra staff. Moreover, line cleaning practices are often seen to be as time better spent serving beer and may incur a loss of turnover.

1.3 The brewing process and the microbiological challenges

Brewing beer is a complex process whereby the all-important and longest step - fermentation - is preceded and proceeded by various process steps. Depending on the brewing process, the raw materials used dictate the organoleptic properties of the beer, explaining the numerous beers styles available. For industrial scale brewing (thousands of hL), each process is controlled to ensure quality and consistency of the product.

Brewing is steeped in tradition. In Germany the *Reinheitsgebot* limits brewing to four essentials, water, yeast, hops and malted barley (Boulton and Quain, 2013). Elsewhere in the brewing world, other raw materials are used (oats, rye, fruit, rice, maize etc). However, the technology to improve the brewing process has seen significant advancement (Mousia *et al.*, 2004). There are seven essential steps, malting, milling, mashing, lautering, wort boiling, fermentation, and conditioning. Each invite different technical and contamination challenges. This section reviews each of the major contamination risks and impacts at each essential brewing step.

1.3.1 The brewing process

The brewing process can be separated into malting, milling, mashing, lautering, wort boiling, fermenting, conditioning, filtration, pasteurisation (or sterile filtration) and packaging (Figure I.1). The aim of brewing is to produce ethanol in a flavourful beverage fit for consumption. By controlling specific conditions such as barley roasting, malt, sugars, oxygen and many more factors, yeast produces ethanol alongside other positive organoleptic compounds.

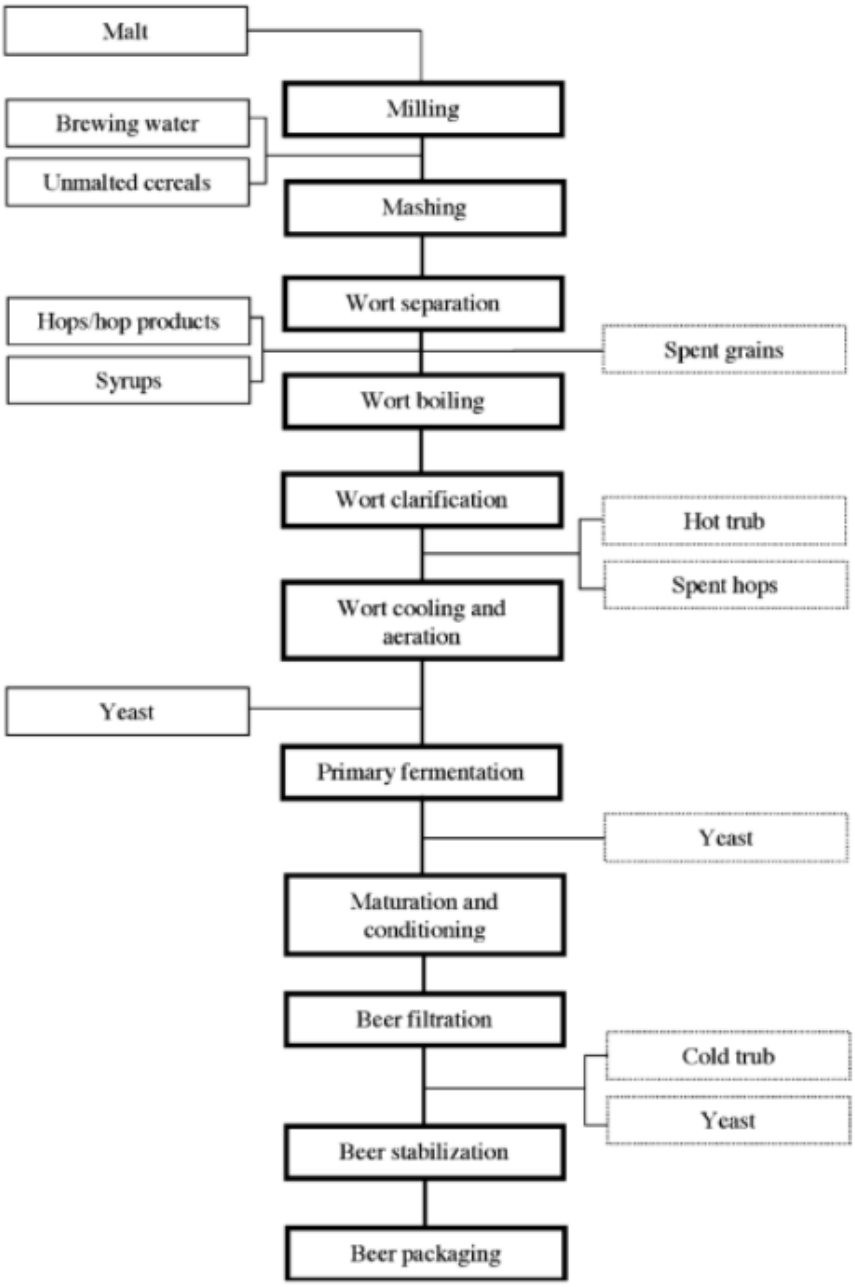


Figure I.1: Schematic of the brewing process, sourced from Willaert (2007b)

1.3.2 Barley and malting

Malting is the controlled germination of barley, a process initiated by 'wetting the grains', known as steeping (Boulton and Quain, 2013, Briggs, 1987). This step initiates the germination of the barley by increasing the moisture content to between 42%-48% (Briggs, 1987). The water (alias; steep liquor) is prone to contamination and requires changing at least once, as the microorganisms from the natural microbiota of barley can cause issues (discussed following) (Briggs, 1987). When the barley germinates, enzymes are triggered making starch reserves available for the developing embryo (Boulton and Quain, 2013). Hydrolases are released during germination, which breakdown the starchy endosperm and release essential nutrition for yeast (sugars) (Briggs, 1987, Kunze, 2004). The germination process is stopped by kilning, with desirable enzymes and starch for later downstream processing for wort production available. Kilning, although primarily used to cease enzymatic processes, can also be used to introduce a range of malt flavours, such as dark roasted malts found in stouts and porters.

The indigenous microbiota of barley is problematic for the brewing process. As aptly put by Lowe *et al.* (2005) 'microbial spoilage as a result of these microorganisms often leads to technological impediments in the malting and brewing processes including raw material spoilage, filtration problems, and deleterious effects on both the fermentation process and final beer'. Contamination of the malt can produce off-flavours in the final product. Without appropriate control during malting, wild yeasts, lactic acid bacteria and Gram negative bacteria can produce haze, off-flavours and impact beer quality (Vaughan *et al.*, 2005). During steeping undesirable microbiota compete for nutrients with the developing embryo (Bokulich and Bamforth, 2013).

Ultimately this inhibits germination and decreases rootlet growth and alpha-amylase activity (Briggs and Mcguinness, 1993).

Moulds are an issue for barley and malt; the microflora is influenced by how the barley is grown and stored (Vaughan *et al.*, 2005). *Fusarium* sp. is a disease causing mould in barley, known as *Fusarium* head blight (FHB) (Nielsen *et al.*, 2014). The mycotoxins produced by *Fusarium* sp. are harmful to both humans and animals (Desjardins, 2006). FHB directly influences the quality of the barley grain, reducing both functionality and seed germination. Consequently, the final beer product will contain off-flavours and clear changes of appearance (Oliveira *et al.*, 2012). As such, the European Union set legislation to control the maximum level of mycotoxins in foodstuff (Commission, 2006). Deoxynivalenol (DON) is the most abundant mycotoxin produced by *Fusarium* sp. and poses a threat to human and animal health (Sobrova *et al.*, 2010). Many studies record the thermal resistance of DON mycotoxins, with notable levels of DON measured up to 350^{o}C (Sobrova *et al.*, 2010). Although DON does not constitute a major threat to health, side-effects can include nausea, vomiting (Perkowski *et al.*, 1990), fever, dizziness and abdominal pain (Sobrova *et al.*, 2010).

Fungal infection of barley can also result in the production of hydrophobic fungal peptides, which cause ‘gushing’. Gushing is when a newly opened container ‘gushes’ with excess gas and foam (Bokulich and Bamforth, 2013). This phenomenon can cause economic loss to brewers (Khalesi *et al.*, 2012). The relationship between grain microbiota and gushing was first noted in 1950s (Garbe *et al.*, 2009). Hydrophobins are described as ‘small surface-active proteins produced by filamentous fungi’ (Sarlin

et al., 2007). Gushing is associated with many fungal species, the most prominent being *Fusarium* sp. Others include, *Aspergillus* sp., *Penicillium* sp. and *Nigospora* sp. (Havlová *et al.*, 2006, Mastanjević *et al.*, 2017). The hydrophobic peptides secreted by fungal contaminants 'serve as nucleation sites for CO_2 bubbles in beer', resulting in foaming and excess gas (Bokulich and Bamforth, 2013). Hydrophobins are extremely thermostable and after the brewing process approximately 10% will remain in the final beer (Mastanjević *et al.*, 2017). Further, treatment of fungicides can often stimulate hydrophobin production from fungi rather than reduce gushing (Havlová *et al.*, 2006). Hence, alternative means of fungal control are necessary.

At this stage of the process, it is inevitable microorganisms will inhabit the surface of barley growing in fields. As discussed, most issues are due to infection with *Fusarium* sp., therefore most effort is focussed on inhibiting its growth. Currently, there are a few accepted methods to achieve this aim. Firstly, reducing the steep temperature helps prevent significant microbial growth (Bokulich and Bamforth, 2013, Petters *et al.*, 1988). Lactic acid bacteria have been shown to have antifungal properties and may support the stability and flavour of the product (Lowe and Arendt, 2004, Schillinger and Villarreal, 2010). Furthermore, *Pichia anomala* (alias: *Wickerhamomyces anomalus*) (Laitila *et al.*, 2007, Laitila *et al.*, 2011) and *Gliocladium roseum* (Knudsen *et al.*, 1995) have been shown to suppress *Fusarium* sp. growth in malt and proposed as a possible bio-control agent during steeping.

1.3.3 Mashing and wort production

The mashing process influences the style and quality of beer produced (Montanari *et al.*, 2005). In the first stage the malt is milled to a 'grist' that is suspended in warm water (Boulton and Quain, 2013). The aim of milling is two-fold: firstly to reduce

particle size for mashing and secondly to release contents of the malt endosperm (Boulton and Quain, 2013). The grist is hydrated and is held at various temperatures.

- 45-50°C: β-glucans and protein hydrolysation
- 62-65°C: maltose production
- 70-75°C: saccharification (α-amylase)
- 75-78°C: inactivation of enzymes, stabilising fermentable sugars (Briggs *et al.*, 2004, Willaert, 2007)

Each temperature optimally activates the desired enzymes. There are three key enzymatic processes which occur during mashing:

- Hydrolysis of gelatinised starch into fermentable sugars
- Hydrolysis of proteins into amino acids and small peptides
- Degradation of β-glucans (Brandam *et al.*, 2003)

The conversion of starch into fermentable sugars is the most important enzymatic process as this determines the ethanol concentration in the beer (Brandam *et al.*, 2003).

1.3.4 Lautering

Lautering ('wort separation') is when malt husk and other insoluble material ('spent grains') are separated from the wort (Willaert, 2007). The aim is to recover as much extract as is possible. Lautering is a filtration process completed through the use of a lauter tun or, increasingly, a mash filter (Boulton and Quain, 2013). Microbes play both a positive and negative role in the Lautering process. *Fusarium* sp. remains the key problem at this point of the brewing process. However, at this stage, it is possible to use lactic acid bacteria (LAB) as a starter culture to inhibit growth of *Fusarium* sp. and other harmful Gram-negative bacteria (Linko *et al.*, 1998). The addition of LAB

as a starter culture has also been shown to improve mash filterability by reducing the molecular weight of β-glucans when applied during the steeping of barley (Suortti, 1993).

1.3.5 Wort boiling

The wort is sterilised by wort boiling and the remaining active enzymes from the malt are denatured. Importantly, hops are added during wort boiling and α-acids are isomerised to iso-α-acids which contribute the bitter flavour to beer (discussed in 1.9.1) (Boulton and Quain, 2013). Depending on the hop varieties, they can be added earlier or later if required for bitterness or aroma (Boulton and Quain, 2013). Some unwanted wort volatiles and hop oils are also removed during boiling ('volatilisation') (Hudson and Birtwistle, 1966). Sterilisation of the wort ensures the only microorganisms fermenting wort sugars is the pitching yeast.

1.3.6 Fermentation

Fermentation is the key transformational step in the brewing process. Post boiling the wort is cooled and yeast is added, which ferments the available sugars to produce alcohol. When producing beer, it is important the correct yeast species/strain is used together with the correct fermentation temperature. For example, for ales *Saccharomyces cerevisiae* is used at 20^{o}C and for lagers *Saccharomyces pastorianus* at 8-12^{o}C (Kodama *et al.*, 2006). Other styles such as lambic beers are produced via 'wild-fermentations' using a diverse range of opportunistic yeasts (e.g., *Brettanomyces* sp.) and bacteria (*Lactobacillus* sp.), resulting in a unique and challenging flavour profile.

Minimising the microbial contamination of fermentation vessels on an industrial scale is a difficult task. As described by Boulton and Quain (2013) for vessel hygiene to be maintained three pre-requisites include:

1) ‘All vessels must be capable of being cleaned in between individual fermentations to remove soiling and avoid the possibility of taints being introduced into the product
2) It may be necessary to disinfect the vessel, prior to filling with wort, to minimise the risk of subsequent microbial contamination and to ensure that all yeast from the previous fermentation is removed
3) It most cases but not all, after the fermentation has commenced the vessel must present a microbiological barrier to the external environment. This is to prevent microbial contaminants gaining entry to the vessel and to confine the yeast within the vessel to minimise the risk of cross-contamination where several yeast strains are used in a single brewery’

These pre-requisites are essential to prevent contamination by spoilage yeast or bacteria. Any batches that become contaminated has an impact on the beer quality, costing time and money.

1.3.7 Conditioning

Conditioning or ‘maturation’ of beer is a well-established process (Derdelinckx *et al.*, 1992, Van Landschoot *et al.*, 2004). The term ‘green beer’ is used to refer to beer directly from primary fermenter (Stewart, 2004). Green beer usually contains debris (protein, polyphenol) from fermentation, brewing yeast and off-flavours (diacetyl) (Krogerus and Gibson, 2013, Pires *et al.*, 2015). Firstly, the green beer is transferred

to the maturation tank, chilled to approximately -1°C to -3°C and held for a few days. During this period, residual sugars and off-flavours are reduced by the yeast. At cold temperatures and due to a lack of available sugars, yeast sediments, leaving behind a clean beer. At this time, priming sugar, flavours, and bitterness can be added.

1.4 Beer styles

"Beer" is an umbrella term to describe a large number of beer styles, each style different from another in aspects of flavour, aroma or appearance. The number of beer styles is increasing due to the popularity of craft breweries creating different and experimental beers. Typically, beer can be categorised into four main styles: lager, stout, keg ale and cask ale, the latter two differ in their packaging, albeit there are a number of less conventional styles such as kolsch, altbier, gose, sake, and sorghum. The brewing process and the materials whilst broadly comparable will differ in terms of hops (variety, addition), malt (style, colour), fermentation temperature, and the yeast(s) used.

1.4.1 Lager

Lager beers are produced using bottom fermenting yeast (*S. pastorianus*), which flocculate and sediment to the fermentation cone at the end of fermentation (Kodama *et al.*, 2006). Lager yeast is traditionally fermented between 8 – 12°C, the product of the fermentation undergoes 'lagering', where the beer is matured or conditioned in the cold for a few weeks. *S. pastorianus* is the typical lager yeast, it is a hybrid of *S. cerevisiae* and *Saccharomyces eubayanus* (Kodama *et al.*, 2006). On average the standard pale lager has an ABV % between 4 – 6 %.

1.4.2 Ale

To produce ales, 'top-fermenting' yeasts are used (*S. cerevisiae*), where the yeast forms a 'head' at the top of the vessel during fermentation by association with rising CO_2 bubbles that rise to the surface (Pavsler and Buiatti, 2009). Average temperature of an ale fermentation is between 20 – 25^{o}C. Ale yeasts belong to *S. cerevisiae* species, with optimum fermentation temperatures between 20 – 25^{o}C but can grow above 37^{o}C (Tornai-Lehoczki and Dlauchy, 2000). Stouts are a type of ale, produced with roasted malt to create a dark or black beer.

1.5 Packaging and ensuring 'commercial sterility'

Packaging in kegs, cans, or bottles (glass, aluminium, or polyethylene terephthalate (PET)) requires the beer to be 'commercially sterile' to eliminate the threat of microbiological spoilage during the shelf life of the beer. In order to achieve this a number of methods are used.

1.5.1 Pasteurisation

Pasteurisation is the common method of heating beer before packaging. Named after Louis Pasteur, who found that after 'pasteurising' beer 'can be transported without detriment or deterioration'. The aim is simply to kill any microorganisms in the beer by applying heat over time, achieving microbial stability. A pasteurisation unit (PU) is equivalent to 1 minute at 60^{o}C (Horn *et al.*, 1997, Vecchio *et al.*, 1951). As the scale is logarithmic, temperatures above 60^{o}C result in a marked increase in PUs.

1.5.2 Tunnel pasteurisation

Tunnel pasteurisation or batch pasteurisation is used for bottle or canned beer. The heat required to pasteurise is applied by spraying the bottle/can with increasingly hot

water (Horn *et al.*, 1997). Figure I.2 demonstrates the various heating and cooling steps during tunnel pasteurisation. (Bhuvaneswari and Anandharamakrishnan, 2014).

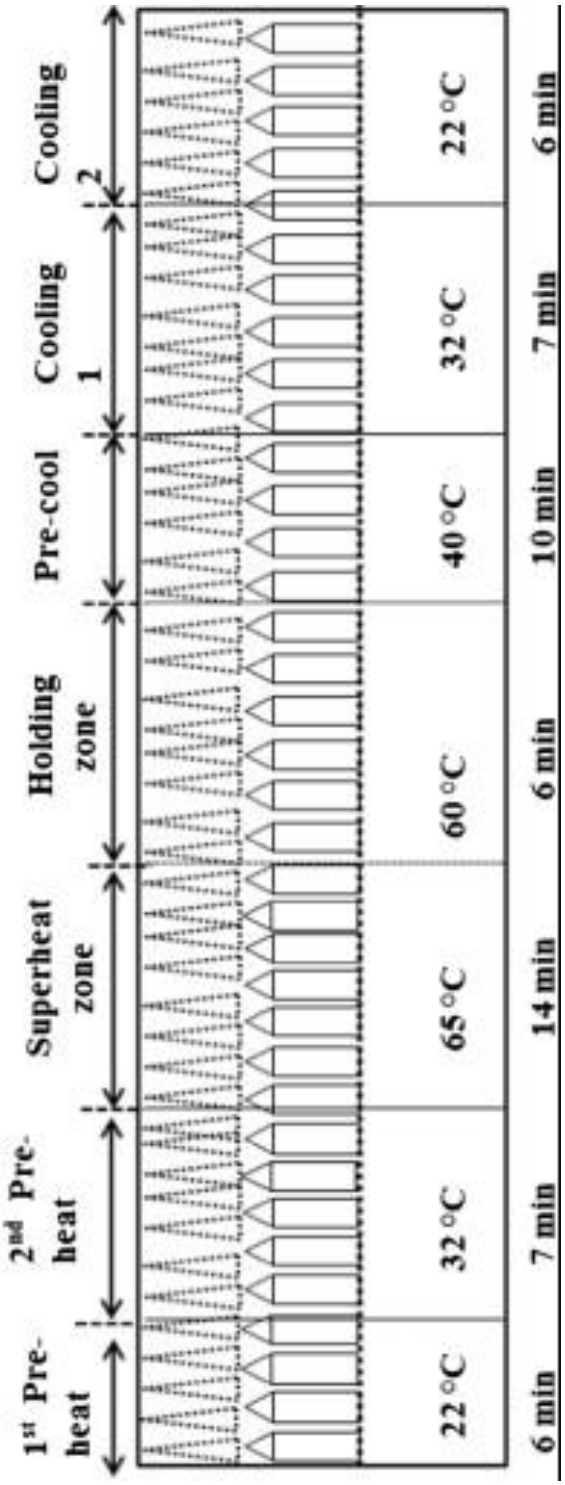


Figure I.2: Schematic of tunnel of pasteurisation, sourced from (Bhuvaneswari and Anandharamakrishnan, 2014)

1.5.3 Flash pasteurisation

Flash pasteurisation is typically used for draught beer and involves the heating of the beer in a heat exchanger to – for example – 72.5^{o}C for 20 seconds (Wray, 2015). This applies 20 PUs to the beer, sufficient for microbial inactivation (Wray, 2015). The pasteurised beer is vulnerable to microbial contamination so downstream processing must be hygienically assured. The beer is held in sterile buffer tanks that 'feed' the keg line.

1.5.4 Sterile filtration

Sterile filtration technology is replacing pasteurisation as it performed cold and does not subject beer to high temperatures that potentially damage flavour. Sterile filtration through 0.2-0.45µm filter removes microorganisms from beer. To minimise blockages multiple layers of filters of decreasing pore sizes are used (Briggs *et al.*, 2004). The technology is used with cans and bottles that are filled aseptically in a sterile environment (Kunze, 2004).

1.5.5 Kegs and kegging

Draught beer is dispensed from (mostly) stainless steel kegs of various capacities ranging from 20 to 163 litres with 50L the most common. Kegs have a single opening with a tamper proof valve with a 'spear' from which beer is removed to the dispense system.

Kegs undergo a separate washing and steam sterilising process before being filled (racked) with pasteurised beer. Initially the keg is cleaned with hot water (70^{o}C) and an alkaline detergent, followed by sterilisation with wet steam. The keg is heated to

105°C for approximately 1 minute and excess moisture is removed by purging with CO_2. This step ensures the removal of oxygen and creates back pressure reducing foam formation on filling.

1.5.6 Cask and cask racking

Compared to keg beer, cask beer is more difficult to manage and requires greater training in cellar management. Cask beer is not pasteurised and is a live product containing viable 'secondary' yeast. Further product carbonation is produced by secondary fermentation of priming sugars or residual sugar left from fermentation. Typically, beer containing $1 – 3 \times 10^6$ yeast cells/mL is racked. Collagen 'finings' are added to the cask to aid the settlement of yeast, which ensures a visually 'bright' beer.

The dispense of cask beer is different from keg in a few important aspects. With cask, a tap is driven into the cask through the keystone and attached to the dispense line. The carbonation of the beer is managed by piercing the cask shive with a peg ('spile') which can be made of 'hard' or 'soft' wood to manage the release of carbon dioxide. Kegged beer is dispensed by the introduction of carbon dioxide, creating a top pressure, and pushing the beer through the dispense line. However, with the peg removed cask dispense pulls beer from the container drawing in air. This exposes the beer to airborne microbial contaminants and oxygen which damages beer flavour. Therefore, once a cask is broached it should be used as soon as possible, ideally within three days.

1.5.7 Small pack: bottle and can

Small pack beer is pasteurised *in situ* using a tunnel pasteuriser and is commercially sterile when leaving the brewery. The majority of small pack beer is sold in the off-

trade (supermarkets etc.). Due to be not being exposed to the environment prior to serving and assuming it has been kept in a cool or refrigerated place and out of direct sunlight, bottle and canned beer is served in the condition intended by the brewer.

1.6 Dispense

There are three main components to a dispense system, the keg, the beer line, and the tap. Keg beer is dispensed through the influx of CO_2 into the keg, pushing the beer through the beer line and dispensed through the tap. However, a relatively simple method of dispense, numerous innovations by brand owners have added complexity, notably temperature and the assurance of 'cold' beer. However, the root of dispense complexity is the design of cellars which are too remote (20-50 m) from taps and are maintained at 12ºC rather than at 2-4 ºC (as in the USA). This has resulted in a diversity of hygienic challenges resulting beer of varying quality at the point of dispense. As such, there has been an increased roll-out of smart dispense systems, which refrigerate the line from keg to tap. Heineken's smart dispense system is one such example.

1.6.1 The cellar

Cellars are the storage place for keg and cask beer, pivotal in maintaining quality beer from delivery to serving. By employing an efficient, effective, and robust cellar management regime, a retailer can expect improved yield, profits, customer satisfaction and reduced waste.

In traditional/'older' public houses, cellars are located beneath the bar(s) where it is cooler. In newer accounts, cellars are on the same level or above the bar. The temperature of a UK cellar is typically between 12ºC and 15ºC. However, it would be

beneficial for retailers to reduce cellar temperatures to <4ºC. However, the costs of installing, maintaining, and running at such an operating temperature are considered prohibitive.

The dispense equipment found in a cellar includes keg couplers, beer lines (bundled together as a 'python'), foam on beer (FOB) detectors, remote coolers (glycol or water) and dispense gases (carbon dioxide or mixed blends of carbon dioxide and nitrogen). All dispense systems face hygienic challenges which are minimised by implementing best practice.

1.6.2 Cooling

Cellar cooling reduces the product temperature and improves the shelf life of keg and cask beer. The average UK cellar temperature is between 11ºC and 13ºC which is ideal for cask beer but not for keg beers which range from (ideally) 2-6ºC (lager) to 6-10ºC (ale). Beer lines in the python are cooled by cold water (glycol) circulating from the remote cooler with additional underbar cooling for lagers.

1.6.3 Keg couplers

Keg couplers are the fitting which links the beer line and CO_2 to the keg. There are six types of keg connectors used in the UK, which vary with brand owner/Brewer. Best hygienic practice requires regular cleaning of couplers and the use of sanitising spray on changing kegs. Regrettably this practice is rarely adopted! It has been reported that keg couplers can act as a source of contamination of kegs (Quain, 2015).

1.6.4 Foam on beer (FOB) detectors

When a keg runs out of beer, FOB detectors prevent the ingress of beer foam into the dispense system by shutting down the line, preventing waste and dispense issues. To

control foam build up, FOB detectors use a float control system, which detects beer flow in a dispense line. When a fresh keg is tapped, the beer lifts the float control system and beer flows freely to the tap. However, as the dispense pressure reduces as the keg empties, the float control lowers and seals the line stopping dispense.

1.6.5 Beer lines

The dispense line is the plastic tubing that connects the keg to the tap. Beer line tubing ranges 5-30 m (or more) and can remain in place for 10 years or more. This can be attributed to the complexity of dispense installations or servicing, with replacement being physically difficult and expensive. Dispense tubing is typically made from mid-density polypropylene lined with nylon with innovations to minimise gas transfer (carbon dioxide out, oxygen in). Microbial contaminants attach to the inner lining of a dispense tube to form biofilms on the surface, which is promoted due to a number of environmental conditions, further detailed in Section 1.13. In the dispense system, tubing offers the largest surface area for microbial attachment.

1.6.6 Fonts, taps and nozzles

At the end of dispense, beer travels through the taps and nozzles and is exposed to the ambient temperatures of the public house. Tap environments will be moist from residual beer, moderate temperature (approx. 20^{o}C), exposed to oxygen and subject to human contact. These conditions are ideal for efficient microbial growth.

A common practice employed in bars is the soaking of tap nozzles in carbonated soda water on the back bar overnight (Quain, 2016) in the mistaken belief that soda water is anti-microbial. Recommended best practice is to soak the tap nozzles in line cleaner overnight and rinse with fresh water prior to use. Quain (2016) demonstrated that it is

more effective to soak nozzles in water with an added (commercially available) sanitising tablet.

1.7 Line cleaning

Cleaning beer lines is essential for a retailer to serve high quality beer. Line cleaning is performed with propriety solutions (about 2%, w/v) of sodium (occasionally potassium) hydroxide containing various additives (wetting agents etc). It is recommended by the British Pub and Pub Association (BBPA) that a line cleaning cycle is performed once a week. Typically, a line cleaning cycle is as follows:

- Beer is chased out with water until water is dispensed from the tap
- Line cleaning solution is pulled through and sits for 30 minutes (typically 'moved' after 15 minutes)
- The line cleaning solution is chased out with water
- Beer is then pulled through

Though this is a simple process, it is an effective method when conducted properly and regularly. In reality, it is one of the many demands of running a bar/pub, and unfortunately line cleaning is a laborious and time-consuming task. Accordingly, 'corners are cut' and the frequency of line cleaning is often 'relaxed' to every two (or more) weeks. Occasionally, lines are cleaned with stronger line cleaning solutions ('bottoming out') which can damage the liner causing 'pits'.

1.7.1 Best hygienic practice

Line cleaning works to a limited degree – cleaning must then be repeated a week later. Although planktonic microorganisms in the beer are chased out by water, the

challenge of line cleaning is to remove the sessile microorganisms in the biofilm attached to surfaces. This is clearly ineffective as a consequence of occurring in one direction (resulting in blind spots), the difficulty of cleaning connectors and – most importantly – the lack of mechanical action.

1.7.2 Sinner's circle

For a cleaning-in-place (CiP) system to be effective, the 'Sinner circle' can be used to design an efficient and cost-effective cleaning routine. The 'Sinner's circle' identifies four key elements essential for effective cleaning (Figure I.3:

1. Time
2. Temperature
3. Chemical action
4. Mechanical action

First proposed by Dr Herbert Sinner in 1959, the Sinner's circle has become a tool for designing effective cleaning protocols for CiP systems. The concept has since been extended to include coverage, as without sufficient coverage of the target area cleaning will not be effective (Tamime, 2009b). The principle of the Sinner's circle can be seen in many domestic applications. For example, to be effective a dishwasher requires heat, time, chemical action, and mechanical action. Other examples include washing machines or manual dish washing. For an industry scale, these are almost universally applied to CiP systems in the food and beverage industry (Tamime, 2009a)

Current line cleaning regimes do not consider all of the ‘Sinner circle’, and therefore it is suggested that it is ineffective in removing biofilms. In particular, the lack of any mechanical action will undermine cleaning.

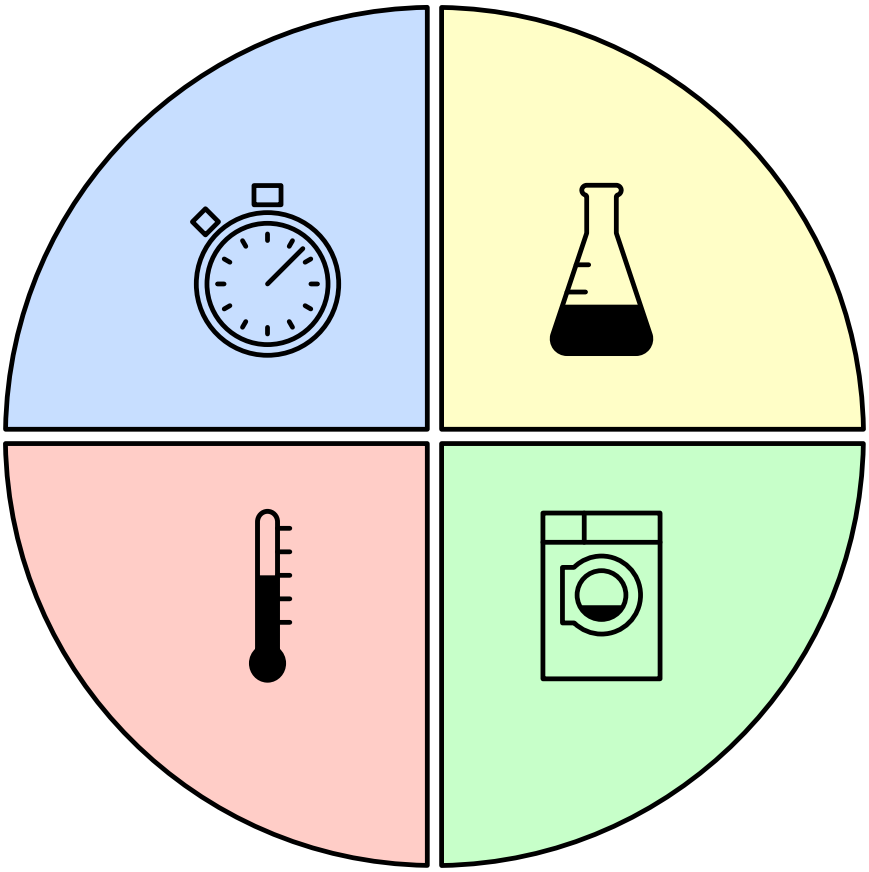


Figure I.3: The four key elements of the 'Sinner circle' - from top left, clockwise; time, chemical action, mechanical actions, and temperature.

1.8 Beer composition

The microbial stability of beer has been overstated. Although there are a number of inhibitory parameters - low pH, low nutrient availability, low oxygen, antimicrobial hop bitter acids, and 4-5% ABV – beer will still spoil through the growth of microorganisms. Although beer does not support the growth of pathogens and is therefore a 'safe beverage', it not immune to spoilage by a supposed narrow selection of yeast and bacteria.

Beer is a complex beverage, rich in organic acids, polyphenols, vitamins and minerals (Buiatti, 2009). Some are sourced from the raw materials and others produced during the fermentation process (Buiatti, 2009). Table I-1 is taken from Buiatti (2009), which is a modified version of the original table published by Hardwick (1994).

Table I-1: Approximate concentrations of a range of substances found in beer, the approximate number of compounds, and further the source of these substances (Buiatti, 2009).

Substances	**Concentration**	**Number of compounds**	**Source**
Water	90-94%	1	--
Ethanol	3-5% v/v	1	Yeast, malt
Carbohydrates	1-6% w/v	~100	Malt
Carbon dioxide	3.5-4.5 g/l	1	Yeast, malt
Inorganic salts	500-4000 mg/1	~25	Water, malt
Total nitrogen content	300-1000 mg/l	~100	Yeast, malt
Organic acids	50-250 mg/l	~200	Yeast, malt
Higher alcohols	100-300 mg/l	80	Yeast, malt
Aldehydes	30-40 mg/l	~50	Yeast, hops
Esters	25-40 mg/l	~150	Yeast, malt, hops
Sulphur compounds	1-10 mg/l	~40	Yeast, malt, hops
Hop derivatives	20-60mg/l	>100	Hops
Vitamin B compounds	5-10 mg/l	13	Yeast, malt

1.8.1 Fermentable sugars

During the brewing process, starch is hydrolysed by malt enzymes (α and β amylase) to produce fermentable sugars (Bering, 1988) which are metabolised by yeast to produce ethanol during fermentation. Sugars are hydrolysed through glycolysis, providing energy for the yeast cell to grow. Carbohydrate levels in beer range between 3 – 61 g/L (Ferreira, 2009). Beer styles can differ too, whereby ales possess on average higher amounts of carbohydrates (15-60 g/L) compared to lagers (10-30 g/L) (Ferreira, 2009). Table I-2 details the approximate carbohydrate levels in beer styles (Buiatti, 2009). Post fermentation, the 'green beer' is conditioned (or matured) at low temperature to improve flavour consistency and remove solids (by sedimentation). Priming sugars and other additions (colour, hop extracts) may be added during conditioning (Briggs *et al.*, 2004).

Table I-2: Carbohydrate concentrations of various beer styles. Data is sourced from (Clement *et al.*, 1992, Uchida *et al.*, 1991, Verachtert and Derdelinckx, 2014)

	Carbohydrate (g/L)		
Beer type	**Total**	**Sugars (including maltotriose)**	**Dextrins**
Beer mean values	2.8 – 61	1.3 – 22	7 – 39
Pilsner	30	3.65	24
Lagers	10 – 30	1 – 7	10 – 20
Ales and stouts	15 – 60	5 – 10	10 – 40
Primed beers	20 – 70	13 – 36	10 – 40
"Lite" beers	2 – 9	1 – 6	1 – 3

Beers can contain monosaccharides, disaccharides, trisaccharides and, dextrin (Buiatti, 2009). Most are residual unfermented carbohydrates from the wort (primarily dextrin), although some may be sourced from priming sugars to add sweetness to the final beer. A breakdown of unfermented carbohydrates in beer can be found in Table I-3 (Baxter and Hughes, 2001). Monosaccharides (glucose, fructose) are quickly metabolised via glycolysis, producing energy (ATP) for growth and the metabolic end product, ethanol. More complex carbohydrates (maltose, maltotriose) require initial transport and enzymatic processing to produce monosaccharides. In theory, depending on the sugar profile or ratio of mono/di/tri saccharides, may determine the spoilage susceptibility of a beer. Importantly, the increasing relevance of low and no alcohol beers high in fermentable sugars (55 g/L) (Ferreira, 2009), and without ethanol, renders low and no alcohol beverages susceptible to spoilage and may be limited to bottle or can.

Table I-3: Breakdown of key sugars of approximate concentrations of unfermented sugars in beer (Baxter and Hughes, 2001).

Carbohydrates	**Concentration (g/L)**
Fructose	0 – 0.19
Glucose	0.04 – 1.1
Sucrose	0 – 3.3
Maltose	0.7 – 3.0
Maltotriose	0.4 – 3.4

Beer is considered as low in nutrition, however little work has been done to understand how the nutritional status can influence the spoilage microflora and how these environmental niches are linked to the spoilability of a beer. Rainbow (1952) reported the more attenuated a beer is, the less potential there is for bacteria to spoil beer. Attenuation refers to the conversion of sugars into ethanol and carbon dioxide during fermentation. Sugars are an essential carbon source for microorganisms, yeasts will

via glycolysis produce ATP required for growth and energy for maintaining intracellular homeostasis (Peretó, 2011). Residual beer carbohydrate levels vary, depending on the style of beer. A typical lager will contain between 10 – 30 g/L, whereas ales may range between 15 – 60 g/L of total carbohydrate, and residual fermentable carbohydrates range between 1 – 7 g/L and 5 – 10 g/L for lagers and ales, respectively (Ferreira, 2009). Market trends also show consumers are switching to low and no alcohol beers (BBPA, 2018). Due to restricted fermentation processes, low and no alcohol beers are high in residual fermentable sugars and are likely to be highly susceptible to spoilage (Blanco *et al.*, 2016). Under anaerobic conditions, yeast will hydrolyse sugars to pyruvic acid, which is metabolised to produced ethanol and CO_2. (Figure I-4).

Interestingly, strains of *Lactobacillus paracollinoides* released monosaccharides, through the hydrolysis of oligosaccharides (Geissler *et al.*, 2016). These heterofermentative lactic acid bacteria strains expressed a diverse carbohydrate metabolism. The presence of heterofermentative LAB may be essential for homofermentative LAB species to effectively spoil beer, which have been reported to favour monosaccharides and disaccharides (Geissler *et al.*, 2016). Homofermentative and heterofermentative LAB species are discriminated by their ability to metabolise hexose and Figure I-5 details the differences between the two types.

During the production of Lambic ales, *Brettanomyces* yeasts are able to utilise malto-oligosaccharides (Verachtert and Derdelinckx, 2014). Lambic ales are produced by spontaneous fermentation, comprising of beer/brewing spoilage microorganisms. *Brettanomyces* species are important during bottle refermentation for lambics, the

breakdown of oligosaccharides supports the growth of *Saccharomyces* sp., which cannot break down maltooligosaccharides (Verachtert and Derdelinckx, 2014). *L. brevis* is commonly found alongside *B. lambicus* during lambic fermentation, and has been shown to produce α-glucosidase (glucoamylase), an enzyme required to break down starch into simple sugars, also produced by *Brettanomyces* sp. (Kumara *et al.*, 1993, De Cort *et al.*, 1994). *B. bruxellensis* is also known to metabolise dextrin, through production of α- and β-glucosidase enzymes (Daenen *et al.*, 2008, Crauwels *et al.*, 2015, Tyrawa *et al.*, 2019). The ability of *Brettanomyces* sp. to breakdown complex sugars, creates a superattenuated beer, which is high in ethanol and low in dextrin (Kumara and Verachtert, 1991, Tyrawa *et al.*, 2019). A beer high in dextrin and oligosaccharides may select for microorganisms capable of producing α- and β-glucosidase enzymes.

Acetic acid bacteria (AAB) are one of a few exceptions when discussing energy production from sugars. Where most microorganisms prefer glucose, acetic acid bacteria prefer ethanol (Chaudhry and Varacallo, 2020). Historically, the ubiquity of AAB in the brewing process is a consequence of high tolerance to hop bitter acids. However, as obligate aerobes, AAB are less common due to the emphasis on the removal of oxygen during the brewing process and in packaged beer (Chaudhry and Varacallo, 2020). However, AAB are associated with spoilage of draught beer during the dispense process (Storgårds, 1997, Storgårds, 2000). Cask beers are at a high risk of AAB spoilage, due to the influx of air/oxygen during dispense and the warmer temperatures that create a favourable environment for AAB to thrive (Bokulich *et al.*, 2012). AAB can also utilise a diverse mix of carbohydrates through the hexose

monophosphate pathway (Paradh, 2015), or the EMP and Entner Dourdoroff pathway (Attwood *et al.*, 1991).

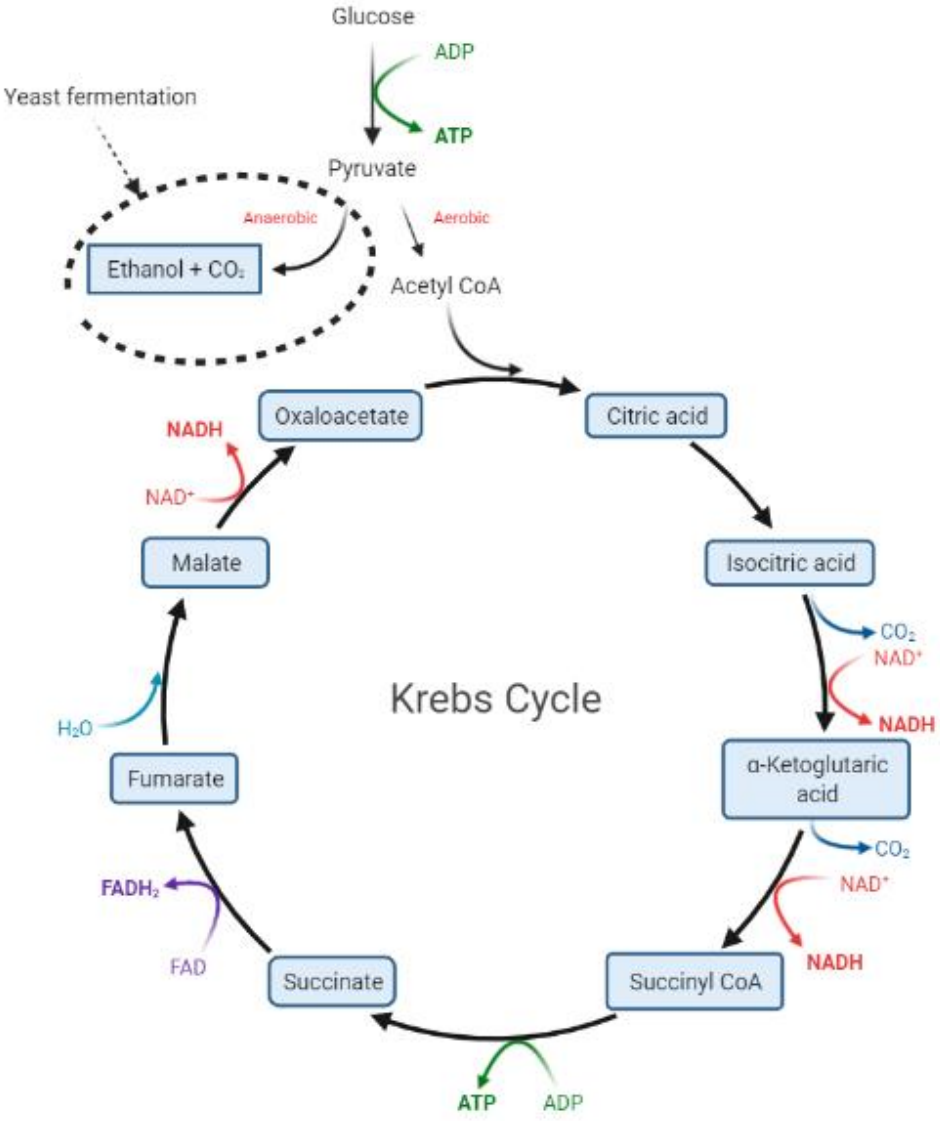


Figure I.4: Schematic of the Krebs cycle and yeast fermentation (Biorender.com)

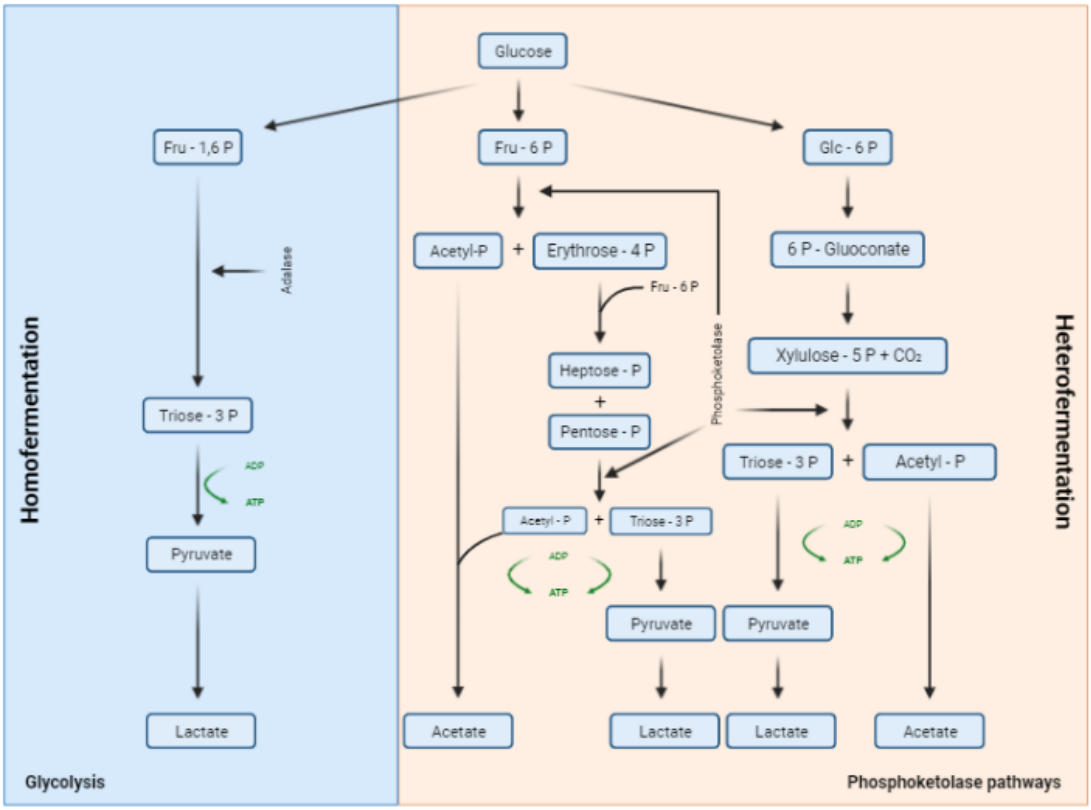


Figure I.5: Metabolic pathways of homofermentative and heterofermentative lactic acid bacteria species (created on Biorender.com)

1.8.2 Organic acids

Beer is often cited as being 'low in nutrients' but there remains an abundance of nutrition available for microbial metabolism. Beer is rich in organic acids, with approximately 200 organic acids present in beer (Buiatti, 2009). Organic acids are typically metabolic by-products of intermediary yeast metabolism (Buiatti, 2009). The organic acids contribute to a beer's acidity and therefore help maintain a level of microbial resistance by predominately limiting growth to acidophiles. Organic acids offer positive physiological benefits (diuretic) and impact on flavour (Montanari *et al.*, 1999, Charalambous, 1981, De Stefano and Montanari, 1996). However, those microorganisms capable of growth in acidic media, are able to exploit organic acids for energy production. A number of publications have reported the relationship between organic acids and energy production for LAB species (Suzuki *et al.*, 2008a, Melchiorsen *et al.*, 2001, Cox and Henick-Kling, 1989, Kolb *et al.*, 1992). For spoilage to occur, microorganisms require to produce energy, and shown by Suzuki *et al.* (2005), pyruvate, citrate and malate are consumed by beer spoilage lactic acid bacteria, with concomitant increases in ATP concentrations. AAB are restricted in their ability to oxidise organic acids lacking a functional Kreb's cycle, although, are still able to oxidise organic acids to CO_2 and H_2O (Paradh, 2015, Seo *et al.*, 2005).

The fermentation of malate is reported to be widespread in LAB, where it is dexcarboxylated into L-lactate (Cox and Henick-Kling, 1989, Kolb *et al.*, 1992). Further, pyruvate is a product of citrolactic fermentation by *Leuconostoc mesenteroides* (Marty-Teysset *et al.*, 1996). Pyruvate is also metabolised by LAB species, mediated by pyruvate formate lyase, which produces ATP, via conversion of acetylphosphate to acetate (Melchiorsen *et al.*, 2001, Suzuki *et al.*, 2005). The same

work also reports that all strains of LAB investigated used pyruvate for ATP synthesis, whereas malate was the least used. The ATP is used for cellular growth, but is required for the ATP-dependent multidrug transporter *horA*, which actively removes imported H^+ ions by iso-α-acids sourced from hops (Suzuki *et al.*, 2005). Hence the presence of organic acids in beer, particularly those required in ATP synthesis, may influence the spoilability of a beer. In Suzuki *et al.* (2005), it is also noted two beer spoiling strains (*L. brevis* and *L. lindneri*), were not able to produce ATP in the presence of hop compounds when using maltotriose as a sole substrate, which compared to *L. paracollinoides,* was able to produce ATP.

1.9 Antimicrobial components

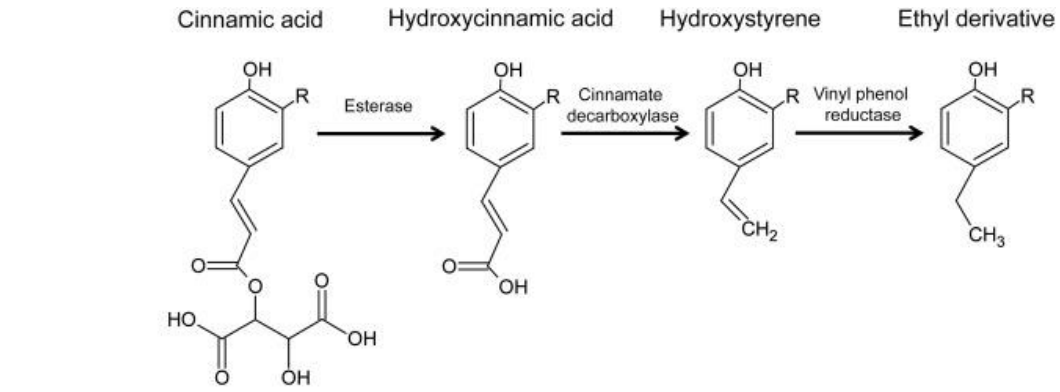
1.9.1 Hop chemistry and antimicrobial activity

Hops (*Humulus lupulus* L.) with water, yeast, and a carbon source, are the essential ingredients in the production of beer (Zanoli and Zavatti, 2008). Documentary evidence suggests hops have been used as early as 1079 A.D. (Corran, 1975). Hop research has been ongoing for over a century, yet much remains to be understood (Schönberger and Kostelecky, 2011). Hops contribute to beer flavour (bitterness), aroma and also act as an antimicrobial agent. The bitterness contributed by hops is a consequence of the content of α-acid (humulones) and β-acids (lupulones) (De Keukeleire, 2000, Boulton and Quain, 2013) . These compounds are not bitter but the wort boiling process converts humulones via thermal isomerisation into isohumulones (iso-α-acids), namely; *cis*-isohumulone and *trans*-isohumulone, dependent upon alcohol function (C-(4)) spatial arrangement (Figure I-6) (De Keukeleire, 2000).

The key α-acids in beer are humulones, cohumulones and adhumulones, of which, each produces a *cis* and *trans* derivative (68:32 ratio) post-thermal isomerisation. Hence, after wort boiling there are six iso-α-acids, each contributing to the bitterness and bacteriostatic capabilities of beer. Commonly, iso-α-acids are described as ionophores, via the exchange of H^+ for cellular divalent cations (Mn^{2+}) and disrupting cytoplasmic ion gradients (Behr *et al.*, 2007, Simpson, 1993a). Such disruptions cause low intracellular pH which interferes with or inhibits enzymatic processes, leading to cell death (Sakamoto and Konings, 2003, Behr *et al.*, 2007). Other antimicrobial actions of iso-α-acids are reported to include:

- Glucose efflux via membrane leakage
- Suboptimal respiratory chain dehydrogenase activity
- Impaired protein, RNA & DNA synthesis
- ATP content depletion
- Reduced L-leucine uptake

(Schurr *et al.*, 2015, Teuber and Schmalreck, 1973, Simpson, 1993a).



R = H	*p*-Coutaric acid	*p*-Coumaric acid	4-Vinylphenol	4-Ethylphenol
R = OCH	Fertaric acid	Ferulic acid	4-Vinylguaiacol	4-Ethylguaiacol
R = OH	Caftaric acid	Caffeic acid	4-Vinylcatechol	4-Ethylcatechol

Figure I.6: Formation of volatile phenols by *Saccharomyces* sp. and *Brettanomyces* sp. *Saccharomyces* sp. encode for cinnamate decarboxylase, producing 4-VP or 4-VG, which is a precursor to 4-EP and 4-EG production by *Brettanomyces* sp. that encodes vinyl phenol reductase (De Keukeleire, 2000).

1.9.2 Ethanol

Ethanol is produced by the fermentation of wort sugars by yeast and is a key antimicrobial component of beer and inhibits the growth of pathogenic microorganisms which renders beer a safe beverage. The antimicrobial activity of ethanol is due to the ability to denature proteins, causing the disruption of the cell membrane (Gold *et al.*, 2020). Further it can cause cells to dehydrate, by drawing water out of the cell through disturbing the osmotic balance, causing cell death. Water molecules are important for the optimum antimicrobial activity of ethanol such that 70% ethanol is commonly used in laboratories as a primary cleaning agent.

1.9.3 Acidity

The pH of an environment is a rate limiting factor for all microorganisms. Microbes can be split into three categories based on their ability to grow at different ranges of pH:

- Acidophile (pH 1 – 5.5)
- Neutrophile (pH 5.5 – 8.5)
- Alkaliphile (pH 7.5 – 11.5)

Most bacteria are neutrophiles with pH 7 the optimum for growth. Typical lager/ale beers will have a pH 4 – 5. Ciders are typically lower pH, ranging between pH 3.5 – 4.5, which has led to the view of landlords/bar staff that draught cider dispense lines do not need to be cleaned as regularly due to increased acidity of the product. Acidity is a consequence of hydrogen ions and organic acids. The antimicrobial activity is similar to the role hops (or more specifically iso-alpha acids) play in beer, via the

impairment of the intracellular ion gradient. The presence of extracellular hydrogen ions in the matrix will disrupt cellular processes, dissipating the intracellular pH balance inside the cell hence impairing energy production. This is caused by an increased influx of organic acids, acidifying the cytoplasm (Dysvik *et al.*, 2020b).

1.10 Beer microbiology and metabolites

All environments on earth are inhabited by some form of microbial life, their ability to adapt to natures' extreme environments and consequential selective pressure giving rise to populations able to survive and proliferate.

1.11 Yeast

Infection of beer with wild yeasts is often associated with the presence of phenolic off-flavours (POF) (Van Der Aa Kühle and Jespersen, 1998). The challenges during the production stages controlling wild yeast infection have largely been accomplished through cleaning, closed vessels and improved hygienic practices, however, wild yeasts are able to have an impact on beer quality at levels as low as one wild yeast cell per 10^5-10^6 yeast culture (De Angelo and Siebert, 1987). Therefore, wild yeasts can impact quality during extended periods of time where the draught lager, cider or ale etc. reside in the dispense line.

1.11.1 *Saccharomyces* sp.

Saccharomyces sp. are the fundamental part of the brewing process. Depending on fermentation type (bottom-fermenting or top fermenting) will dictate the species employed. Importantly, different strains of *Saccharomyces* sp. can influence the products flavour, haze and aroma (Stewart, 2016). According to Stewart (2016),

‘Germany, for example, most of the beer is produced with only four individual yeast strains and approximately 65% of it is produced with a single strain’..

Spoilage of beer by *Saccharomyces* sp. are well documented (Fleet, 1992, Hemmons, 1954, Wiles, 1950). Hemmons (1954) conducted an ‘exploratory survey’ of wild yeasts in draught beer in London. 41 (35%) of the 118 isolates belonged to the *Saccharomyces* sp., of which 26 were *S. cerevisiae*. Hemmons’ work focussed on ales and bitters. To further emphasise the presence of *Saccharomyces* sp. in spoilt beer, Wiles (1950) analysis of 22 spoiled draught beer samples, were found to contain *Saccharomyces* sp., with most containing *S. cerevisiae* and all containing *S. carlsbergiensis*.

Wild yeasts, in particular wild *Saccharomyces cerevisiae*, spoil beer through multiple routes; haze formation, POF (phenolic off flavour) production, super-attenuation (Bokulich and Bamforth, 2013). Super-attenuation is caused by the production of glucoamylases, which can hydrolyse dextrin and release fermentable sugars into the media. Thus, this can result in the over-conditioning of a beer, causing frothing and results in the loss of beer. For example, *S. cerevisiae* var. *diastatitcus* is a microorganism capable of hydrolysing dextrin (Andrews and Gilliland, 1952). Moreover, diastatic variants are most commonly POF+, thus will also product phenolic off flavours in beer (Štulíková *et al.*, 2021). Yeasts generate POF through the decarboxylation of *p*-coumaric acid and ferulic acid in beer to 4-vinylphenol (4-VP) and 4-vinylguaiacol (4-VG), respectively, through the *POF1* (alias: *PAD1*) gene (Meaden and Taylor, 1991). The breakdown/conversion of *p*-coumaric and ferulic acid is mediated by the enzymes ‘cinnamate decarboxylase’ and ‘ferulic decarboxylase’, encoded by *POF1* (Coghe *et al.*, 2004, Goodey and Tubb, 1982).

Meaden and Taylor (1991) further discussed the presence of conserved *POF1* genes within brewing strains considered POF⁻, where brewing strains of *S. cerevisiae* were transformed to express *POF1*, which produced aromas characteristic of POF. The *POF1* gene is widespread amongst wild yeast strains, and has been reported in wort-contaminating bacteria (Van Beek and Priest, 2000). Although POF spoils lager beers, the decarboxylation of *p*-coumaric & ferulic acid have a positive role and are responsible for flavours in Belgian ales and German wheat beers (Bokulich and Bamforth, 2013).

1.11.2 *Brettanomyces* sp.

Brettanomyces sp. (namely, *B. bruxellensis* (Gilliland, 1961), *custerianus* (Shimotsu *et al.*, 2015), *lambicus* (Smith and Divol, 2016), and *anomalus* (Comitini *et al.*, 2020)), are known beer contaminants. *Brettanomyces* sp. (teleomorph *Dekkera*) are associated with wine making and lambic beers, and are sourced naturally from grape skins or within barrels (Wedral *et al.*, 2010). During the 1920s, *Brettanomyces* was the name given to the microorganisms required to produce English stock beers, and later the yeast for Lambic ales in Belgium (Henschke *et al.*, 2007). Stock beers were originally matured over months/years, and finally mixed with young beers to create an 'aged' flavour. Aged tastes was first described by Claussen (1904) who identified the aged action was from *Brettanomyces*.

Brettanomyces sp. are fundamental in winemaking and produce 4-ethylgluaiacol (4-EG) and 4-ethylphenol (4-EP), which are key contributors to wine aroma (Wedral *et al.*, 2010). In beers, 4-EG and 4-EP production is indicative of *Brettanomyces* contamination and an indicator of infected beer. Aromas from 4-EG and 4-EP resemble bandages, smoke and sweat, of which are (obviously) undesirable

characteristics in beer (Bokulich and Bamforth, 2013). Despite being once considered fundamental to beer production (in the 1920s), *Brettanomyces* contamination in the modern age is a major concern in beer production (Henschke *et al.*, 2007). Evidence suggests small changes in the concentration of 4-EG and 4-EP are noticeable by the consumer (Vigentini *et al.*, 2008).

It can be argued that wild *Saccharomyces* sp. and *Brettanomyces* sp. work in tandem during beer spoilage. *Saccharomyces* sp. produce 4-VP and 4-VG that are the precursors to 4-EP and 4-EG production, respectively, by *Brettanomyces* sp. (Figure I-1) (Steensels *et al.*, 2015). Hence, during the production of the volatile phenols 4-EG and 4-EP, by yeast with a *POF1*/*PAD1* gene producing 4-VG and 4-VP must precede *Brettanomyces* spoilage. The role of *Brettanomyces* in the biotransformation of 4-VP and 4-EG to 4-EP and 4-VG, respectively, by the production of a vinylphenol reductase has been studied (Edlin *et al.*, 1995, Granato *et al.*, 2015, Godoy *et al.*, 2009). Vinylphenol reductase was originally identified as Zn/Cu dismutase in *B. bruxellensis*, described as the key enzyme in ethyl phenol production (Granato *et al.*, 2015). Further work confirmed its role in POF production by cloning the *DbVPR* (encoding vinylphenol reductase) gene into a non-ethyl producing *S. cerevisiae*, supporting *B. bruxellensis* and its associated vinylphenol reductase role in phenolic off flavours in beer (Romano *et al.*, 2017).

1.11.3 *Rhodotorula* sp.

Rhodotorula sp. significance as a beer spoilage organism is arguably understated. A number of studies have identified *Rhodotorula* sp. from beer (Hemmons, 1954, Hutzler *et al.*, 2013, Turvey *et al.*, 2016, Shinohara *et al.*, 2021), with the most common *Rhodotorula mucilaginosa*. A key phenotype of the *Rhodotorula* genus is

pink/red coloured colonies. *Rhodotorula* sp. are an increasingly important fungal pathogen, in particular for immunosuppressed patients (Nunes *et al.*, 2013). However, in the context of the work reported here, it is the biofilm formation of *Rhodotorula* sp. that is the most problematic trait of this yeast. Several publications discuss the biofilm formation of *Rhodotorula* sp. (Gattlen *et al.*, 2011, Gharaghani *et al.*, 2020, Jarros *et al.*, 2020), with one specifically identifying *R. mucilaginosa* with significantly increased biofilm formation potential compared to other *Rhodotorula* sp. (Nunes *et al.*, 2013). Further, *Rhodotorula* sp. have been identified as a biofilm former on conveyer belts and bottle/can warmers in breweries (Banner, 1994, Storgårds, 2000).

It is currently unclear what the exact impact of *Rhodotorula* sp. on beer would be. However, *Rhodotorula* sp. contain a diverse carbohydrate metabolism, and in some recent work was found to be able to use a variety of sugars, on par with *S. cerevisiae* strains (Misihairabgwi *et al.*, 2015). *Rhodotorula* sp. are an oleaginous yeast due to their ability to accumulate intracellular lipids, and possess a broader metabolism and pH range (Spagnuolo *et al.*, 2019). The role of lipids on the final product beer have been related to impacting beer foam stability (Roberts *et al.*, 1978). Lipids collapse beer foam by the 'spreading particle mechanism', which increases coalescence of bubbles creating larger bubbles, thus resulting in a less visually appeasing foam (Evans and Sheehan, 2002). Thus, it is hypothesised a *Rhodotorula* sp. infection could result in foam instability of draught beer.

1.11.4 *Pichia* sp.

Pichia sp. are common beer spoilage microorganisms and are known to produce haze, film and off-flavours (Jespersen and Jakobsen, 1996). Although most require an aerobic environment, some are capable of anaerobic growth (Campbell and Msongo,

1991). *Pichia* sp. are able to spoil unpasteurised draught beer typically forming haze and surface films (Boulton and Quain, 2013). Much of *Pichia* sp. beer spoilage potential, in particular *Pichia anomala*, is a consequence of its ability to thrive in extreme environmental conditions (Passoth *et al.*, 2006). *P. anomala* can survive:

- Low and High pH
- High osmotic pressure
- Anaerobic conditions
- Low water activity

(Fredlund *et al.*, 2002, Passoth *et al.*, 2006)

Pichia sp. are described as opportunistic contaminants and are particularly prevalent in unhygienic sampling ports and other areas. Generally, during fermentation *Pichia* sp. are not an issue due to improved hygienic standards and therefore growth is limited. Issues of *Pichia* sp. spoilage are typically in barrel fermented beer where the head space permits the influx of oxygen into the beer enabling aerobic opportunistic contaminants to thrive. Similarly, beer dispense lines in the on-trade also provide conditions for *Pichia* to thrive.

Other yeast genera including *Candida*, *Torulaspora*, *Issatchenkia*, *Debaryomyces*, *Zygosaccharomyces, Schizosaccharomyces,* and *Kloeckera* possess spoilage potential (Timke *et al.*, 2008a, Van Der Aa Kühle and Jespersen, 1998). Although, more often spoilage is limited due to a combination of factors such as oxygen limitation, competitive inhibition, ethanol toxicity and storage conditions (Bokulich and Bamforth, 2013).

1.12 Bacteria

1.12.1 *Lactobacillus* sp.

Of the Gram-positive bacteria, lactic acid bacteria (LAB) are the most prevalent beer spoilage organisms. LAB are found widely in nature, plant matter and in human beings (Bokulich and Bamforth, 2013). However, most LAB struggle to spoil beer due to the antimicrobial activity of hop-derived compounds (Simpson and Smith, 1992). A comprehensive list of potential LAB beer spoilage organisms can be found in Bokulich and Bamforth (2013). Here, only the primary beer spoilage organisms will be reviewed. Both *Lactobacillus brevis* and *Pediococcus damnosus* exhibit the largest spoilage potential, with a number of reports of these microorganisms within finished beer (Fujii *et al.*, 2005, Preissler *et al.*, 2010, Suzuki *et al.*, 2006, Tsuchiya *et al.*, 1993). Riedl *et al.* (2019a) reported from 2010-2016, between 41 – 53% of brewery samples sent to Weihenstephan Research Centre contained *L. brevis* (Riedl *et al.*, 2019a, Schneiderbanger *et al.*, 2018).

LAB contamination is, in part, due to it being a part of barley's natural microbiota, coupled with its ability to survive the malting and mashing processes (not wort boiling) (Vaughan *et al.*, 2005). LAB contamination is characterised by acidification, haze formation and diacetyl production (Bokulich and Bamforth, 2013). Diacetyl causes a buttery off-flavour in beer and is indicative of spoiled beer. Brewing yeast do produce diacetyl during fermentation, but much of this is removed during conditioning. Although LAB sp. do also produce lactic acid, diacetyl is notably more potent with a much lower threshold before being noticeable (0.15ppm) when compared to lactic acid (300ppm) (Sakamoto and Konings, 2003, Hough *et al.*, 2012).

P. damnosus has similar impacts on beer, however, is also capable of significant exopolysaccharide production which in severe cases can result in a slimy beer (Van Oevelen and Verachtert, 1979).

Ethanol tolerance is widespread amongst LAB species (Gold *et al.*, 1992), moreover, many LAB sp. possess hop-resistance genes, which further support the proliferation in beer (Behr *et al.*, 2006, Bergsveinson *et al.*, 2014, Pittet *et al.*, 2011). The ability of *L. brevis* to resist hop bitter acids is derived from the expression of hop-resistance genes *horA, horC* and *hitA* (Suzuki *et al.*, 2006, Sakamoto *et al.*, 2001). *horA* is an ATP-binding multidrug transporter, which pumps out antimicrobial acids sourced from hops (e.g., *trans*-isohumulone, (see 1.9.1) and *horC* enacts to aid hop-resistance by encoding for a proton motive force-dependent multidrug efflux system (Simpson, 1993b, Haakensen *et al.*, 2008, Suzuki *et al.*, 2006). Evidence suggests the role of *hitA* is to prevent the dissipating pH gradient of bacteria, by importing Mn^{2+} in place of H^{+}, which is increased intracellularly as a response to hop-derived isohumulones (Hayashi *et al.*, 2001) (Figure I-2). Therefore, *hitA* enables the spoilage-bacteria to maintain cellular homeostasis, prevent the cell undergoing an apoptotic or necrotic state and allow essential enzymatic processes continue without disruption. Together, the efflux systems encoded by the discussed genes, can nullify the impact of iso-α-acids, and permit the hosts proliferation within beer.

1.12.2 *Pediococcus* sp.

Pediococcus damnosus is another significant beer spoilage organism, and was responsible for ~12% of spoilage incidents reported in Germany between 1980 and 2002 (Behr *et al.*, 2016). Similarly to *L. brevis, P. damnosus* has been shown to possess a functional *horA* gene, which gives reason to its significant beer spoilage

capabilities (Suzuki *et al.*, 2006). Analysis of the genebank database suggests that only *L. brevis* and *P. damnosus* were shown to possess all three proposed hop-resistance genes (*horA, horC,* & *hitA*), providing insight into why these organisms are potent beer spoilage organisms (Uniprot, 2018). A number of species from this genus have been identified in beer/breweries such as, *P. claussenii* (Dobson *et al*., 2002), *P. inopinatus* (Iijima *et al.*, 2007), *P. pentosaceus* (Pinto *et al.,* 2004), *P. mevalovorus* (Kitahara Kukua, 1958), and *P. cerevisia*e (Van Oevelen and Verachtert, 1979).

Pediococcus sp. are associated with haze formation, diacetyl production, and exopolysaccharide production into beer. Exopolysaccharide excretion into beer causes an oily/slimy consistency to the beer, often referred to as 'ropiness'. However, similarly to *Lactobacillus* sp., diacetyl production is the main spoilage product from *Pediococcus* sp. *Pediococcus* are homofermentative lactic acid bacteria and therefore will only produce lactate from hexoses.

1.12.3 *Acetobacter* sp.

Gram-negative bacteria are major beer spoilage organisms. However, technological advances in brewery design have enabled the near-complete exclusion of oxygen in packaged beer, hence removing the spoilage of aerobic Gram-negative bacteria (Sakamoto and Konings, 2003). Subsequently, the industry's success inhibiting aerobic Gram-negative bacteria has invited new challenges in regard to obligate anaerobic bacteria (Jespersen and Jakobsen, 1996). Acetic acid bacteria can be sourced from a range of environments, but are typically found in sugary or alcohol based solutions (Swings and De Ley, 1981), including beer, wine, grapes, sake, and vinegar.

When the management of oxygen in breweries was significantly less effective, gram-negative acetic acid bacteria (AAB) were of great concern. Acetic acid bacteria include the species *Acetobacter aceti, A. pasteurianus* and *Gluconobacter oxydans*.. AAB spoil beer by oxidising ethanol, producing acetate, essentially creating vinegar, this process is mediated alcohol dehydrogenase and aldehyde dehydrogenase (Figure I-7) (Mamlouk and Gullo, 2013). In industry, AAB are both beneficial and spoilage organisms. Oxidation mechanisms of AAB can be exploited across a range of biotechnological applications (Mamlouk and Gullo, 2013). The formation of acetic acid from AAB oxidation has application in white, malt, wine, and cider vinegars (Drysdale and Fleet, 1988). However, in beer, acetate formation is indicative of beer spoilage. *Acetobacter* sp., via the citric acid cycle, over-oxidise ethanol to acetic acid, reducing the pH and creating a vinegar aroma (Drysdale and Fleet, 1988).

Acetobacter sp. are reliant on oxygen for proliferation and metabolism, and therefore are essentially irrelevant in the brewing process. Despite this, at the point of dispense where oxygen is reintroduced, *Acetobacter* sp. can be found in beer (Cosbie *et al.*, 1941, Kulka *et al.*, 1948). These findings challenge the current perception that *Acetobacter* sp. spoilage is insignificant. In particular, cask beer remain susceptible to AAB contamination as air is drawn into the container on dispense, with environmental wild yeasts and AAB (and other bacteria) introduced (Boulton and Quain, 2013). The notoriety of AAB is derived from the ability of AAB to resist iso-α-acids, use ethanol as a carbon source, and capable of survive low pH environments. Thus, in the presence of oxygen it is predicted AAB would rapidly spoil the beer.

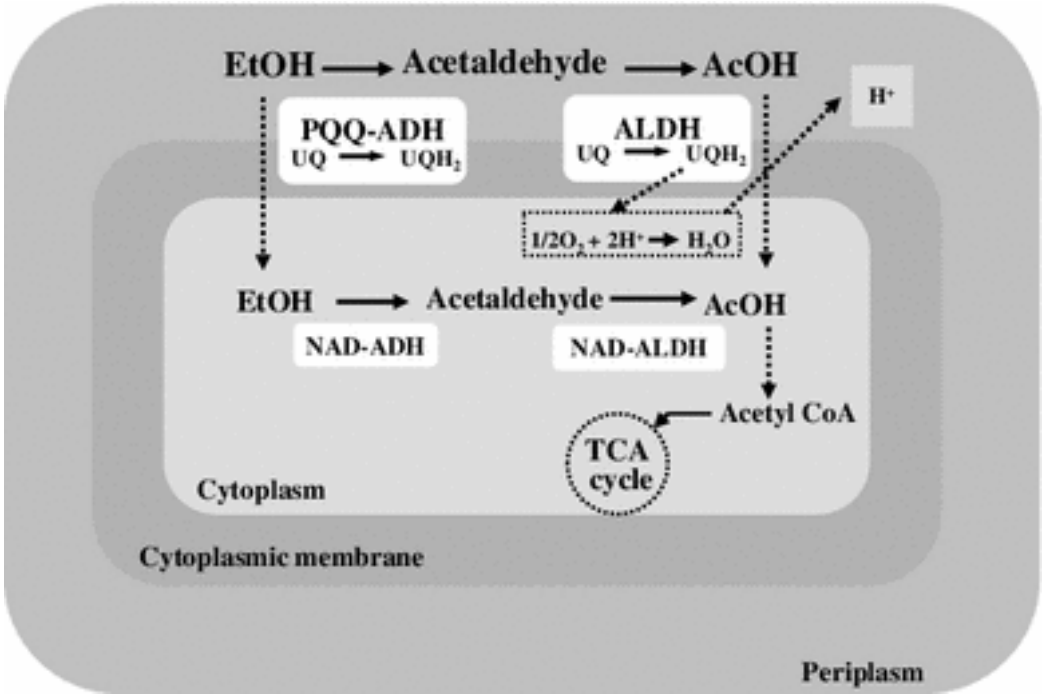


Figure I.7: Ethanol oxidation by acetic acid bacteria at the outer surface of the cytoplasmic membrane (Mamlouk and Gullo, 2013).

1.12.4 *Gluconobacter* sp.

Gluconobacter sp. are common beer spoiling organisms and are like *Acetobacter* sp. in much of their spoilage mechanisms and environment requirements for survival. Both *Acetobacter* and *Gluconobacter* are described as acetic acid bacteria due to their production of acetic acid. The most common isolate is *Gluconobacter oxydans*, *G. cerevisiae* has been isolated from a brewery (Spitaels *et al.*, 2014), however, examples of other species are limited.

Like *Acetobacter* species, being a strict aerobic bacterium, *Gluconobacter* sp. are also restricted in their capacity to spoil beer during the fermentation process. However during dispense, will oxidise ethanol producing a vinegar aroma through the production of acetic acid (Drysdale and Fleet, 1988).

1.12.5 *Pectinatus* and *Megasphaera* sp.

P. cerevisiiphilus and *P. frisingensis* are beer contaminants, the former being first described by Lee *et al.* (1978) after it was isolated from bottled beer incubated at 30^{o}C. The latter, *P. frisingensis*, was more recently isolated from breweries, where taxonomic analysis characterised a second species of *Pectinatus* growing in beer, which was using different substrates and excreting a wider range of products (Schleifer *et al.*, 1990). *Pectinatus* spoilage is often characterised via volatile fatty acid production (propionate and acetate), resulting in off flavours (Tholozan *et al.*, 1997). Propionic acid is the most abundant product of *Pectinatus* sp. contamination (Takahashi, 1983, Haikara *et al.*, 1981). Propionic acid synthesis is mediated by succinate oxidoreductase, reducing fumarate to succinate and this occurs in both *P. cerevisiiphilus* and *P. frisingensis* (Tholozan *et al.*, 1994, Hettinga and Reinbold, 1972). In beers with a low alcohol content, *Pectinatus* sp. have been shown to

proliferate and to be acid-tolerant (Haikara *et al.*, 1981, Satokari *et al.*, 1998). Despite spoiling beer, *Pectinatus* sp. are unable to contaminate wort as they are unable to assimilate maltose. Interestingly, *Pectinatus* sp. possess the necessary metabolic machinery to utilise lactate, a by-product of Gram-negative LAB, thus the presence of *L. brevis* could facilitate/aid the growth of *Pectinatus* (Haikara and Helander, 2006).

Alongside *Pectinatus* sp., *Megasphaera* sp. have become prominent beer spoilers as a consequence of improved filling technology and the reduction in dissolved oxygen in small pack beer (Haikara and Helander, 2006). *M. cerevisiae, M. paucivorans* and *M. sueciensis* are three key species from this genus responsible for beer spoilage (Paradh *et al.*, 2011a). *M. cerevisiae* is the major beer spoilage species from this genus and is mostly abundant in low-alcohol beer, where *M. cerevisiae* was reportedly responsible for 3-7% of beer spoilage in Europe between 1980-2002 (Paradh *et al.*, 2011a). In breweries, there is a need for rapid identification of spoilage organisms, as current quality control methods use conventional microbiology methods that take days, or require forcing, which takes similar length of time (4 days) (Mallett *et al.*, 2018). However, in cases involving *Megasphaera* sp. and *Pectinatus* sp., identification via conventional culturing methods are time consuming as both genera require an incubation time of up to 14 days at 30^{o}C (Lee, 1994). Associated beer spoilage include turbidity, hydrogen sulphide and fatty acid off flavours (Satokari *et al.*, 1998).

Obligate anaerobes, such as *Megashaera* sp., *Pectinatus cerevisiiphilus*, *P. frisingensis, Zymophilus raffinosivorans, Z. paucivorans* and *Zymomonas mobilis,* have all been isolated from breweries, and described as beer spoilage organisms (Dadds and Martin, 1973, Schleifer *et al.*, 1990, Van Vuuren, 1999). Improvements in

packaging technology has increased the prevalence of anaerobes in packaged beer, as dissolved oxygen is now very low coupled with minimal head space. Further, it is becoming increasingly common for beers to be sterile filtered, which coupled with significantly reduced oxygen content in packaging and bottling process, permits the growth (if present) of these genera (Jespersen and Jakobsen, 1996). These obligate anaerobes, mentioned above, can spoil beer through the production of:

- Propionic acid
- Acetic acid
- Succinic acid
- Mercaptan – drains or rotten garbage (scents)
- Dimethyl sulphide – sweetcorn, tomato sauce
- Hydrogen sulphide – boiled/rotten eggs (scents)
- Turbidity

(Lawrence, 1988, Jespersen and Jakobsen, 1996).

1.13 Biofilms

In nature, microorganisms exist as communities that communicate forming multicellular aggregates, referred to as biofilms (Flemming and Wingender, 2010). Since their first description in 1936, biofilms have increased in significance and it has become progressively evident biofilms are profoundly different in growth and environmental resistance to planktonic counterparts (Costerton *et al.*, 1995, Zobell and Anderson, 1936). For example, evidence has shown how low levels of alcohol (1-6%) can promote biofilm formation in bacteria rather than act as an antimicrobial (Knobloch *et al.*, 2002). In the 17th century, Van Leeuwenhoek examined the plaque

of his own teeth, investigating “animalcules”, since accepted the first description of biofilms. The accepted theory of biofilms states bacteria will form in matrix-enclosed biofilms that adhere to surfaces in nutrient-sufficient aquatic ecosystems (Donlan and Costerton, 2002, Costerton *et al.*, 1978). Within biofilms, the microorganisms exist amongst extracellular polymeric substances (EPS) (containing polysaccharides, proteins, nucleic acids and lipids), which permit stability, surface adhesion and act as a scaffold for forming a polymer network connecting transiently immobilised cells (Flemming and Wingender, 2010). A brief overview of the EPS constituents’ functions can be found below, but for a more detailed discussion of EPS constituents’ the reader is directed to Flemming and Wingender (2010).

As briefly described earlier (Section 1. Background), within beer dispense lines there exist conditions that match the conditions required for biofilm formation. Firstly, a solid surface (dispense line), nutrient sufficiency (beer, sugars, ethanol, oxygen) and an aquatic ecosystem (beer). Further to this, the recalcitrance of biofilms to cleaning and sanitation has been well documented (Somers and Lee Wong, 2004, Gibson *et al.*, 1999). It is these conditions supporting biofilm formation and the documented inertness to chemical sanitation that compromise the hygiene of dispensed draught beer.

1.13.1 Biofilm formation

Cell-surface attachment and cell-cell interactions are the essential early steps required for biofilm initiation, followed by biofilm maturation (O'toole *et al.*, 2000). The molecular mechanisms differ dependent on the genus and/or species initiating biofilm formation. There are five key steps for biofilm formation, which are common throughout all biofilms (Figure I-8):

- Attachment
- Irreversible attachment
- Biofilm scaffold development
- Maturation
- Dispersion

(Salas-Jara *et al.*, 2016)

Biofilm formation is initiated by cell-surface contact in the presence of a liquid environment. Often, where biofilm formation takes place is often determined by gravity, diffusion or fluid dynamic forces (Storgårds, 2000). Upon surface interaction, organic and inorganic molecules are adsorbed to the surface forming a 'conditioning film' (Kumar and Anand, 1998). The conditioning film enables a heightened concentration of nutrients to accumulate, and hence within biofilms nutrient transfer is more efficient than planktonic bacterium (Kumar and Anand, 1998). Attachment is conducted via the formation of various molecular bonds (hydrophobic, dipole, van der Waals etc.) that together cause irreversible attachment (Dunne, 2002, Garrett *et al.*, 2008). Adhered cells will producing exopolysaccharides (EPS), which act as a scaffold to stabilise the biofilm and produce a three dimensional structure (Danese *et al.*, 2000, Watnick and Kolter, 1999).

As described by Flemming *et al.* (2007), the EPS matrix is the 'house of biofilm cells'. The EPS matrix is responsible for a cells immediate environment and living conditions within a biofilm. Often, descriptions of the EPS are limited and do not reflect the importance of the matrix within the biofilm. Indeed, without the EPS a biofilm would lose essential processes such as adhesive properties, water retention, charge and

stability and would be unregulated and ineffective (Flemming and Wingender, 2001, Flemming *et al.*, 2007). Within the EPS matrix, there exists a conglomerate of proteins, glycoproteins, glycolipids and extracellular DNA, where polysaccharides are commonly a minor element (Frølund *et al.*, 1996). Studies have shown the extracellular polymers, excreted during adhesion, improve the adhesion to metal surfaces (Characklis and Marshall, 1990). This has relevance to brewing process and packaging, where metal vessels, processing equipment, and distribution mains are used throughout, as is the case generally across the food and beverage industries.

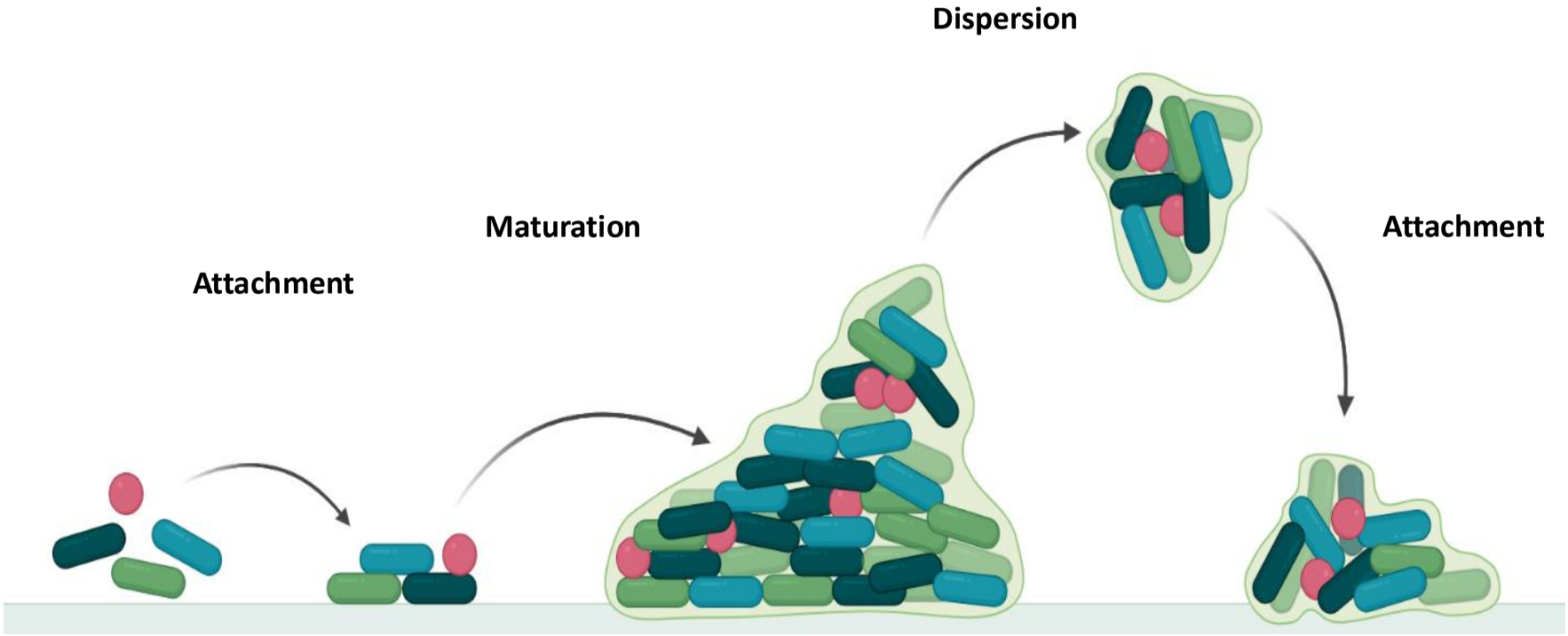


Figure I.8: A basic schematic of the lifecycle of a biofilm (Biorender.com)

1.13.2 Yeast and multi-kingdom biofilms

Research and understanding of yeast biofilms is commonly extrapolated from work with bacterial biofilms. The review in 1.13 is mostly literature sourced from research on bacterial biofilms. Information about biofilm formation by yeasts remains in its infancy in comparison to bacterial biofilms (Gattlen *et al.*, 2011), although it is accepted that the general stages of biofilm formation remain the same. Yeast biofilms have been reported in a range of food 'realms', a review by Zara *et al.* (2020) discusses yeast biofilms in brewing, vegetables, syrups and many more.

Yeast biofilm formation is understood to differ mostly during the maturation phase. For example, *C. albicans* and *S. cerevisiae* develop into spherical cells creating a monolayer and pseudohyphae during maturation (Vopálenská *et al.*, 2010). What makes yeast biofilms particularly problematic is their innate ability to grow on a range of materials (plastics (Reynolds and Fink, 2001), steel (Brugnoni *et al.*, 2007, Brugnoni *et al.*, 2014)) and also mature as a secondary coloniser using a developed bacterial biofilm (Douglas, 2002) or fungal biofilm (Webb *et al.*, 2000) as a surface. More recently there is increasing interest in regard to yeast biofilm and brewing (Brányik *et al.*, 2004, Riedl *et al.*, 2019b), and growing interest in food industries employing 'clean-in-place' (CIP) systems (Goode *et al.*, 2010, Zara *et al.*, 2020a).

However, does examining these microbial kingdoms accurately represent a real-world perspective? It is difficult to imagine a sessile community, which has inhabited through opportunity, being exclusively bacteria or yeast. Lactic acid bacteria and yeasts have already shown to coexist during rice wine production (Haruta *et al.*, 2006),

and a further report has shown how *Lactobacillus* sp. and *Saccharomyces* sp. form mixed species biofilms in the same culture (Kawarai *et al.*, 2007)

1.13.3 Quorum sensing

One of the most striking insights into biofilm communities is the ability for cell communication. The polymicrobial aggregate requires general signalling molecules that can be interpreted across species, genera and/or kingdom to allow synchronised responses to environmental stresses. To communicate, bacteria produce chemical signal molecules, Gram-positive bacteria excrete autoinducer peptide (AIP) and Gram-negative bacteria produce N-acyl homoserine lactones (AHL). These autoinducers enable bacteria regulate phenotype expression, biofilm formation and virulence factors (Miller and Bassler, 2001). This form of cell-cell communication is termed 'quorum sensing' and broadly describes a range of functions achieved through the excretion of autoinducer and signalling molecules. Quorum sensing was first described 29 years ago, where the authors investigated cell communication between *Vibrio fischeri* and *Vibrio harveyi* (Nealson and Hastings, 1979).

Gram-negative, Gram-positive and eukaryotic biofilm quorum sensing has been extensively studied, and has been excellently reviewed in Miller and Bassler (2001). However, as discussed by Miller and Bassler (2001), more recent studies investigating quorum system invest significant time identifying novel quorum sensing circuits enabling intergenera or interkingdom communication. Eukaryotic recognition of bacterial quorum sensing has already been documented, whereby a eukaryotic host is demonstrated to be stimulated by quorum sensing molecules from a pathogen and responds appropriately (Givskov *et al.*, 1996, Rasmussen *et al.*, 2000, González and Keshavan, 2006). Interkingdom/intergenera biofilms in brewing are more likely to

synchronise via autoinducers to ensure/prolong survival in the present environment. Studies in the field of oral disease have investigated this phenomenon. In biofilms, *Candida albicans* have been shown to change the virulence of *Steptococcus mutans*, where it aided the pathogenesis of *S. mutans* (Sztajer *et al.*, 2014) and other works have also shown how interkingdom communication can enhance biofilm formation (Bamford *et al.*, 2009). In humans *Pseudomonas aeriginosa* and *C. albicans* are shown to communicate and possibly promote virulence of microbial communities (Burns *et al.*, 1999, Hermann *et al.*, 1999, Hogan and Kolter, 2002).

1.13.4 Horizontal gene transfer

Horizontal gene transfer (HGT) is the transfer of genetic material (e.g. antibiotic resistance) between organisms, (Burmeister, 2015). Burmeister (2015) concludes that all genes can be transferred, which suggests that this is possible for those encoding ethanol resistance and hop resistance. HGT in biofilms is known to occur at a heightened rate due to enclosed environment compared to their planktonic counterparts (Madsen *et al.*, 2012). The genes released are in the form of plasmids, which are independent replicons, and typically contain genes to support the survival of their hosts (Madsen *et al.*, 2012).

Conjugation - a mechanism of bacterial HGT - was first observed between bacteria and how bacteria transfer genetic information via direct contact (Figure I-5). However, it is becoming more apparent that conjugation is not unique to bacteria, but that the conjugative mechanism enables the transfer of genetic information within the biological kingdom (Syvanen and Kado, 2001). Evidence of interkingdom conjugation has been published, where the transfer of bacteria/yeast plasmids was identified (Hayman and Bolen, 1993). It is such findings that provide credence to the idea that

brewing biofilms containing spoilage organisms, are through conjugative mechanisms transferring genetic information (e.g., *horA*) to a neighbouring cell to support the fitness of the biofilm and/or polymicrobial population.

1.13.5 Biofilms and industry

Biofilms cost industry potentially billions of dollars per year, in the US, biofilms cause millions of infections and are reported to incur a cost of 11 billion dollars per annum. Whether it is for sanitation, equipment replacement or loss of production. Established or 'mature' biofilms can cause irreparable damage to equipment, corroding steel (Chongdar *et al.*, 2005, Procópio, 2019) and damaging the product (food or drink). Biofilms have been reported in a number of industries, water waste treatment (Rittmann, 2004), oil and gas (Xu and Gu, 2015), dairy (Teh *et al.*, 2015), food industry (Kumar and Anand, 1998), medical equipment (Reid, 1999) dental (Marsh *et al.*, 2011), breweries (Quain and Storgårds, 2009) and dispense (Storgårds, 2000).

In the food and beverage industry, biofilm formation compromises hygiene, causes food spoilage, resulting in significant economic losses (Holah and Kearney, 1992, Kumar and Anand, 1998). The importance of removing biofilms relates to brand damage. If a food or beverage is spoiled and is recalled from sale it can significantly harm a brand, impacting on profit, market share, reputation, and consumer loyalty. Hence most companies will follow a strict cleaning procedure, which are continually being monitored to confirm and improve cleaning effectiveness (Goode *et al.*, 2010).

1.13.6 Biofilms in brewing

Biofilms have been extensively studied in the last 20 years; however, within brewing, research is scarce. Research into biofilms often use methods that do not replicate the

microorganism's natural environment (e.g., artificial, synthetic, or chemically designed media), for brewing it is important biofilms are studied in their environment. Biofilms provide microorganisms with a protective shield against harsh environments, whilst simultaneously providing a nutrient rich microenvironment, supporting proliferation. Such characteristics render biofilms as a much higher risk of product contamination. Therefore, preventing, or minimising biofilm formation is paramount in process and dispense design and hygiene. As discussed, at the point of dispense, there exists the correct environmental conditions that support biofilm formation (Section 1.13.7). Coupled with a recalcitrance to chemical treatment and anti-microbial constituents, biofilms can be detrimental to draught beer hygiene and quality. Furthermore, batch fermentation with long production runs (6-10 days), provides opportunity for contaminants to develop (Fratamico *et al.*, 2009).

1.13.7 Biofilms in beer dispense systems

Microbial biofilms in dispense systems are a direct consequence of infrequent and/or inadequate cleaning of the end to end dispense system by on-trade staff (Walker *et al.*, 2007). The brewing industry ideally should be mindful of developing dispense systems to minimise 'dead-legs', which are often nutrient-rich environments ideal for biofilm formation and proliferation of its planktonic counterpart. Contaminant control at the on-trade level, arguably the most important point from brewery to consumer, is not to the standard set by breweries during the production of beer. It has been observed that there is a correlation between beer quality and the frequency of line cleaning (Quain, 2007). Beer quality is defined as meeting consumer needs with no unwanted surprises (i.e. off flavours, diacetyl) (Bamforth *et al.*, 2011). Beer quality issues can be in part attributed to lack-of-knowledge regarding dispense hygiene amongst untrained on-trade staff, whereas in breweries, professionals tackle and improve

process hygiene (Jurado, 2003). Commercial sterility defines a food/beverage item to be safe for consumption but is not completely sterile. Here, low levels of microorganisms may be present but in insufficient numbers to compromise quality over the shelf life of the product. Alongside this, the inhospitable composition of beer prevents the growth of pathogens, thus achieving commercial sterility is innately aided by the product. Leaving the brewery, draught beer will contain low-levels of microorganism (< 50 cfu/L), which begin to inhabit dispense lines (Quain and Storgårds, 2009).

Despite strict regulations on food safety in regard to hygiene, stringent procedures are not in place for draught beer at the point of consumption (Quain, 2015). Draught beer microbiology has been sporadically investigated, with the recent use of a forcing test a tool in understanding microbial growth in draught beer (Mallett *et al.*, 2018). However, studies characterising genus and species inhabiting draught beer via molecular methods are limited. Yeast inhabitants include *Saccharomyces* spp., *Brettanomyces* spp., *Pichia* spp. and *Candida* spp. (Quain, 2015). Gram-positive inhabitants include LAB (*L. brevis* and *P. damnosus*) and Gram-positive inhabitants consists primarily of AAB (*Acetobacter* spp., & *Gluconobacter* spp.) (Bokulich and Bamforth, 2013). These organisms are the major contaminants of draught beer, a more comprehensive list can be found in Quain (2015).

Biofilms in dispense systems have been reported on various occasions. Biofilm formation can be driven by the age of the material, as beer lines can be in place for decades, exposure to strong caustic chemicals causes irreparable damage. Further, the role of the dispense conditions play a key role on the biofilm formation rate and

microflora (Jevons and Quain, 2021). It has been noted aged materials can be an important factor in promoting biofilm formation in draught beer dispense, and functional materials such as nylon can reduce biofouling (Heger and Russell, 2021). More recently work by Selna *et al.* (2021) revealed pre-conditioning with hop-derived terpenes reduced biofilm formation in beer lines by *Lactobacillus* sp.

1.13.8 Biofilms in packaging

In the brewing process, there are a number of points where the conditions support biofilm formation. Accordingly, process design is optimised to assure cleaning, minimise areas that are difficult to clean and reduce 'dead space'. Beer during packaging not subject to pasteurisation are susceptible to biofilm formation (Storgårds, 2000). The susceptibility of packaging lines to biofilm formation is due to continual exposure to moisture and nutrients (Storgårds *et al.*, 2006) and aerosols, difficult to clean solid surfaces and beer residues (Hofmann and Fischer, 2015).

Investigations of biofilms in the brewing process and packaging is limited. Process biofilms have been shown to develop between 2-12 hours in areas supportive of biofilm formation, however, areas less supportive (dry surfaces) are also susceptible to biofilm maturation (Storgårds *et al.*, 2006). Common brewery isolates, such as *L. brevis* and *Acetobacter* sp. are readily isolated in breweries, particularly in pasteurisers, and these organisms have been shown to attach to stainless steel, Buna-N and Teflon (Czechowski and Banner, 1992). Adhesion to stainless steel by microorganisms was also shown to be influenced by environmental conditions, such as a low pH (pH 3) (Bittner *et al.*, 2016). Further conclusions by Bittner *et al.* (2016) suggest that despite daily cleaning procedures, microbial adhesion and biofilm formation at bottling plants persists. Banner (1994) investigated biofilms on conveyer

systems and showed that contamination was associated with small pack filler heads and bottle warmers. Microorganisms such as LAB, AAB, enterobacteria and yeasts have been isolated from the surfaces of bottling and dispense equipment (Devolli *et al.*, 2016). In clinical environments there is a growing activity to explore functional coatings to minimise the attachment of organisms, and such coatings have been suggested for use within breweries (Priha *et al.*, 2015, Page *et al.*, 2009) including photocatalytic metal-ion doped and non-dope TiO_2 coatings on stainless steel. Functional coatings have been discussed for other industries such as for cosmetics and food (Fierascu *et al.*, 2021), but for the brewing industry no reduction in biofilm was reported by Priha & Raulio Priha *et al.* (2015).

1.14 Draught beer quality

1.14.1 Definition

Draught beer quality is the assessment (ideally measurement) of its current state compared the expected flavour, haze, and aroma. During the brewing process numerous controls and quality assurance practices are in place to ensure consistency and assure quality. A quote by Bamforth in Kellershohn (2016) succinctly defines a quality in beer as:

"Quality is the achievement of consistency and the elimination of unwanted surprises"

Despite the significant controls in the brewery, once in the hands of the retailer draught beer is vulnerable on dispense to contamination by microorganisms. Beer is exposed to airborne microbial contaminants and, more significantly, biofilms attached to dispense lines and associated surfaces. To tackle this problem, methods are necessary

to quantify beer quality ex-dispense. Central to this is regular cleaning of the dispense lines which need to be effective at minimising or better still the removal of biofilms. The ultimate aim of improvements in the hygienic practices of beer dispense is to serve customers with a consistently high-quality beer as intended by the brewer.

1.14.2 Methods to quantify beer quality

Traditionally and unsurprisingly, assessment of beer quality was a qualitative analysis, by an experienced brewer or Trade Quality technician who would assess the haze and aroma of the beer and pronounce on its quality. This technique is still in use today, under the banner of 'Cask Marque' – a commercial organisation – initially focussed on cask beer but now extending to include keg beer and it is from this analysis public houses proudly, with no hint of irony, display the Cask Marque badge to prove their beer is of a high quality. The lack of consistency in the analysis fundamentally renders this certification questionable, as such alternative approaches have been proposed as an alternate method to quantify beer quality.

A more robust, quantitative alternative to the qualitative Cask Marque approach is required. In the late 19th Century, brewers in Burton-on-Trent applied the 'forcing' approach to assess whether beer brewed in the cooler months would be of satisfactory quality to be sold during the summer. This method including collecting a small sample of the beer, incubating it at room temperature and assessing its quality by assessment of haze, flavour, and aroma. The forcing method was 'reinvented' to assess the quality of draught beer by Mallett *et al.* (2018). The method requires the incubation for 4 days at 30^{o}C of 25mL of draught beer (in duplicate) sampled from the trade. Absorbance is measured at 660nm at 0 h and 96 h, with the change in absorbance reported in 'bands' between A-D (A = excellent, B = acceptable, C = poor and D unacceptable). By

measuring the increase in turbidity, enables a retrospective analysis of the initial microbial loading in the dispensed beer. The larger the initial inoculum, the greater the increase in turbidity. The method provides an insight into draught beer quality and can be used proactively to improve line hygiene and quality.

1.14.3 The consequences of complex draught beer dispense systems

Beer dispense is fundamentally a simple process requiring a container (cask) of beer and a tap. However, in the UK draught beer dispense is a complex process involving a pressurised keg, a motive gas (carbon dioxide but increasingly blended as 'mixed gas' with nitrogen), lengthy (average of 25 metres) dispense tubing ('line'), cooling (in the cellar, under the bar) to a branded tap. Beer lines are bundled together ('python') with cold water circulating to provide cooling).

Draught beer dispense is increasing subject to 'innovation', typically cooling technologies, and increased dispense speed. Heineken's Smart dispense system delivers end to end cooling from the keg to tap and reportedly requires a 6-week cleaning cycle (by Heineken dispense technicians) rather than the recommended weekly cleaning (by bar staff). The control of temperature is designed to be a 'two birds with one stone' scenario. By reducing the temperature - to meet market trends for extra cold beer - the growth rate of contaminating microbes is reduced, improving beer quality and consistency. Simply put, microbes do not choose the environment it can thrive in, the environment chooses which microbes survive. Therefore, in the case of dispense, the reduced temperatures will facilitate the growth of previously insignificant contaminants sourced in beer and dispense lines. As discussed, biofilms or 'sessile' state is the preferred mode of survival for microorganisms, contrary to historical understanding of microbial behaviour. Evidence shows biofilms continue

to grow in low temperatures, increasing in maturity and hence difficulty to remove (Jevons and Quain, 2021). Therefore, it is conceivable that long-term issues regarding beer line hygiene could arise due to colder line conditions.

1.14.4 The influence of beer style and composition on spoilage microflora

The limited and historical research on draught beer microflora has extrapolated the findings across all beer styles. For example, the notion that *Lactobacillus* sp. are key beer spoilers of lager is correct, but such a conclusion does not necessarily apply to other beer styles.

"Everything is everywhere, but the environment selects" Baas Becking (1934)

The quote by Baas Becking in 1934 is a well-known in the field of microbial biogeography. When discussing its relevance to beer, it is important to first understand the plethora of beers available first discussed in Section 1.4. Between ABI InBev, Heineken, and Carlsberg there are approximately 1300 beer brands brewed by these three companies alone. Irrespective of the accuracy of that information the number is directional in appreciating the vast number of beers and consequently styles brewed. Once comprehended it is clear the illogical nature of using data from approximately 60 years ago often focussed on a single beer style and extrapolating this data across all these beers and many more.

Different styles and brands of beers range in their bitterness, ethanol concentration, sugar profiles, pH, and trace nutrients. These differences will result in microbial contaminants unique to a particular beer style or brand.

1.14.5 Draught beer quality: A new hope

For the most part, the economic challenges facing the industry are out of the breweries control. In this aspect the best path forward is to follow the laws and find novel avenues to generate revenue. A key factor however, not yet discussed, is the increased incidence of poor draught beer quality. An element that is completely within the industries control but not necessarily an area of focus.

A recent trade audit in the East Midlands published in 2019 identified approximately 70% of beers sampled had suffered some degree of microbial contamination (Mallett and Quain, 2019b). In other words, 70% of the beers were not in the condition set by the brewery, with likely changes to flavour, haze, and aroma. 'Off-beer' is not a pleasant experience, and anecdotal evidence suggests consumers will react one of three ways; 1) ask for a replacement drink 2) replace drink with a different beer/brand or 3) change bars. All three result in a revenue loss for both the on-trade retailer and the brewer. It should be the responsibility of the retailer to maintain the lines as best as possible. However due to the presumed economic burden of a robust best-practice regime for dispense lines, it is not uncommon for retailers to conduct fortnightly or three-week cleaning cycles on a traditional beer line, above the recommended weekly-cleaning cycle by the Brewers' Association.

1.15 Aims and objectives

Beer is innately a complex environment, consisting of numerous inhospitable elements to the vast majority of microorganisms. Amidst evolving dispense conditioning, the on-trade microflora has likely adapted to the new challenges of dispense, such as extra cold lines. Prior work in this field has used conventional methods and hence the

primary focus of this work was to firstly investigate the ‘culturable’ microbiome from draught beer and compare this to the microbiome identified using a ‘culture-independent’ method. Thus, mitigating the phenomenon of ‘non-culturability’. As dispense technology has advanced, there are likely novel beer spoilers unknown in the field. Current methods utilise agar media designed to target a host of historical beer spoilers, LAB sp., AAB sp., and wild yeasts. By employing next-generation sequencing tools – this work will aim to reveal the entire microbiome (yeasts and bacteria), quantify, and investigate numerous styles of beers.

The objectives of this work include:

1) Quantify spoilage of four draught beer styles from the on-trade using the forcing method
2) Plate on-trade samples onto industry standard media (WLN and Raka-Ray agar), and select colonies for identification, prioritising abundance
3) Identify the ‘microbiome’ using Oxford Nanopores Next-Generation sequencing tool
4) Discuss differences between the culture-dependent and culture-independent identification methods.

Biofilms are a huge problem in industry, and this remains true for beer dispense systems. Biofilms are likely harbouring an undefinable number of previously unidentified microorganisms in draught dispense systems. Further, the formation of biofilms will be influenced by the dispense parameters and cleaning regime of the account. Thus, this work will aim to design a method to quantify draught dispense

biofilms and investigate the impact of dispense parameters on biofilm formation and maturation.

The objectives of this work include:

1) Design a simple method in 96-well plates, which can closely replicate the environment / conditions experienced during draught dispense to quantify biofilm formation
2) Test reproducibility of the assay
3) Quantify the impact of draught dispense environmental parameters (time, temperature, nutrition, oxygen, etc.) on biofilm formation.
4) Quantify the effectiveness of line cleaning solutions on biofilm removal and microorganism recovery post-cleaning

Finally, previous work by Dr David Quain and Dr James Mallett revealed beers of the same style and similar key parameters (pH, %ABV) reveal key differences in ‘spoilability’. The driving elemental niches behind these differences are yet to be determined. Identifying these other factors and their relationship to ‘spoilability’ may in future prove valuable in determining a beer’s suitability to draught dispense or designing beers with increased microbiological stability. Hence, this work will aim to begin tackling this rather complex question in hope of providing a foundation for future work.

The objectives of this work include:

1) Quantify key environmental parameters important to a beers spoilability
    a. ABV%, pH, fermentables, free amino nitrogen

b. Impact of increasing sugar complexity on spoilability by inoculating microorganisms sourced from different styles of on-trade beer, into a YPD-modified medium
c. Use a ‘spoilage screen’ to identify the differences in spoilability between commercially available lagers

2) Quantify the sugar profile of commercially available lagers and ales
3) Track sugar assimilation during spoilage using anion chromatography
4) Track the use of organic acids key in the Kreb’s cycle using LC-MS techniques during forcing and relate to spoilage using the forcing method

Chapter II: Culture-based microflora of draught beer

Foreword

Work in this Chapter was completed during 2018 to the year end and was prepared for publication during late 2021 and 2022. Aims of this work was to identify the 'culturable' microflora of various draught beer styles using industry standard agars from samples collected from the on-trade

This work was submitted on 5th April 2022 for publication to the Journal of Applied Microbiology and was accepted on the 8th September 2022.

I was responsible for the production of all data in this work and lead author of the work under the supervision of Dr David Quain.

Abstract

Aims: To determine whether the culture dependent spoilage microflora found in draught beer are influenced by beer style and/or the public house.

Methods and Results: Four beer styles – lager, ale, stout, and cask ale - were sampled twice from five different public houses (accounts) in four different locations. The microbiological quality of the dispensed beers was determined by incubating at 30ºC and measuring the increase in absorbance. Beer quality varied between accounts with some being 'excellent' and others being 'acceptable' to 'poor'. Beer quality by style ranged from 90% (cask beer) to 67.5% (keg ale). Using DNA based identification of microflora, 386 colonies from agar plates were identified with 28 different microorganisms from five genera of yeast and six of bacteria. Seven microorganisms were found in all beer styles with *Brettanomyces bruxellensis, B. anomalus* and *Acetobacter fabarum* representing 53% of the identified microorganisms. The microflora of draught beer spoilage resembled that reported in the production of Belgian Lambic sour beers.

Conclusions: In this work, draught beer in UK public houses was of variable quality. Culture-based analysis of draught beer suggests that core microflora vary with style. A subsequent, limited study of culture independent microorganisms suggests that the microflora of draught beer may be more expansive when analysed using culture independent methods.

Significance and impact of the study: The quality of draught beer is of commercial importance and is important to the Consumer. However, draught beer quality and

microflora has received little attention. Here, we report the core and diverse microflora found in different styles of draught beer.

Keywords

microflora, identification, draught, beer, spoilage, styles,

2.1. Introduction

Beer is a relatively low alcohol (4-8% alcohol by volume/ABV) beverage produced by fermentation with yeast (*Saccharomyces* species). Globally there are around 100 diverse beer styles (Papazian, 2017), which are primarily packaged into bottles and cans ('smallpack', typically ≤ 0.5 l) or, less frequently, large pack (stainless steel kegs and casks - ≥ 20 l). The global market is split into on-trade (pubs, bars, clubs, restaurants, hotels) and off-trade (shops, supermarkets). Broadly, small pack beers are purchased in off-trade accounts whereas in on-trade accounts beer is served as 'draught' dispensed from kegs into glasses. Beer in cans, bottles and kegs are pasteurised (or sterile filtered) and are 'commercially sterile'. Kegs are pressurised vessels, dispensed with a top pressure of carbon dioxide or a blend of carbon dioxide and nitrogen. Cask beer is a traditional UK format, without gas top pressure and unpasteurised with secondary fermentation to provide carbon dioxide or 'condition'.

In the UK, draught beer in the on-trade accounted for 41.1% of total beer sales (46.0 million hl) in 2019. Of this, keg lager is the predominant style (64%) with 16% cask ale, 12% keg ale and 7% keg stout. In 2020, during the Covid pandemic, on-trade sales declined by 55%, off-trade increased by 19% with the overall market declining by 14% (British Beer & Pub Association, 2021). Production methods, raw materials (varieties of malt and hops, adjuncts) and water differ between beer styles (Papazian, 2006). Notably, fermentation uses *S. cerevisiae* (ales and stout) or the hybrid yeast *S. pastorianus* (lager). With more than 800 compounds in beer (Cortacero-Ramırez *et al.*, 2003), beer styles have a common 'backbone' but are differentiated by signature flavours and aromas and, increasingly, by analysis of chemical composition (Anderson *et al.*, 2021). Visually, styles range from straw/golden (lager), amber (ale)

to black (stout) and, with draught beer, the serving temperature range from 2-6°C (lager), 4-8°C (stout), 6-12°C (ales) and 11-14°C (cask ale).

The dispense of keg beer in pubs in the UK is complex, involving lengthy chilled dispense 'lines' of varying age and quality from the cellar (11-13°C) to the tap in the bar (Quain, 2015). Although commercially sterile (< 1 cfu l^{-1}) in keg, draught beer post dispense contains (non-pathogenic) microorganisms that range from < 10^3/mL (where best hygienic practices are applied) to > 5 x10^4 ml^{-1} (Mallett *et al.*, 2018, Quain, 2015). The hygiene (and quality) of beer dispense is managed by cleaning dispense lines with cleaning solutions containing sodium hydroxide, wetting agents etc. Although the best practice in the UK for line cleaning is weekly, many accounts clean every two or three weeks. The cleaning process is essentially static and lacks mechanical action to clean effectively (Jevons and Quain, 2021). Accordingly, line cleaning as practiced in most public houses is not successful and the lines quickly become recontaminated with microorganisms.

Beer is considered inhospitable to microorganisms as it is typically low in nutrients (oxygen, sugars, amino acids etc), high in ethanol (4-8% ABV) with a low pH (< 4.2) and antimicrobial bitter substances contributed from hops. Despite this, draught beer is susceptible to spoilage by a limited but diverse range of microorganisms (Quain, 2015), including aerotolerant gram-positive bacteria (*Lactobacillus*, *Pediococcus*) (Suzuki 2015), aerobic gram-negative bacteria (*Acetobacter*, *Gluconobacter*) (Kubizniaková *et al.*, 2021), facultatively aerobic yeasts (*Saccharomyces*) and aerobic yeasts (*Brettanomyces, Pichia, Rhodotorula*) (Powell and Kerruish, 2017).

Despite the dominant market share of draught beer in the 20th century, there have been only sporadic publications on the microbiology of draught beer (Quain, 2015). Early studies focussed on the identification of yeasts recovered from spoilt draught cask beer (Wiles, 1950) or samples from public houses (Hemmons, 1954). Subsequent studies from the University of Birmingham (Casson, 1985, Harper, 1981, Harper *et al.*, 1980, Hough *et al.*, 1976) extended to the microflora of keg beer and the role of line cleaning in managing beer microbiology. All these reports used conventional selective microbiological methods, reporting plate counts for beer spoilage and environmental microorganisms. Whilst informative as to the microflora of draught beer, this approach is difficult to relate to quality, as the quantification of microbial colonies on agar plates does not relate to growth in beer (Mallett *et al.*, 2018).

Beer quality can be assessed by 'forcing' beer post dispense at 30°C which allows indigenous microorganisms to grow which after four days are quantified by measurement of turbidity (Mallett *et al.*, 2018). Depending on the increase in absorbance, four quality bands are recognised ranging from 'excellent' to 'unacceptable'. Using this approach, a trade audit of 237 samples of lager and keg ale in 57 public houses showed draught beer quality to be variable, with lager of better quality than ale (Mallett and Quain, 2019).

The aim of this work was to evaluate the microflora of four draught beer brands available across the United Kingdom. The four beers - cask ale (SC1) and keg lager (SL3), ale (KA1) and stout (ST1) – were sampled from five different accounts per brand in four different locations. The 20 on-trade accounts were sampled on two occasions a month or so apart. Culture-based microflora from each of the 40 samples

were obtained after forcing the draught beer sample, plating on selective agar, extraction of DNA, amplification (16S rRNA for bacteria and ITS for yeast), sequencing and BLAST identification.

2.2. Materials and methods

2.2.1. Trade sampling from 20 accounts

The beer brands and the licensed public houses ('accounts') used in this work are anonymised using codes to provide continuity with past studies (Jevons and Quain, 2021, Mallett and Quain, 2019, Mallett *et al.*, 2018). The four beer styles were sampled twice from five different accounts in four different locations, four to six weeks apart between August 2018 and February 2019. Sampling was typically between 13.00 and 16.00 on Mondays (3x), Wednesday, Thursday, or Friday (3x). The samples were taken from public houses in Nottingham city centre (stout ST1), Derby city centre (ale KA1), Burton-on-Trent town centre (cask ale SC1) and three local villages (lager SL3). Samples (half pint) of draught beer were purchased as a 'customer' and, accordingly, the efficacy and frequency of line cleaning and other hygienic practices in the public house were not known. Samples (250 ml) were transferred to sterile Duran bottles, kept cold (4-6°C), and processed within 12 hours.

2.2.2. Forcing test and quality index

The microbiological loading of beer post dispense was determined by incubating the beer at 30°C for 96 hours and measuring the increase in absorbance at 660 nm (Mallett *et al.*, 2018). Cycloheximide (4 mg l^{-1}) was added to samples of unpasteurised cask beer (SC1) to suppress the growth of indigenous 'secondary' brewing yeast (Lin, 1975). Samples were assessed in triplicate. The increase in absorbance reflected the microbiological quality of the beer and was categorised as A/excellent (Δ A_{660} 0-0.3),

B/acceptable (Δ A_{660} 0.31-0.6), C/poor (Δ A_{660} 0.61-0.9) and D/unacceptable ((Δ A_{660} > 0.91).

For each brand, a 'quality index' was calculated from the sum of the individual scores (n=10) for each quality band (where A = 4, B = 3, C = 2, D = 1) divided by (number of samples x 4) x 100.

$$\text{Quality index (\%)} = \frac{\Sigma\, quality\ score}{number\ of\ samples\ x\ 4} \text{ x } 100$$

Accordingly, if all samples are in the excellent band (A), the quality index is 100% whereas if all samples are in quality band B (acceptable) the index is 75%.

2.2.3. Culture-dependent microbiological analysis

2.2.3.1. (i) Plate culture

Post forcing, samples were diluted (10^{-4}) in sterile RO water and plated onto industry standard agar media (n=3 per media type) (in a Class 2 cabinet). WL Nutrient (WLN) Agar (Oxoid, CM0309) was used for aerobic microorganisms and Raka Ray (RR) (Oxoid, CM0777) for anaerobes and plates were incubated at 30^{o}C for 4 days and 7 days, respectively. WLN and RR prepared according to the manufacturer instructions. RR plates included cycloheximide (10 mg l^{-1}) and were incubated in anaerobic jars with an anaerobic sachet (Thermo Scientific™ AN0035A).

2.2.4. ii) Master Cultures

Single colonies were selected and transferred to fresh agar plates of WLN or RR to create master cultures. Five colonies per account were selected, reflecting abundance.

to select the primary spoilage organisms of the account/beer style. Plates were incubated as above.

2.2.5. DNA extraction, sequencing, and identification

2.2.5.1. (i) total DNA extraction.

DNA extraction was using phenol/chloroform (Legras and Karst, 2003). After identification by microscopy, a colony of bacteria or yeast was added to 0.4g glass beads (Sigma-Aldrich, G8772), 400µl phenol/chloroform (Acros Organics, 327111000) and 400µl lysis buffer (Tris 10mM, pH 7.6, EDTA 1mM, NaCl 100mM, Triton X-100 2% w/v, sodium dodecyl sulphate (SDS) 1% w/v). The mixture was vortexed for 4 minutes and then centrifuged for 5 minutes at 2250 x g. The supernatant was transferred to a separate tube containing 500µl chloroform-isoamyl alcohol (Sigma-Aldrich, 25666), inverted 2-3 times and centrifuged for 2 minutes at 17,000 x g. The supernatant was transferred to ethanol (99.8%) in a separate tube (approx. 2:1 ratio ethanol: supernatant). This was centrifuged for 5 minutes at 17,000 x g, the ethanol decanted, and the tube allowed to air dry. The DNA was suspended in TE buffer/ddH_2O (30-50µl) and stored at -20^oC or used directly for PCR.

2.2.5.2. (ii) Amplification and purification of bacterial and Yeast loci

PCR was carried out in 50µL reaction volumes containing 50-250 ng of yeast/bacteria DNA, 1X buffer (PCRBio), 1.5 mM $MgCl_2$, 200 µM dNTP mix (New England Biolabs, N0447S), 1 unit/µL of PCRBio taq DNA polymerase and 0.5µM of each oligonucleotide primer. Where yeast DNA was amplified, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990) were used and for bacteria, 16S rRNA primers 63F (5'-CAGGCCTAACACATGCAAGTC-3') and 1387R (5'-GGGCGGWGTGTACAAGGC-3') (Marchesi *et al.,* 1998) were used for bacterial

DNA. Amplification was performed using a Techne TC-512 thermocycler using the parameters: 5 min at 94ºC followed by 34 cycles of 30 s at 92ºC, 45 s at 54ºC and 1 m 15 s at 72ºC, ending with a final extension of 5 min at 72ºC. PCR amplicons were purified using Jena Bioscience PCR purification kit (Jena Bioscience, PP-201L).

2.2.5.3. (iii) Sequencing and bioinformatic analysis.

Purified amplicons were prepared for sequencing as required by the service providers (https://www.sourcebioscience.com). Sequences were analysed using NCBI Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the genus and species.

2.2.6. PCR GeneDisc analysis

Culture independent analysis of spoilage microorganisms was performed using PALL PCR GeneDisc technology. The yeast GeneDisc technology delivers multiplex quantitative PCR for yeasts including *Saccharomyces, Brettanomyces* and *Candida/Pichia* and the spoilage bacteria GeneDisc includes *Lactobacillus* species (*L. paracollinoides, L. backii, L. brevis*) and *Pediococcus* but not species of *Acetobacter*. As the primer sets and targets are proprietary, it is not known which sequences are used to distinguish between the species. Limit of sensitivity of the method used is approximately 50 cfu ml^{-1}. Draught beer samples were obtained during February 2022 from a local public house (L7) used in previous studies (Jevons and Quain, 2021, Mallett *et al.*, 2018). Samples were forced (96 hours, 30°C) and the culture analysed directly using GeneDisc at https://www.murphyandson.co.uk.

2.3. Results

The four beer styles - cask ale and keg lager, ale, and stout – were sampled in five different accounts per style in four different locations. The microbiological quality of ten beer samples per style was assessed retrospectively by forcing, enabling spoilage

organisms to grow (Table II-1). Cask ale samples were of the best quality as determined by the quality index (90%) with samples either ‘excellent’ or ‘acceptable’. Both lager and stout samples had a quality index of 75%, whereas samples of keg ale had a quality index of 67.5% with individual samples ranging from ‘acceptable’ to ‘poor’.

Table II-1: Beer quality post dispense

Lager SL3 (4% abv)					Stout ST1 (4.4% abv)				
Account	**£/pint**	**08/03/18**	**09/05/18**	**Quality Index (%)**	**Account**	**£/pint**	**10/19/18**	**11/19/19**	**Quality Index (%)**
B7	3.00	C	B		**N5**	3.30	A	A	
V5	3.80	B	B		**N8**	4.00	B	B	
V6	3.40	B	C	75	**N14**	4.00	A	C	75
V8	3.60	B	B		**N15**	3.40	D	C	
V13	2.90	A	A		**N16**	3.50	A	B	
Average	3.34	15	15		**Average**	3.64	16	14	

Ale KA1 (3.6% abv)					Cask ale SC1 (4.2% abv)				
Account	**£/pint**	**10/29/2018**	**11/29/2018**	**Quality Index (%)**	**Account**	**£/pint**	**01/04/19**	**02/04/19**	**Quality Index (%)**
D2	2.00	C	B		**B2**	3.23	A	B	
D10	2.00	B	B		**B10**	3.20	A	A	
D13	2.00	B	C	67.5	**B11**	3.40	A	A	90
D19	3.40	C	B		**B12**	3.30	A	B	
D20	3.80	B	B		**B13**	3.70	B	B	
Average	2.64	13	14		**Average**	3.37	19	17	

Approximately 100 culture dependent colonies per beer style were processed with 385 identified using BLAST. The abundance of identified colonies by beer style is presented in Table II-2. with the 'top 10' representing 89% of the total. The samples of the four styles were purchased from twenty public houses on two occasions. The samples were obtained as 'consumers' and nothing is known of hygienic practices in the accounts to assure the microbiological quality of the beer. Accordingly, the prevalence of microorganisms reported in Table II-2 would be impacted by hygienic interventions such as line cleaning which in turn would reflect frequency and efficacy. Given these unknown variables, the only constant across this study is beer style. As the beers are brewed by four global brewers with sophisticated quality management systems, beer quality would be consistent.

Across the four styles, 28 different microorganisms were identified, with stout (19) and lager (17) more diverse than cask ale (12) and keg ale (10). In all, five genera/10 species of yeast and six genera/18 species of bacteria were identified. The recovery of individual microorganisms in both samples from an account – separated by four weeks – was found on 49 occasions and reflected abundance.

By far the most prevalent microorganisms were the yeasts *Brettanomyces bruxellensis* and *B. anomalus* together with acetic acid bacteria (*Acetobacter fabarum*, *A malorum*, *Gluconobacter oxydans*). These microorganisms were ubiquitous in all four beer styles, accounting for 67% of the 386 identified colonies. Together, *Brettanomyces bruxellensis* and *B. anomalus* represented 46% of the identified microorganisms in cask ale, 42% in keg ale and 32-35% in lager and stout. The acetic acid bacteria (*A.*

fabarum, A. malorum, G. oxydans) accounted for 41% of the recovered microorganisms in cask ale, 29/31% in lager/stout with 11% in keg ale.

Yeasts predominated in keg ale (82%), stout (58%) and cask ale (51%) but not in lager (43%). Although dominated by *Brettanomyces* species, other yeasts were found in all four beer styles albeit at low levels (2-8%, *Saccharomyces cerevisiae;* 1-2%, *Pichia membranifaciens).* Notably, *Rhodotorula mucilaginosa* represented 24% of the identified colonies in keg ale and 14% in stout but was not found in cask ale or lager. Of the four beer styles, yeasts were the most diverse in stout with species of *Saccharomyces* (x3) *Brettanomyces* (x2), *Pichia* (x2), *Rhodotorula* and *Candida*.

Stout was also the most diverse in bacteria with species of *Acetobacter* (x4), *Gluconobacter* (x2), *Weissella and* lactic acid bacteria *(Lactobacillus, Leuconostoc, Pediococcus*). Of the identified microorganisms, *Acetobacter* and *Gluconobacter* species accounted for 18% of the microorganisms in keg ale, 33% in cask ale, 35% in stout and 37% in lager. The lactic acid bacteria *Secundilactobacillus paracollinoides* (basonym *Lactobacillus paracollinoides) and Levilactobacillus brevis* (basonym *Lactobacillus brevis*) were found in lager (20% of the recovered colonies), but with low abundance in stout (6%), cask ale (1%) and absent in keg ale. *Leuconostoc fallax* and *Gluconobacter frateurii* were in lager and stout. Both *Pichia manshurica* and *S. paracollinoides* were in lager SL3, *Acetobacter pasteurianus* in cask ale and *Pediococcus damnosus* in stout.

Table II-2: Microorganisms found in different draught beer styles

Microorganism	Colonies identified in each beer style					
	Lager (SL3)	Stout (ST1)	Ale (KA1)	Cask ale (SC1)	Total	Found in Lambic*
Brettanomyces bruxellensis	19 (5)	18 (1)	10 (2)	30 (5)	77	*1-9*
Brettanomyces anomalus	13 (3)	13 (3)	32 (4)	15 (2)	73	*5-7*
Acetobacter fabarum	16 (2)	15	8 (2)	16 (2)	55	*1,8*
Rhodotorula mucilaginosa	-	13	24 (2)	-	37	*1*
Acetobacter malorum	2	6	2 (1)	19 (4)	29	*3*
Gluconobacter oxydans	11 (3)	7	1	5	24	
Saccharomyces cerevisiae	2	3	8 (1)	4 (1)	17	*1,3,5-8*
Levilactobacillus brevis	13 (2)	2	-	1	16	*1,2*
Saccharomyces uvarum	3 (1)	1	5	-	9	*9*
Pichia membranifaciens	2	1	2	1	6	*2,5-7*
Leuconostoc fallax	3 (1)	2	-	-	5	
Acetobacter lovaniensis	-	1	3	1	5	*4*
Secundilactobacillus paracollinoides	4	-	-	-	4	
Acetobacter pasteurianus	-	-	-	4	4	*4,6*
Pichia manshurica	4 (1)	-	-	-	4	
Acetobacter cerevisiae	-	1	1	1	3	*3,4*
Acetobacter persici	-	-	3	-	3	
Gluconobacter frateurii	2 (1)	1	-	-	3	
Acetobacter sicerae	2	-	-	-	2	
Acetobacter tropicalis	2	-	-	-	2	
Candida boidinii	-	1	-	-	1	
Saccharomyces bayanus	-	1	-	-	1	*5,7*
Pichia fermentans	-	1	-	-	1	
Acetobacter estunensis	-	-	-	1	1	
Acetobacter indonesiensis	1	-	-	-	1	
Gluconobacter albidus	1	-	-	-	1	
Pediococcus damnosus	-	1	-	-	1	*2,6-8*
Weissella cibara	-	1	-	-	1	
Total	100	89	99	98	386	

* (*1*) Bokulich *et al*. 2012; (*2*) Bossaert *et al.* 2021; (*3*) Bossaert *et al*, 2022 (*4*) De Roos *et al.* 2018: (*5*) De Roos and De Vuyst, 2019: (*6*) De Roos *et al*. 2020; (*7*) Spitaels *et al*. 2014; (*8*) Spitaels *et al.* 2015; (*9*) van Oevelen *et al*. 1977.

2.4. Discussion

2.4.1. Draught beer quality

Draught beer inevitably contains microorganisms, of which the magnitude and diversity impacts on quality. Forcing of draught beer samples post dispense for 96 hours at 30^{o}C enables a retrospective analysis of quality through measurement of turbidity. Beer of 'excellent' quality contains low levels of microorganisms and on forcing the turbidity increases marginally (Δ A_{660} 0-0.3). As quality declines, microbiological quality ex-dispense and (on forcing) the associated turbidity increases through 'acceptable' to 'poor' and 'unacceptable' (Mallett *et al.*, 2018).

The quality of cask ale was notably superior to lager and stout with keg ale exhibiting the poorest quality (Table II-1). Unlike keg beers, cask ale is not pasteurised. The beer contains brewer's yeast (*S. cerevisiae*) which contributes carbon dioxide ('condition') through secondary fermentation in cask. Once broached, air (oxygen, microorganisms) enters the cask, and consequently the beer has a limited shelf life (three days). Accordingly, cask beer is not found in all public houses as it requires enhanced cellar management skills to assure good quality beer. Given this, it is no surprise that cask ale in this survey exhibited an enhanced quality index of 90%. With the keg beers, the quality index of lager SL3 (75%), stout (75%) and keg ale KA1 (67.5%) demonstrated that beer quality varied markedly between individual accounts. For example, the samples of stout ST1 ranged from both being excellent (account N5) to unacceptable and poor (N15). The quality index of both lager SL3 and keg ale KA1 from the five accounts was like that reported for the same brands in a previous, wider survey (Mallett and Quain, 2019) with SL3 at 75% and KA1 at 68.3%. This suggests that the susceptibility to microbial spoilage is influenced by beer composition.

In agreement with previous studies (Mallett and Quain, 2019, Mallett *et al.*, 2018), beer quality was found to vary by brand and by licensed account. The quality of draught beer is dependent on numerous factors including turnover together with regular and effective implementation of hygienic practices (such as line cleaning). As sampling was covert, these considerations are not identified at an account level but will have been reflected through the measurement of beer quality.

2.4.2. Microflora in draught beer

The genera reported in Table II-2 have been reported in draught cask and keg beer between 1950 and 2013 (Quain, 2015). In these studies, identification of microorganisms was based on morphological and biochemical tests whereas the work reported here used DNA sequence for identification. Accordingly, this report confirms the diversity of draught beer microflora and extends it to include the impact of different beer styles.

Two yeasts (*B. bruxellensis, B. anomalus)* and a bacterium *(A. fabarum)* predominated in all four styles. Other 'core' microflora but at a much lower loading included *A. malorum, G. oxydans, S. cerevisiae and P. membranifaciens.* Style specific microorganisms included *A. pasteurianus* (cask ale), with *S. paracollinoides* and *P. manshurica* in lager. The yeast *R. mucilaginosa* was present at comparatively high abundance in keg ale and stout.

Irrespective of beer style, *Brettanomyces* yeasts were found in draught beer in all 20 accounts and in 39 of the 40 samples that were taken during the six months of this study. Such ubiquity was surprising as, other than a report in 1961 of *Brettanomyces*

in ‘trade samples’ (cask beer) (Gilliland, 1961), reports of the yeast in draught keg beer are limited (Hough et al., 1976; Harper et al., 1980; Harper, 1981; Casson, 1985, Quain, 2015). Indeed, there are few reports of *Brettanomyces* in the brewing process (Manzano *et al.*, 2011, Pham *et al.*, 2011) or of spoiling beer (Crauwels *et al.*, 2017, Day and Helbert, 1971, Gilliland, 1961, Shimotsu *et al.*, 2015b).

Brettanomyces was named by Claussen in 1904 who reported that the ‘secondary fermentation effected by *Brettanomyces* is indispensable for the production of the real type of English beers’ and suggested that the yeast ‘exists as a general infection’ in breweries at the time (Claussen, 1904). Over a century later, whilst retaining the reputation as a spoilage yeast of fermented beverages (Steensels *et al.*, 2015) the yeast is viewed more positively for its contribution to the spontaneously fermented sour beers, such as Belgian Lambics (De Roos and De Vuyst, 2019) and American coolship ales (Bokulich *et al.*, 2012).

Of the 28 different microorganisms found in this work, 13 were acetic acid bacteria with 10 species of *Acetobacter* with three species of *Gluconobacter*. In addition to *A. fabarum*, both *A. malorum* and *G.oxydans* were found in all four beer styles. Despite their abundance in draught beer (Table II-2), only *G. oxydans* (formally *Aeromononas oxydans*) has been recognised in beer (Ault, 1965), with *A. fabarum* reported in ‘dinner beer’ (Wieme *et al.*, 2014) and sour beer (Bokulich *et al.*, 2012, Spitaels *et al.*, 2014).

Acetic acid bacteria oxidise ethanol to acetic acid (Kubizniaková *et al.*, 2021), and although aerobic, *Gluconobacter* species are ‘capable of developing at very low oxygen tensions’ (Ault, 1965). Whilst in the past (1940s) much feared, acetic acid

bacteria are no longer a concern in brewing with closed vessels, packaging in kegs, significantly improved hygiene and oxygen stringently minimised to assure flavour stability of the product. However, the threat of acetic acid bacteria and associated vinegar taint in cask beer (Ault, 1965) remains a concern (Kubizniaková *et al.*, 2021, Quain, 2015). Ingress of oxygen occurs during the dispense of cask beer and 46% of the microflora identified in the cask ale samples were acetic acid bacteria. At packaging, the specification for oxygen in keg beers is < 100 μg l^{-1} (Rod White, personal communication), with any additional oxygen pick-up during dispense limited to gas permeation through beer dispense tubing and the beer/air interface at the keg connector and tap nozzle. Given this, as noted by Harper *et al.* (1980) 'the prevalence of acetic acid in (keg) beer is surprising' reflecting the 'microaerophilic conditions in the pipes'. Indeed, the preponderance of *Acetobacter* and *Gluconobacter* in this work – with 35-37% of the microflora in stout and lager with 17% in keg ale – suggests that in draught beer, oxygen is more available to microorganisms than anticipated.

Rhodotorula mucilaginosa was found in keg ale and to a lesser extent in keg ale but not in lager and cask ale. In brewing, this aerobic yeast, which is unable to ferment, has been recovered from sugar syrups or 'primings' (Wiles, 1950), pitching yeast (Brady, 1958), fermentation (Pham *et al.*, 2011) and sour beer (Bokulich *et al.*, 2012). Outside of brewing, *R. mucilaginosa* is 'considered one of the top 10 yeast species causing food spoilage' and – of relevance to draught beer - can grow at refrigerated temperatures and low pH (Robinson, 2014). The growth of the yeast in stout and keg ale may reflect both styles being compositionally more nutritious than the more 'attenuated' lager and cask beer.

The yeast genus *Saccharomyces* contains seven natural and two hybrid species (Sampaio *et al.*, 2017). *S. cerevisiae* – a facultative anaerobe - was found in all four beer styles, with *S. uvarum* in three styles and the hybrid *S. bayanus* on one occasion in stout. Brewing strains of *S. cerevisiae* are used to produce ale (keg and cask) and stout. The hybrid yeast *S. pastorianus* is used in the fermentation of lager but was not found in the work reported in Table II-1. Although keg beers are 'commercially sterile', the microbiological specification (< 1 cell l^{-1}) will – over time - contribute low levels of organisms to the draught beer microflora. However, a more immediate contribution to account microflora is from cask beer (Hemmons, 1954, Wiles, 1950) which contain brewing yeast (> 10^3 cells l^{-1}). *S. cerevisiae* was identified in four samples of cask beer (Table II-2). This is unexpected as cycloheximide was added to the samples to supress the growth of brewing strains of *Saccharomyces* during forcing to assess quality. Cycloheximide is used selectively to inhibit *Saccharomyces* species in culture media but not non-*Saccharomyces* 'wild' yeasts. However, it has been reported that 'a few strains' of *Saccharomyces* can grow in the presence of cycloheximide (4 mg l^{-1}) (Lin, 1975).

S. uvarum is taxonomically close but genetically distinct to the hybrid *S. bayanus* (Sampaio *et al.*, 2017), with both species the most distantly related to *S. cerevisiae* in the genus. Despite no reported presence in the production or spoilage of beer, *S. uvarum* was identified in the three keg beers albeit at low abundance. Like *S. bayanus*, *S. uvarum* is involved in wine and cider fermentations and is cryotolerant growing well at low temperatures (e.g., 8°C). This physiology may contribute to its presence in draught beer which – depending on style – is dispensed at 2-12°C.

The non-brewing yeast *S. cerevisiae* var. *diastaticus* was not identified in this work. It is a sub-species of *S. cerevisiae* and, with the method used here, was not identified due to the similarity of the ribosomal DNA internal transcribed spacer (ITS) regions used in differentiation (Kurniawan *et al.*, 2022). *S. diastaticus* is a spoilage yeast which is amylolytic and 'super-attenuates' beer through hydrolysis of starch oligosaccharides (dextrins) with glucoamylase to produce glucose (Andrews and Gilliland, 1952). As a contaminant, it is a bigger concern in smallpack causing elevated levels of carbon dioxide through fermentation and the 'peaking' of cans or exploding bottles. The yeast has a markedly lower profile in draught beer where it has been reported in both keg and cask beer (Casson, 1985, Harper, 1981, Hough *et al.*, 1976).

A supplementary investigation was performed in Spring 2022 using a multiplex Real Time Quantitative PCR platform (PALL PCR GeneDisc) with proprietary primer sets and targets (Suiker *et al.*, 2021). Analysis of forced samples of lager (3), keg ale (1) and cask beer (4) showed *B. bruxellensis* in all samples, *S. cerevisiae* in all but one (cask beer SC4), and lager yeast *S. pastorianus* in two (of the three) lager samples together with cask beer (SC7). Four samples – lager (2), keg ale and cask ale - contained *S. diastaticus.* Accordingly, it likely that of the 17 isolates of *S. cerevisiae* reported in Table 2 some are the amylolytic spoilage yeast *S. diastaticus.*

Pichia species – notably *Pichia membranifaciens* – have long been reported in draught beer (Casson, 1985, Harper, 1981, Hough *et al.*, 1976, Wiles, 1950). In this work, *P. membranifaciens* was found in all four beer styles with *P. manshurica* only in lager (four colonies) and *P. fermentans* in stout (one colony). These yeasts form films on

the surface of liquids and are nutritionally fastidious consuming glucose and ethanol. Like *Rhodotorula, Pichia* are aerobic yeasts, providing more evidence than oxygen is available to microorganisms in dispense systems.

Lactic acid bacteria in general but notably *Levilactobacillus brevis* (basonym *Lactobacillus brevis* has been reported as the most prevalent spoilage bacteria found in beer. This is based on a long-term survey of beers and beer in process from breweries in predominately Germany between 1980-2002 (Suzuki, 2015) and using PCR between 2010-13 (Suzuki, 2015) and 2010-16 (Schneiderbanger *et al.*, 2018). Despite this insight, there are surprisingly few reports of lactic acid bacteria in draught beer (Ault, 1965, Casson, 1985, Quain, 2015, Storgårds, 1997). In alignment with the above surveys, Table II-2 shows *L. brevis* and *S. paracollinoides* (Suzuki *et al.*, 2004) were primarily found in lager. However, a supplementary study with the culture independent GeneDisc technology (Table II-4) suggests *S. paracollinoides* and *L. brevis* to be common in keg ale and cask ale as well as lager. Further, using culture-based methodology, *Pediococcus damnosus* was found in stout (Table II-2) but with the culture independent method was present as '*Pediococcus'* in lager, keg ale and stout (Table II-4). As discussed below, there is a gap between the identification of microorganisms using culture-dependent and culture independent methods.

2.4.3. Microflora in draught beer and Belgian sour beer

Nine of the ten most abundant microorganisms found in draught beer (Table II-2) from 20 public houses in the English Midlands have also been reported in the Belgian beer Lambic. Whilst the brewing process to produce the fermentable extract ('wort') for Lambic is fundamentally the same as for ale and lager beers, it contains more unfermentable glucose oligosaccharides from the use of unmalted wheat in the grist.

Rather than using pure cultures of *Saccharomyces* yeasts, Lambic is 'spontaneously' fermented by exposure to diverse microflora from the air during overnight cooling of wort in an open vessel ('coolship') and, more significantly, from the organisms associated with wooden barrels (for a review on Lambic production see Bongaerts *et al.*, 2021). The process for Lambic production in wood casks takes up to three years with, in the first six months, three phases of microbial succession followed by lengthy maturation period. Key microbial players over time are enterobacteria and acetic acid bacteria (initial fermentation), *Saccharomyces* species (alcoholic fermentation), lactic and acetic acid bacteria (acidification) followed by lactic and acetic acid bacteria together with *Brettanomyces* species (maturation).

The commonality of microflora in Lambic and draught beer suggests that there are similar processes at work. It is suggested that the headline composition of Lambic post alcoholic fermentation is comparable with the beer styles evaluated in this work. Accordingly, the acidification and maturation phases in the production of Lambic mirror the spoilage of draught beer. Both processes take a long time. Whilst Lambic can remain in cask for up to three years, draught beer is exposed to microbial biofilms in dispense lines for a decade or more (Quain, 2015). Whilst semantics, it is suggested that 'production' of Lambic and 'spoilage' of beer are effectively the same.

2.4.4. Biofilms in beer dispense

The attachment of microorganisms to the internal surfaces of dispense tubing and ancillary equipment has long been recognised by bar staff and dispense technicians. What are now recognised as biofilms were initially reported in draught beer dispense in the 1980s (Casson, 1985, Harper, 1981, Harper *et al.*, 1980) together with a handful of subsequent studies (Jevons and Quain, 2021, Thomas and Whitham, 1997, Walker

et al., 2007). As noted above, the level of sessile and planktonic microflora in beer are managed by regularly cleaning the dispense lines with alkali-based propriety solutions. Even with weekly cleaning, the process is ineffective and must be repeated reflecting the recalcitrance of biofilm and a lack of mechanical action.

The microorganisms in draught beer reflect the microflora in the dispense system. Although planktonic, microorganisms in dispensed keg lager, ale, stout, and cask ale are able to form biofilms when incubated in microplates (Jevons and Quain, 2021). Indeed, many of the microorganisms found in this work have been identified in dispense biofilms including acetic acid bacteria (Kubizniaková *et al.*, 2021, Thomas and Whitham, 1997). *Lactobacillus* species (Walker *et al.*, 2007), *Pediococcus* (Heger and Russell, 2021, Thomas and Whitham, 1997) and *Saccharomyces* species (including *S. diastaticus*) (Riedl *et al.*, 2019b, Walker *et al.*, 2007). Elsewhere biofilm formation has been reported for *Brettanomyces bruxellensis* from beer in wine (Dimopoulou *et al.*, 2019), *R. mucilaginosa* (Riedl *et al.*, 2019b) in beer, some (but not all) 20 brewery isolates of *L. brevis* in beer (Riedl *et al.*, 2019a) and five species of *Lactobacillus* from beer (Wang *et al.*, 2020).

The prevalence of aerobic microflora in draught beer suggests that sufficient oxygen is available to support growth at low temperatures. The dispense tap will allow oxygen ingress as will, to a less extent, the attachment of keg couplers. Less obvious, is the permeation of oxygen through the wall of dispense tubing (Jevons and Quain, 2021). The composition and incorporation of barrier layers in dispense lines can reduce the ingress of oxygen by 20-80 fold (https://www.micromatic.com/5-16-inch-id-barriermaster-flavourlock-tubing-549BF). Reduced access to oxygen, together with

smoother internal surfaces, reduces biofilm formation in draught beer lines (Heger and Russell, 2021, Thomas and Whitham, 1997).

Post fermentation by *Saccharomyces* yeasts, beer is nutritionally depleted. Nutrients remain unutilised by brewing yeast or where the fermentation is incomplete. Further some brands are 'primed' post fermentation with sugars (maltose, glucose, fructose) to enhance sweetness. However, irrespective of production methods and styles there are clearly macro- and micronutrients in beer to enable growth by spoilage microorganisms. The nutritional requirements of such microflora are satisfied directly or via extracellular enzymes degrading unavailable substrates such as glucose oligosaccharides by *S. diastaticus* (Andrews and Gilliland, 1952) and *B. bruxellensis* (Crauwels *et al.*, 2017). Further proline – which *Saccharomyces* species are unable to utilise and accordingly is the most abundant amino acid in beer - can be used as a nitrogen source by *B. bruxellensis* (Crauwels *et al.*, 2015). Presumably, in the mixed community of beer microflora, there is the trading of metabolic by-products by one microorganism as substrates for another. Such a relationship is recognised between *Lactobacillus* and *S. cerevisiae* (Xu *et al.*, 2021).

Line cleaning is ineffective and, because dispense systems are in place for many years, biofilms are long established. Although microorganisms are lost from the biofilm by dispense through shear and dispersal, the biofilm is exposed to fresh beer supporting biofilm growth. Continuing sources of microbial infection into the dispense system involve poor hygienic practices including soaking tap nozzles in soda water (Quain, 2016) and keg couplers not being sanitised. Other contributions include the dispersal of microorganisms by bioaerosols from the movement of air (Masotti *et al.*, 2019) and

insect vectors transporting microorganisms with the fruit (or vinegar) fly *Drosophila melanogaster* interacting with *Brettanomyces* (Dweck *et al.*, 2015).

2.2.5. Culture based microflora – not the complete picture

Although directional, the microflora identified in four styles of draught beer using culture-based methods provides an incomplete picture. The limited recent investigation using the culture independent PALL GeneDisc technology suggests that *Candida/Pichia* yeasts (Table II-3) and *S. paracollinoides*, *L. brevis*, *Pediococcus* (Table II-4) are more prevalent in draught beer than found using culture based identification (Table II-2). This is likely to reflect the use of selective isolation media, manual selection of colonies, and the viable but nonculturable state reported for acetic acid bacteria (Spitaels *et al.*, 2014), *Lactobacillus* (Wang *et al.*, 2020), *Brettanomyces* (Suzuki *et al.*, 2008b).

Table II-3: Culture independent yeasts found in different draught beer styles. Present (grey), absent (white).

Beer	Style	*S. diastaticus*	*S. pastorianus*	*S. cerevisiae*	*B. bruxellensis*	*Candida/Pichia*
SL6	Standard lager					
PL3	Premium lager					
PL13	Premium lager					
PKA1	Premium keg ale					
SC4	Standard cask					
SC7	Standard cask					
SC9	Standard cask					
SC10	Standard cask					

Table II-4: Culture independent bacteria found in different draught beer styles. Present (grey), absent (white).

Beer	Style	*S. paracollinoides*	*L. backii*	*L. brevis*	*Pediococcus*
SL6	Standard lager				
PL3	Premium lager				
PL13	Premium lager				
PKA1	Premium keg ale				
SC4	Standard cask				
SC7	Standard cask				
SC9	Standard cask				
SC10	Standard cask				

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Conflict of interest

The authors declare no conflict of interest.

Chapter III: Next generation sequencing of the draught beer microbiome

3.1. Introduction

Draught beer contains a complex mix of yeast and bacteria, where the style and dispense method are influential in determining the microflora fingerprint. Spoilage microorganisms are prevalent in the on-trade, causing economic loss to breweries, to the extent where major breweries are bringing the responsibility for line-cleaning and dispense in-house. A beer distributed by the brewer, whether in small pack or keg, is of a quality set by the brewer and is 'commercially sterile'. The specification of the meets the brewers' standards for haze, taste, and aroma. Seton in 1912 described the problems associated with on-trade beer at the time:

"The national beverage as it is served over the counter of many public-houses in England today, has not the flavour and appearance commensurate with the care bestowed upon its manufacture by the brewer"

This quote is 110 years old at the time of writing and is arguably as relevant now as it has ever been. Mallett and Quain (2019) conducted an on-trade audit in 2019, identifying that approximately 70% of beers were subject to some degree of microbial contamination. As beer is dispensed it is introduced to an abundance of beer spoilage yeast and bacteria (e.g., *Lactobacillus* sp., *Acetobacter* sp., *Brettanomyces* sp. and *Saccharomyces* sp.). Each will uniquely impact a beer, metabolising available nutrients to create haze, off flavours and aromas. Our knowledge of beer spoilage microorganisms is largely driven by research sporadically published throughout the 20th Century (Seton, 1912, Zobell and Anderson, 1936, Wiles, 1949, Wiles, 1950, Rainbow, 1952, Hemmons, 1954, Hemmons, 1955, Gilliland, 1961, Ault, 1965, Harper *et al.*, 1980, Lawrence, 1988). Although these works were key and

inspirational sources of information, they are limited in scope or by the available technology. The scope was often restricted to focus on a single style of beer, a single kingdom, or a single genus. Further, these works were published decades before the availability of next-generation sequencing, and therefore were reliant on conventional microbiological plating and identification.

Spoilage organisms have arguably been viewed through a relatively narrow lens, with work often focused on one beer style, or one microbial species of significance. One prime example of this is the significance of *Lactobacillus brevis* in lager beers. Chapter II highlighted the increased presence of *Lactobacillus* sp. in lager style, whilst conversely ale style beers exhibited an absence or lower abundance. Suzuki (2015) excellently reviews the impact of lactic acid bacteria on beer, and further a number of works can be found which solely focuses on LAB sp. in lager (Adams *et al.*, 1989, Van Vuuren *et al.*, 1979, Rachon *et al.*, 2022, Kajala *et al.*, 2018, Fernandez and Simpson, 1995). Although the work has value and merit, it could be argued there is a wider world of spoilage microorganisms that possess more significance in other styles of beers. Moreover, much of this work has relied on culture-based approaches and does not consider the potential *in-situ* diversity driven by the environmental niches of a beer style. The use of selective agar media will innately drive the selection of the targeted microorganism(s), such as *Lactobacillus* sp., and miss the detail. Non-culturability is where a microorganism cannot be cultured using conventional plate methods and accordingly will not be identified. Quality control methods for breweries will employ conventional microbiology techniques, using four industry standard media:

- Lysine agar (Fowell, 1965)
- Lin's Copper Media Agar (LCMA) (alias: copper sulphate agar) (Taylor and Marsh, 1984)
- WL Differential (WLD) (Green and Gray, 1950, Greenspan, 1965)
- Raka-ray (RR) (Saha *et al.*, 1975)

These media are specifically designed to target predetermined microorganisms. Lysine agar is focused on the isolation of wild yeasts, LCMA targets non-*Saccharomyces* wild yeasts, WLD can cultivate a range of yeasts and bacteria, and finally Raka Ray is designed to target *Lactobacillus* sp. LCMA and WLD are supplemented with cycloheximide (~ 4mg l^{-1}) that enacts to inhibit residual brewing yeast from fermentation (De Angelo and Siebert, 1987). Further, these media were designed in the 1950s to 1980s, and since there have been several fundamental changes to the brewing or dispense processes (Fowell, 1965, Green and Gray, 1950, Greenspan, 1965, Saha *et al.*, 1975, Taylor and Marsh, 1984). These include the shift from cask to keg beers and the focus on reducing oxygen across the process and in final package. Accordingly, strict aerobic bacteria have been removed as a threat to quality. Beer dispense is colder ('extra cold') with lines chilled with ice-cold water or glycol. Recently, the advent of no and low alcohol beers (NABLAB) and the lack of ethanol will invite novel beer spoilers previously not experienced (Quain, 2021). More recently, Heineken announced the Heineken 0.0 beer would be on draught beer lines across the UK in 2022. These changing parameters will ultimately change the dynamic of beer spoilers. New stresses such has low temperature lines will promote the selection of microorganisms which are primary biofilm colonisers (Jevons and Quain, 2021). The absence of alcohol will invite novel challengers, and further the

increased sugar concentration of NABLABs will inevitably increase the rate of spoilage. Despite these new challenges, there has been little-to-no change to QC practices in the industry. As recently as 2018, Safety and Local Supplier Approval (SALSA) food safety auditors required small breweries to have their core brands monitored at least once a year for microbiological stability using the above four media. Herein lies the current problem, reliance of unrelated media to promote beer spoilers offers no relation to the real-world, hence there must be a focus to move QC methods from culturing on agar media towards next-generation methods or *in-situ* quality methods (Mallett *et al.*, 2018)

Dispense in the on-trade can have a profound impact on the phenotype of the microflora. Depending on the style, dispense may be anaerobic (kegged lagers and ales) or aerobic (cask ale) and served at varying temperatures (lagers 1-8^{o}C, cask (12-15^{o}C). These parameters impart selective pressures on the microbiome, it has been previously reported that dispense conditions can have a profound impact on microflora and rate and maturation of biofilm formation (Jevons and Quain, 2021). For cask ales, as the beer is dispensed with an influx of air/oxygen (Jevons and Quain, 2021) acetic acid bacteria are the dominant genera. Furthermore, Jevons & Quain (in review; Chapter II)) have described how style directly influences the microflora. Lager maintained a large presence of *Lactobacillus* sp., however, its significance diminished in the three other styles (stout, keg ale, and cask ale). *Brettanomyces* sp. and *Acetobacter* sp. were present across all samples and were responsible for over half of all identified species. The work employed conventional isolation of microflora on selective agar plates prior to PCR based identification. The dominant presence of *Acetobacter* sp. underlined the flaws of conventional microbiology. *Acetobacter* sp. is

a historical beer spoiler, and its significance has diminished due to oxygen restriction throughout the brewing process and in pack (Vriesekoop *et al.*, 2012). By using aerobic agar plates, oxygen is available and enables conserved *Acetobacter* sp., possibly sourced from biofilms, to proliferate and possibly misrepresent their significance *in situ*. Although importantly, the change in environment and conditions renders much of the microbiome undetectable using selective agars. This highlights the requirement for methods not innately supportive of microorganisms that would otherwise not grow *in situ*.

There exist thousands of beer brands, with no two beers the same with subtle variations in pH, ABV %, residual sugars, bitterness, trace nutrients etc. These differences are more obvious between different beer styles, which can then be further differentiated by the dispense method and beer line management of the account. Yet, it is assumed that beer spoilage microorganisms are consistent irrespective of these differences. Our prior work ventured to answer this question, using conventional microbiological methods, and demonstrated key differences between different styles of beer. Whereby, ales repeatedly exhibited a 'yeastier' microflora, compared to lagers that supported a greater concentration of bacteria (e.g., lactic acid bacteria).

Culture-based microbiology, specifically on agar plates, is limited in a few aspects. First and foremost is time, culture-based methods often take days, and up to 14 days for strict anaerobic bacteria (e.g., *Megasphaera* sp., *Pectinatus* sp.). Secondly, using selective media will target microorganisms supported by the media and not those reflecting the original environment. Accordingly, it is becoming more common, and affordable, for research in environmental microbiology to use next-generation

sequencing tools for microbiome analysis. This enables researchers to avoid the limitations of conventional microbiology and go straight to extraction and sequencing of DNA.

The sequencing market has been dominated by second-generation sequencing tools (Mikheyev and Tin, 2014); such as Illumina sequencing by synthesis platform (Nakamura *et al.*, 2011) and Roche 454 pyrosequencing (Margulies *et al.*, 2005). Second-generation sequencing technology is largely limited to a few hundred bases, which although beyond the scope of this work can result in mismapping and misalignment (Mckenna *et al.*, 2010). More recently, Oxford Nanopore Technologies (ONT) MinION next-generation sequencing offers an affordable platform with long read sequencing capability. The MinION next-generation sequencer is an 'iPhone sized' device capable of microbiome analysis. The MinION uses nanopores to channel single DNA strands, with the nanopore embedded in an electrically resistant membrane (Jurkowski, 2020). As the bases pass through the channel each base causes a unique electrical displacement in the ion current, which allows for interpretation of the base or base modification (Jurkowski, 2020).

Recent work has demonstrated the applicability of MinION technology for studies of the microbiome relevant to brewing quality control (Shinohara *et al.*, 2021). Whilst further publishing barcoded primer designs for yeast identification and multiplex analysis using MinION. However, spoilage microorganisms sourced from beer as - previously discussed - are a complex mix of yeast and bacteria. In this work we investigate the application of ONT's MinION platform for the microbiome analysis

of beer sourced from the on-trade and quantifying the abundance of microorganisms, and differentiating the microbiome fingerprint between beer styles, and accounts.

3.2. Methods and materials

3.2.1. Sample selection

Between October and November 2020, four beer styles were purchased from 12 accounts and collected in sterile 250mL Duran bottles. Table III-1 details the styles and the accounts sampled.

Table III-1: Style of beer samples and the location

Style	Accounts
Standard lager (SL3)	V6, V8, V13
Stout (ST1)	N5, N8, N14
Keg ale (KA1)	D2, D10, D13
Cask ale (CA1)	B10, B12, B13

3.2.2. Forcing

The microbiological quality of draught beer ex dispense was determined – in triplicate - using the forcing method as described by (Mallett *et al.*, 2018). Draught beer (25 mL) in 30 mL plastic universal tubes was incubated at 30^{o}C for 96 hours. Absorbance at 660nm was determined at 0 and 96 h using a Jenway 7315 spectrophotometer. Cycloheximide (4mg/L) was added to cask beer before forcing to inhibit the growth of primary brewing yeast.

3.2.3. DNA extraction

DNA extraction was using a conventional phenol/chloroform method (Legras & Karst, 2003). 200µL of forced samples was added to 0.4g glass beads (Sigma-Aldrich,

G8772), 400µl phenol/chloroform (Acros Organics, 327111000) and 400µl lysis buffer (Tris 10mM, pH 7.6, EDTA 1mM, NaCl 100mM, Triton X-100 2% w/v, sodium dodecyl sulphate (SDS) 1% w/v) (Legras & Karst, 2006). The mixture was vortexed for 4 minutes and then centrifuged for 5 minutes at 2250 x g. The supernatant was transferred to a separate tube containing 500µl chloroform-isoamyl alcohol mixture (Sigma-Aldrich, 25666), inverted 2-3 times and centrifuged for 2 minutes at 17,000 x g. The supernatant was transferred to 100% ethanol in a separate tube (approx. 2:1 ratio ethanol: supernatant). This was centrifuged for 5 minutes at 17,000 x g, the ethanol decanted, and the tube is allowed to air dry. The DNA was suspended in TE buffer/ddH_2O (30-50µl) and stored at -20^oC or used directly for PCR.

3.2.4. Amplification and purification of bacterial and yeast loci

PCR was carried out in 50µL reaction volumes containing 50-250 ng of yeast/bacteria DNA, 1X buffer (PCRBio), 1.5 mM $MgCl_2$, 200 µM dNTP mix (New England Biolabs, N0447S), 1 unit/µL of PCRBio taq DNA polymerase and 0.5µM of each oligonucleotide primer. Where yeast DNA was amplified, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White, TJ. *et al.* 1990) were used and for bacteria, 16S rRNA primers 63F (5'-CAGGCCTAACACATGCAAGTC-3') and 1387R (5'-GGGCGGWGTGTACAAGGC-3') (Marchesi, JR. *et al*. 1998) were used for bacterial DNA. Amplification was carried out using Techne TC-512 thermocycler using the following parameters: 5 min at 94^oC followed by 34 cycles of 30 s at 92^oC, 45 s at 54^oC and 1 m 15 s at 72^oC, ending with a final extension of 5 min at 72^oC. PCR amplicons were purified using Jena Bioscience PCR purification kit (Jena Bioscience, PP-201L).

3.2.5. Native barcoding of amplicons

Native barcoding of amplicons (version: NBA_9093_v109_revH_12Nov2019) was conducted using the 'Native barcoding amplicons (with EXP-NBD104, EXP-NBD114 and SQK-LSK109)' kits from Oxford Nanopore Technologies.

3.2.6. MinION sequencing using MinKNOW software

Barcoded amplicon sequences were interpreted by Oxford Nanopores MinKNOW software. The software recommends computers running a sequencing run use a computer containing a minimum of an i5 processor, 512GB SSD and 8GB RAM. Each sequencing run lasted 24 hours, sequences were 'basecalled' into FASTQ files by the MinKNOW software. A minimum threshold of 7 was applied to ensure only high-quality reads were used for identification using the EPI2ME workflow. Each sample was required to have a minimum of 50,000 reads before being considered for use in this work.

3.2.7. EPI2ME workflow – 'What's in my pot?'

Basecalled FASTQ files were analysed using the 'What's in my pot?' (WIMP) taxonomic assignment workflow available on the EPI2ME software available through Oxford Nanopore. The workflow uses the NCBI database to identify the sequenced reads.

3.2.8. Data analysis

Each sample required a minimum of 50,000 reads per sample and a 1% cut-off for minimum abundance was applied prior to plotting of the data. Further, a 99% identification cut-off was applied to all data, ensuring identification was accurate for each read reported in this work.

3.3. Results

3.3.1. Quality scores

Beer quality and overall Quality Index (QI) were calculated according to Mallett *et al.* (2018) (Table III-2). The QI of standard lager (SL3) and stout (ST1) were both 75% with keg ale (KA1) and cask ale (SC1) both 91.6%. Higher price did not result in increased quality, with ST1 average of £3.97 per pint generally of worse quality than KA1 average price of £1.99. Beers SL3 (V13) and ST1 (N14) were the poorest quality beer, both scored as C/poor.

Table III-2: Quality score and index of sampled beers. Each sampled beer was forced as according to Mallet *et al*. (2018). The same samples were subject to microbiome analysis using Oxford Nanopore's MinION next-generation sequencing platform

Lager SL3				Stout ST1			
Account	**£/pint**	**07/10/2020**	**Quality Index (%)**	**Account**	**£/pint**	**08/10/2020**	**Quality Index (%)**
V6	3.45	C		**N5**	3.1	A	
V8	3.8	B	75%	**N8**	4.3	B	75%
V13	3	A		**N14**	4.15	C	
Average	3.42	9		**Average**	3.97	9	

Ale KA1				Cask ale SC1			
Account	**£/pint**	**06/10/2020**	**Quality Index (%)**	**Account**	**£/pint**	**08/10/2020**	**Quality Index (%)**
D2	1.99	A		**B10**	3.6	B	
D10	1.99	A	91.6%	**B12**	3.3	A	91.6%
D13	1.99	B		**B13**	3.9	A	
Average	1.99	11		**Average**	3.6	11	

3.3.2. MinKNOW quality score

Sequenced reads were filtered to only include high quality reads, to improve the accuracy of identification. An example of this can be found in Figure III-1, anything of a ‘low quality’ is removed and is considered a failed read and is not used for identification.

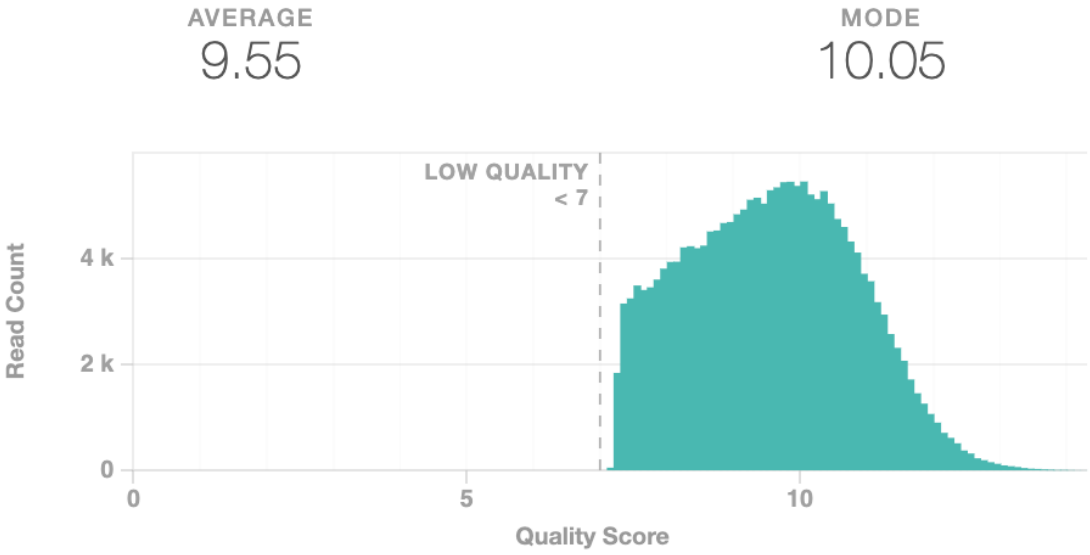

Figure III-1: Quality scores of sequenced reads from the MinKNOW software. Low quality scores were filtered out of the run to ensure only high-quality reads were used for microorganism identification

3.3.3. Lager

Without the need for sub-culturing on agar plates, lager exhibited demonstrable differences in the microbiome fingerprint compared to their cultured counterparts. Lager from three of the five accounts sampled in Chapter II, were sampled again for the microbiome analysis, enabling real-time quantification of the sequenced reads. Figure III-2 (V6) was found to be high in *Pectinatus* sp. (48.5%), along with other obligate anaerobic bacteria; *Megasphaera* sp. (3.0%), and *Megamonas* sp. (3.6%). These bacteria are technically difficult and time consuming to culture. Accounts V8 and V13 exhibited growth of *Actinomyces* sp., 4.6% and 15.6% respectively. *Actinomyces* sp. are an important research focus in the biotechnological field due to their expansive secondary metabolism potential for antibiotic production, effective against a broad spectrum of microorganisms (Dimri *et al.*, 2020). However, the major spoilage organisms were *Brettanomyces* sp. at 47.0% (account V8) and *Saccharomyces* sp. at 64.9% (account V13). V8 and V13 exhibited a much 'yeastier' microbiome, versus V6, which was more diverse.

From account V6, 11 genera account for over 1% each of total reads. Ranging from the expected *Levilactobacillus* sp. (alias; *Lactobacillus* sp.) to lesser known *Secundilactobacillus* sp. and *Selenomonas* sp. The key spoiler of the *Levilactobacillus* genera is *Levilactobacillus brevis*, which accounted for over 90% of the reads in this genus. *Levilactobacillus koreensis, L. suantsaii,* and *L. zymae* were also identified at lower levels (Table III-4). Of the *Secundilactobacillus* (*Lactobacillus*) genus; *S. paracollinoides* was identified, in agreement with previous work (Chapter II).

Bacillus sp. were identified in account V6. This is of interest as some species of this genus are considered pathogenic to humans (Bottone, 2010). *B. cereus* was the most abundant species identified, which is most associated with food poisoning (Schoeni and Lee Wong, 2005). Although at a low abundance (1%), its presence is indicative of poor hygiene management. In support of this, account V6 recorded the beer with the poorest quality score of the investigated beers (C/poor) (Table III-1).

As previously mentioned *Pectinatus* account for half of the total reads of account V6 (Figure III-2), but only *P. frinsingensis* was identified at the species level. *Selenomonas* sp. were the third most abundant genera in this account (3.9%). Two species were identified; *S. ruminantium, S. sputigena,* alongside a third unidentified species described as *S. sp.* oral taxon 920. *Pectinatus, Selenomonas, Megamonas,* and *Megasphaera* are slow growing anaerobic bacteria, typically missed by (aerobic) agar plate methods used in brewing.

Accounts V8 and V13 had yeast rich microbiomes which were not diverse at the species level. In an environmental study this is unlikely and raises questions about the accuracy of the yeast database underpinning this work. However, the strong correlations between V8 and V13 are clear, with both exhibiting similar microbiomes (Figure III-2; Table III-4-5)

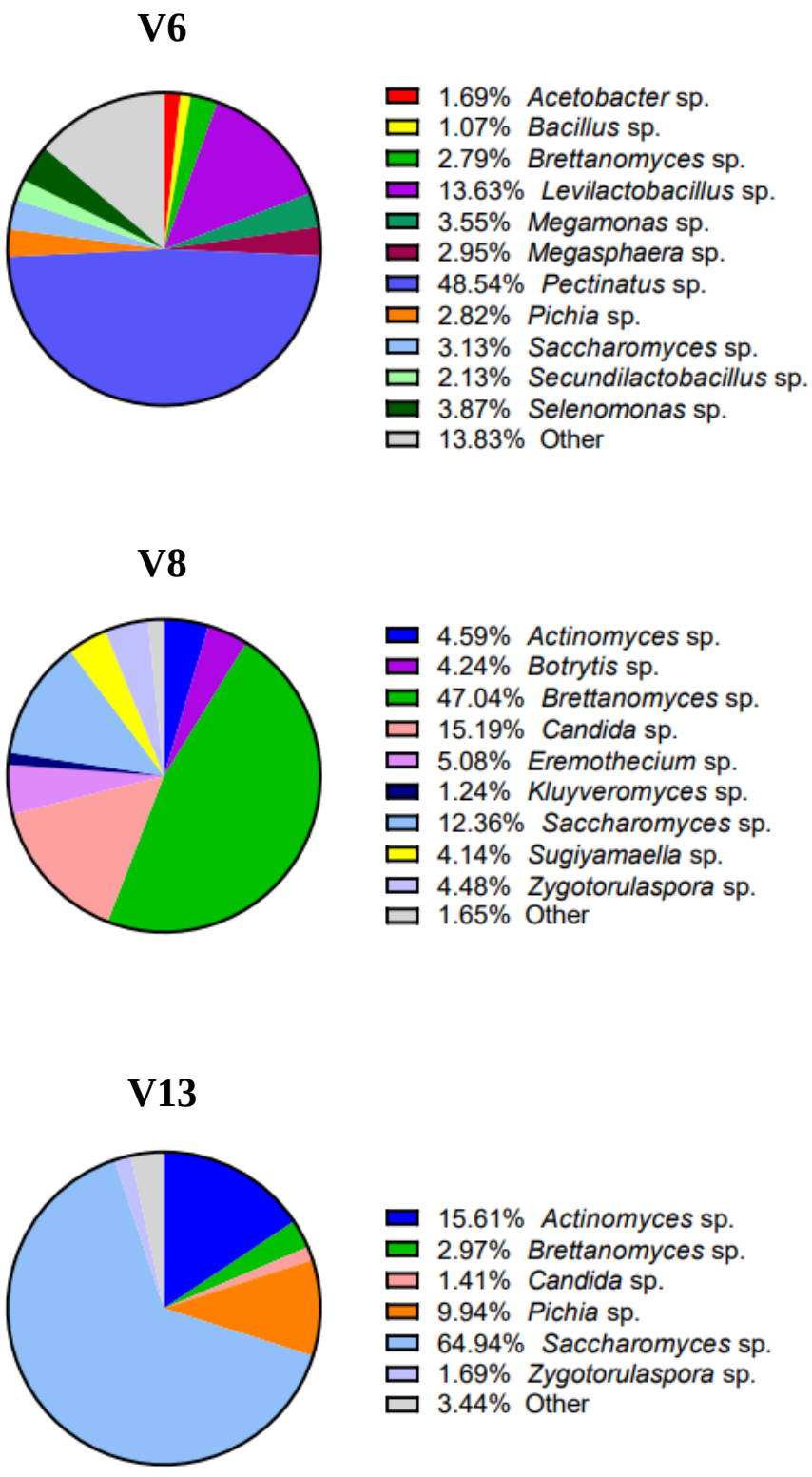

Figure III-2: Microbiome of standard lager (SL3) sourced from three on-trade accounts; V6, V8, and V13. Microorganisms <1% abundance are collated into the 'other' category. Each run consisted of a minimum of 50,000 reads.

Table III-3: Species identified in the most abundant genera in account V6

Genus	Species
Acetobacter	*A. orientalis A. persici, A. pomorum, A. senegalensis, A. aceti*
Bacillus	*B. cereus, B. thuringiensis, B. velezensis, B. subtilis*
Brettanomyces	*B. nanus*
Levilactobacillus	*L. brevis, L. koreensis L. suatsaii, L. zymae*
Megamonas	*M. funiformis, M. hypermegale*
Megasphaera	*M. stantonii, M. elsdenii*
Pectinatus	*P. frinsingensis*
Pichia	*P. kudriavzevii*
Saccharomyces	*S. cerevisiae*
Secundilactobacillus	*S. paracollinoides*
Selenomonas	*S. ruminantium, S. sputigena, S. sp.* oral taxon 920

Table III-4: Species identified in the most abundant genera in account V8

Genus	Species
Actinomyces	*A. oris*
Botrytis	*B. cinerea*
Brettanomyces	*B. nanus*
Candida	*C. dubliniensis*
Eremothecium	*E. gossypii*
Kluyveromyces	*K. lactis, K. marxianus*
Saccharomyces	*S. cerevisiae*
Sugiyamaella	*S. lignohabitans*
Zygotorulaspora	*Z. mrakii*

Table III-5: Species identified in the most abundant genera in account V13

Genus	Species
Actinomyces	*A. oris*
Brettanomyces	*B. nanus*
Candida	*C. dubliniensis*
Pichia	*P. kudriavzevii*
Saccharomyces	*S. cerevisiae*
Zygotorulaspora	*Z. mrakii*

3.3.4. Stout

Stout samples were taken from three accounts across Nottingham, and subject to analysis using the MinION next-generation sequencing tool. Earlier work (Chapter II) suggests that a variety of yeasts would be expected, which dominate the microbiome. This theme continues, with wild yeasts dominating the microbiome for each account, with *Pichia* sp. and *Saccharomyces* sp. the key spoilers.

Accounts N5 and N8 exhibited a high abundance of *Pichia* sp. and *Saccharomyces* sp. (Figure III-3; Table III-6-7). For account N5, *Pichia* sp. represented 84% of total reads, with *Saccharomyces* sp. at <1.%. Lesser-known beer spoilage, *Candida* sp. and *Sugiyamaella* sp. accounted for approximately 5% each of the total reads in this account. The accounts N5 and N8 were comparable in the in their microbiomes, with the same yeasts being identified.

For account N14, there was a notably more diverse microbiome (Figure III-3; Table III-8), with a much wider range of bacterial genera and species identified. However, *Saccharomyces* sp. was the most dominant, accounting for 44% of reads, with *Acetobacter* sp. the second-most abundant with 14%. Beer is often cited as not supporting the growth of pathogens and the risk is minimised by application of good hygienic practices. However, in this account the *Escherichia* genus represented >2% of the total reads for this sample, with *E. coli* accounting for 99% of sequenced *Escherichia* sp. (Figure III-3; N14). Alongside *E. coli, E. albertii, E. fergusonii,* and *E. marmotae* were also identified (Table III-8). Although the viability of *E. coli* in this sample cannot be determined, it is reasonable to assume that the number of reads

(1420) implies the DNA was extracted from viable cells. The identified microbiome coincided with also being the poorest quality beer sample (C/poor) (Table III-1).

Lactic acid bacteria, *Pediococcus* sp. and *Secundilactobacillus* sp. were identified in N14. Although, *Pediococcus* sp. were only identified at a low abundance (1%), a broad range of species were identified; *P. damnosus, P. claussenii, P. inopinatus, P. pentosaceus, P. acidilactici, P. pentosaceus*. *P. damnosus* was the most dominant species of the genus, in agreement with the literatiure on beer spoilage (Sakamoto and Konings, 2003). *Secundilactobacillus* (*Lactobacillus*) *paracollinoides* has previously been identified in this work (Chapter II) but was most abundant in standard lager (SL3) and stout (ST1). This theme continues, as both *S. paracollinoides* and *S. malefermentans* were identified in ST1 accounting for a notable 8% of the microbiome (Table III-8).

A broad range of species were identified as *Acetobacter,* irrespective of abundance. As the forcing method is not strictly anaerobic, their abundance is possibly amplified by the introduction of oxygen post-dispense (Table III-6, Table III-8). As ethanol metabolism is a primary carbon source for acetic acid bacteria, in the presence of oxygen it is understandable how a wide number of species are identified. Acetic acid bacteria are historically ubiquitous with beer and spoilage. This work would suggest their potency to spoil beer remains and in the presence of oxygen a broad spectrum of *Acetobacter* sp. will thrive.

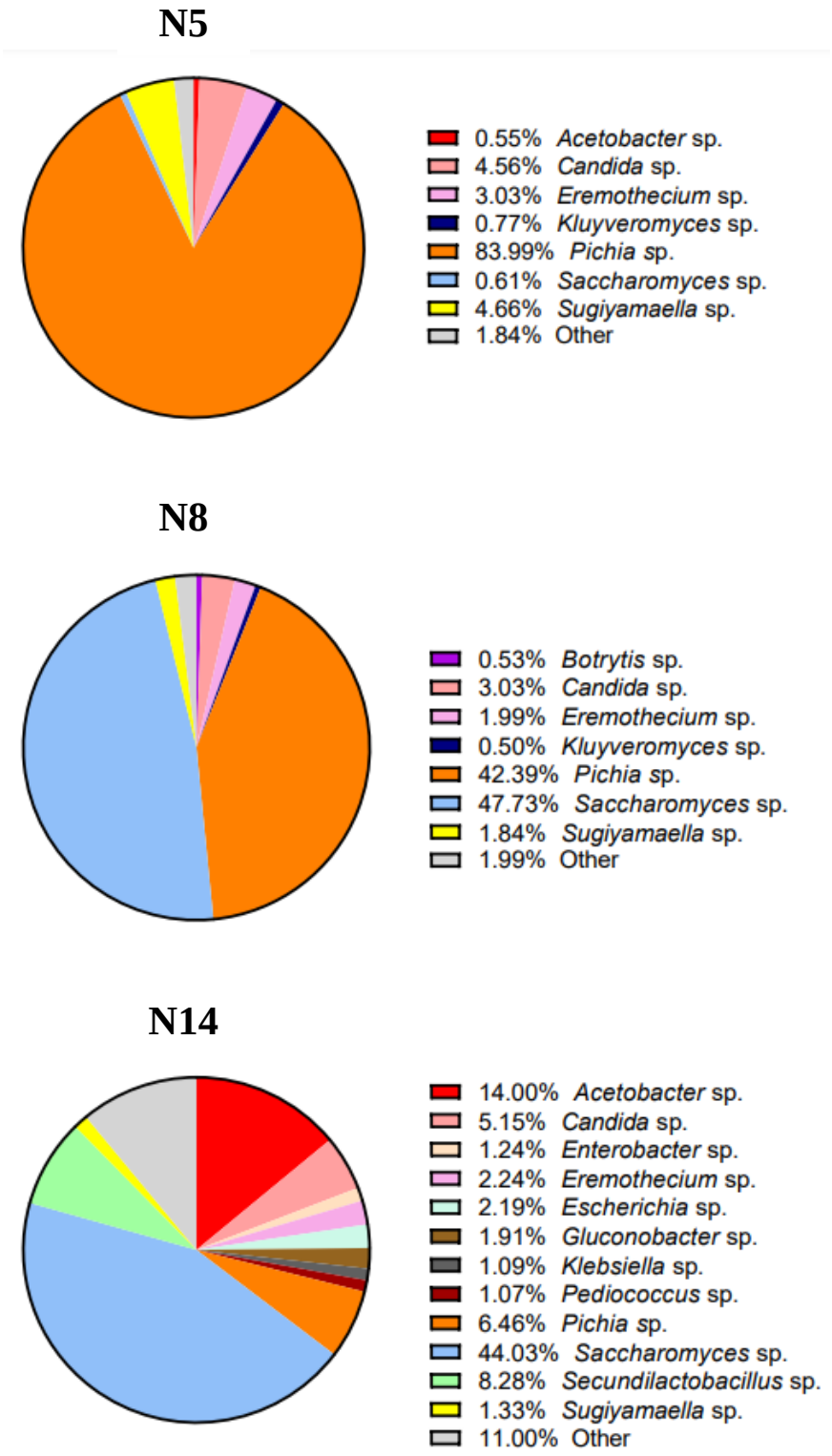

Figure III-3: Microbiome of stout (ST1) sourced from three on-trade accounts; N5, N8, and N14. Microorganisms <1% abundance are collated into the 'other' category. Each run consisted of a minimum of 50,000 reads.

Table III-6: Species identified in the most abundant genera in account N5

Genus	Species
Acetobacter	*A. persici, A. orientalis, A. pomorum, A. aceti, A. tropicalis, A. senegalensis, A. ascendens*
Candida	*C. dublinensis*
Eremothecium	*E. gossypii*
Kluyveromyces	*K. lactis, K. marxianus*
Pichia	*P. kudriavzevii*
Saccharomyces	*S. cerevisiae*
Sugiyamaella	*S. lignohabitans*

Table III-7: Species identified in the most abundant genera in account N8

Genus	Species
Botrytis	*B. cinerea*
Candida	*C. dubliniensis*
Eremothecium	*E. gossypii*
Kluyveromyces	*K. lactis, K. marxianus*
Pichia	*P. kudriavzevii*
Saccharomyces	*S. cerevisiae*
Sugiyamaella	*S. lignohabitans*

Table III-8: Species identified in the most abundant genera in account N14

Genus	Species
Acetobacter	*A. persici, A. pomorum, A. orientalis, A. aceti, A. senegalensis, A. tropicalis, A. oryzoeni, A. ascendens, A. pasteurianus*
Candida	*C. dubliniensis*
Enterobacter	*E. asburiae, E. cloacae, E. hormaechei, E. bugandensis, E. kobei, E. ludwigii, E. roggenkampii, E. sichuanensis*
Escherichia	*E. coli, E. albertii, E. fergusonii, E. marmotae,*
Gluconobacter	*G. albidus, G. oxydans*
Klebsiella	*K. pneumoniae, K. quasipneumoniae, K. aerogenes, K. oxytoca, K. variicola, K. michiganensis,*
Pediococcus	*P. damnosus, P. claussenii, P. inopinatus, P. pentosaceus, P. acidilactici, P. pentosaceus*
Pichia	*P. kudravzevii*
Saccharomyces	*S. cerevisiae*
Secundilactobacillus	*Lactobacillus paracollinoides, L. malefermentans*
Sugiyamaella	*S. lignohabitans*

3.3.5. Keg ale

Visual observation of forced keg ale (KA1) (D2 and D13) exhibited a highly flocculant, sticky and difficult to break up yeast flocs (Figure III-4). From previous work, it was anticipated the microbiome would favour the preponderance of yeasts in this beer style. Indeed, wild yeasts were the most abundant genera in each of the accounts, with D13 exhibiting the most diverse microbiome, with 12 genera >1% abundance. D13 supported several possible pathogens; *Corynebacterium* (2.3%), *Cutibacterium* (4.2%), *Delftia* (1.3%), and *Staphylococcus* (3.6%). Further, account D13 had the poorest quality beer (B/acceptable) of the accounts (Table III-1). On the other hand, accounts D2 and D10 were almost identical in their microbiomes with only subtle differences between the two accounts. Both were graded 'A/excellent' (Table 1), with a very limited number of genera identified above the 1% threshold (Figure III-4; Tables III-9-10).

For each account *S. cerevisiae* was the most abundant microorganism, representing 60%, 58%, and 38% of the identified microbiome, respectively (Figure III-4). Moreover, *Pichia* species (*P. kudriavzevii*) was the second most abundant genus for D2 (14.8%), and third most abundant in D10 (12.8%) and D13 (8%). *Actinomyces oris* was the second most abundant in accounts D2 (22%) and D10 (19.5%). *P. kudriavzevii* has previously been identified in beer (N'guessan *et al.*, 2011), however, typical yeast spoilers from this genus include *P. membranifaciens, P. manshurica,* or *P. fermentans which* were not identified. In the previous work, *Brettanomyces* sp. was a key spoilage yeast (Chapter II); *B. bruxellensis* and *B. anomalus* were the key spoilers from this work, here only *B. nanus* was identified in two accounts (D2, D10), accounting for approximately 1% of the total reads in each.

Acetobacter sp. were previously identified in abundance using conventional microbiology in Chapter II. Similar to stout (ST1) (Tables III-5-7), a broad range of *Acetobacter* sp. were identified including *A. persici, A. pomorum, A. orientalis, A. aceti, A. senegalensis, A. tropicalis, A. oryzoeni, A. ascendens, A. pasteurianus,* and *A. tropicalis*. These were sourced from either accounts D2 or D13, although abundance was low, 1.5% and 3.3%, respectively.

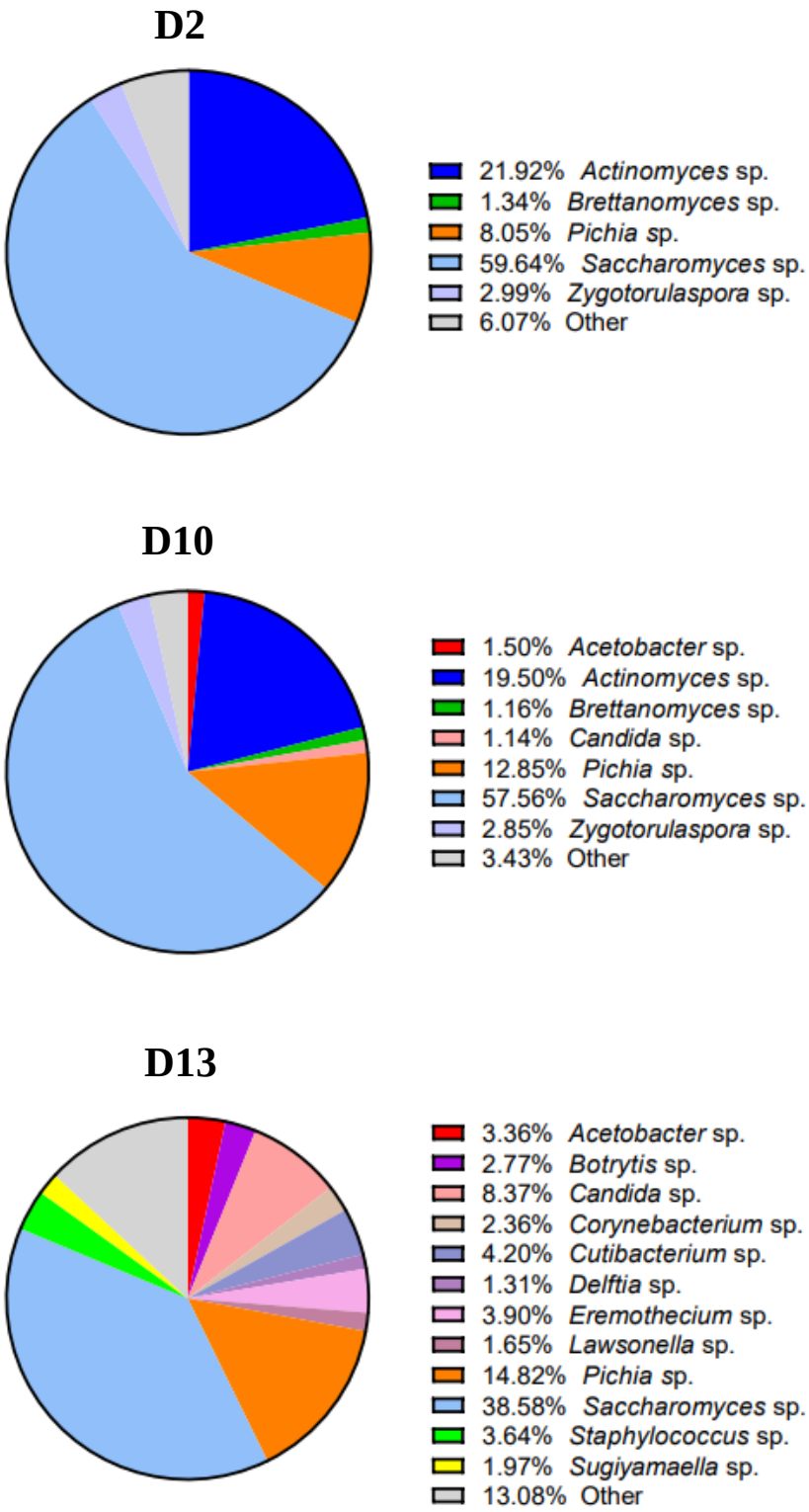

Figure III-4: Microbiome of keg ale (KA1) sourced from three on-trade accounts; D2, D10, and D13. Microorganisms < 1% abundance are collated into the 'other' category. Each run consisted of a minimum of 50,000 reads.

Table III-9: Species in the most abundant genera in account D2

Genus	Species
Actinomyces	*A. oris*
Brettanomyces	*B. nanus*
Pichia	*P. kudriavzevii*
Saccharomyces	*S. cerevisiae*
Zygotorulaspora	*Z. mrakii*

Table III-10: Species identified from the top isolated genera in account D10

Genus	Species
Acetobacter	*A. pomorum, A. persici, A. orientalis, A. senegalensis, A. aceti, A. tropicalis, A. oryzoeni, A. pasteurianus*
Actinomyces	*A. oris*
Brettanomyces	*B. nanus*
Candida	*C. dubliniensis*
Pichia	*P. kudriavzevii*
Saccharomyces	*S. cerevisiae*
Zygotorulaspora	*Z. mrakii*

Table III-11: Species identified in the most abundant in account D13

Genus	Species
Acetobacter	*A. pomorum, A. orientalis, A. persisci, A. senegalensis, A. aceti, A. tropicalis, A. oryzoeni, A. pasteurianus, A. ascendens*
Botrytis	*B. cinerea*
Candida	*C. dublinensis*
Corynebacteria	*C. segmentosum, C. geronticis, C. cystitidis, C. efficiens, C. atypicum, C. camprrealensis, C. diphtheriae, C. striatum, C. ureicelerivorans, C. renale,*
Cutibacteria	*C. acnes, C. granulosum, C. avidum*
Delftis	*D. tsuruhatensis. D. acidovorans*
Eremothecium	*E. gossypii*
Lawsonella	*L. clevelandensis*
Pichia	*P. kudriavzevii*
Saccharomyces	*S. cerevisiae*
Staphylococcus	*S aureus, S. epidermidis, S. caprae, S. haemolyticus, S. capitis, S. lugdunensis, S. cohnii, S. muscae, S. delphini, S. auricularis,*
Sugiyamaella	*S. lignohabitans*

3.3.6. Cask Ale

Unlike keg beer, cask ale is exposed to air during the dispense process which is drawn into the cask as beer leaves it. Accordingly, cask beer has a headspace of air which are ideal conditions for aerobic acetic acid bacteria to thrive. *Acetobacter* sp. were present in each of the three samples, 1% (B10), 16.6% (B12), and 12% (B13) (Figure III-5; Table III-11-13). Similar species were identified as previous samples, with a diverse range found all samples, irrespective of their abundance. *Saccharomyces* sp. were the key spoiler of each account, which is consistent with the expectations of this style. Cask ale requires conditioning prior to dispense, which is a secondary fermentation step completed by residual brewing yeast in pack.

Actinomyces oris was isolated in two of the three accounts (B10 and B12), with 19.7% and 14.5% abundance respectively. *Brettanomyces nanus* was isolated from each account, with B13 exhibiting the highest abundance (10%). In Chapter II, *Brettanomyces bruxellensis* and *B. anomalus* were the key spoilers, however, their significance is seemingly diminished when culture independent methods are used. *Pichia* sp. was present in all samples, albeit with B10 (2.2%) and B12 (4.5%), their abundance was much lower than in keg ale samples (Figure III-4). Account B13 exhibited a diverse range of spoilers, with 10 genera isolated with over 1% abundance in the sample. Interestingly, *Staphylococcus* sp. - *S. aureus* and *S. agnetis* - were identified in sample from account B13. This is of note as *S. aureus* is a pathogen, and is the most common cause of infection in hospitalised patients (Archer, 1998).

As anticipated from previous work, an increased abundance of acetic acid bacteria (AAB) were isolated from cask ale samples. As with other beer styles, a broad range

of AAB species were identified (Table III-12-14). This is a direct consequence of the dispense method as discussed through this thesis but acts a validation of the methods and the sequencing platform used to complete this work.

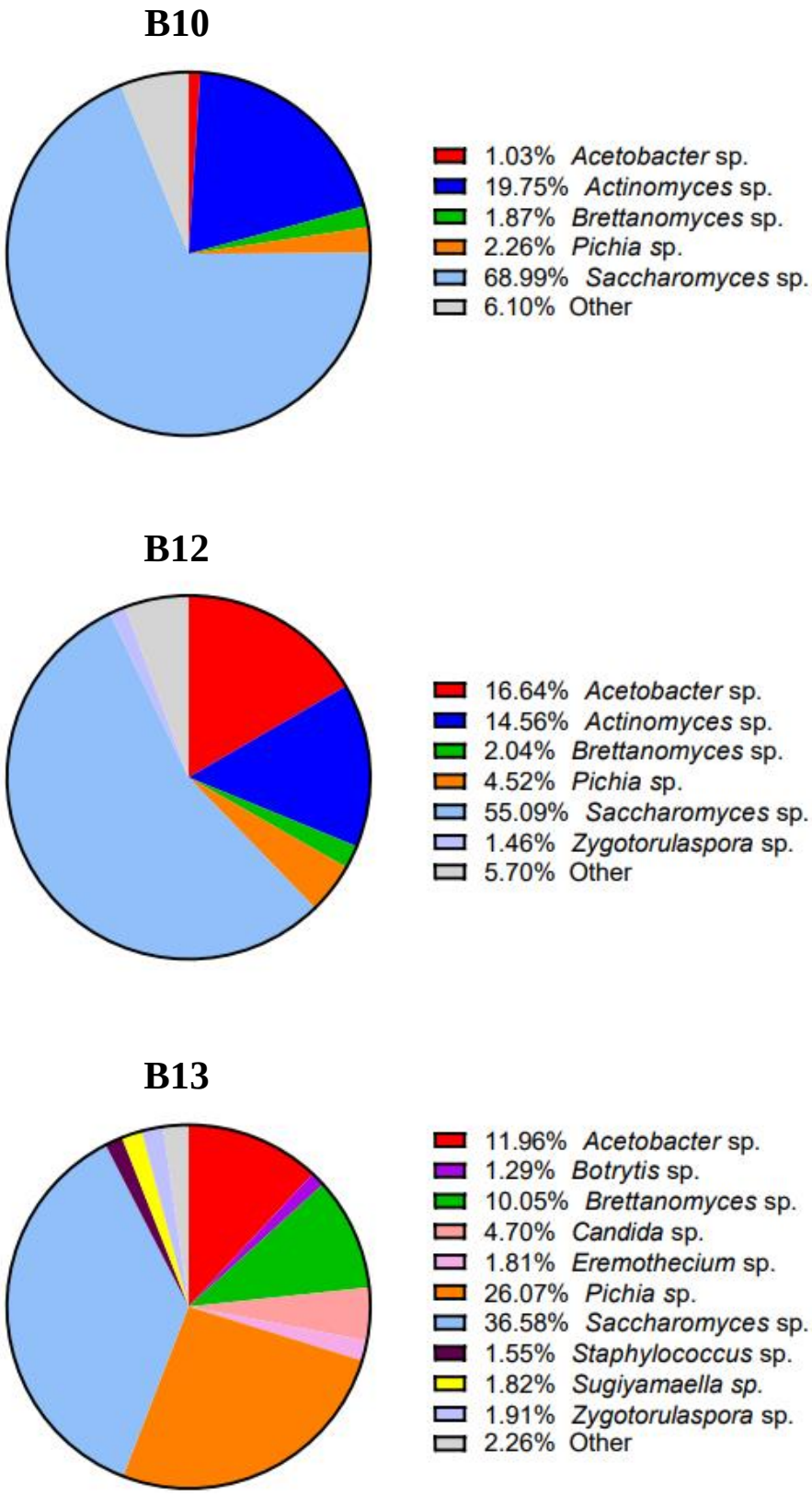

Figure III-5: Microbiome of Stout (SC1) sourced from three on-trade accounts; B10, B12, and B13. Microorganisms <1% abundance are collated into the 'other' category. Each run consisted of a minimum of 50,000 reads.

Table III-12: Species identified in the most abundant genera in account B10

Genus	Species
Acetobacter	*A. orientalis, A. aceti, A. persici, A. pomorum, A. senegalensis, A. oryzoeni, A. tropicalis, A. pasteurianus*
Actinomyces	*A. oris*
Brettanomyces	*B. nanus*
Pichia	*P. kudriavzevii*
Saccharomyces	*S. cerevisiae*

Table III-13: Species identified in the most abundant genera in account B12

Genus	Species
Acetobacter	*A. orientalis, A. persici, A. aceti, A. pomorum, A. senegalensis, A. tropicalis, A. oryzoeni, A. pasteurianus*
Actinomyces	*A. oris*
Brettanomyces	*B. nanus*
Pichia	*P. kurdiavzevii*
Saccharomyces	*S. cerevisiae*
Zygotorulaspora	*Z. mrakii*

Table III-14: Species identified in the most abundant genera in account B13

Genus	Species
Acetobacter	*A. pomorum, A. orientalis, A. aceti, A. senegalensis, A. persici, A. oryzoeni, A. ascendens, A. pasteurianus, A. tropicalis*
Botrytis	*B. cinerea*
Brettanomyces	*B. nanus*
Candida	*C. dublinensis*
Eremothecium	*E. gossypii*
Pichia	*P. kudriavzevii*
Saccharomyces	*S. cerevisiae*
Staphylococcus	*S. aureus, S. agnetis*
Sugiyamaella	*S. lignohabitans*
Zygotorulaspora	*Z. mrakii*

3.4. Discussion

3.4.1. Dispense hygiene, quality, and the microbiome

Dispense hygiene is a critical consideration in the quality of beer served in public houses. For retailers that have a strict and effective cleaning regime, the consistency and quality of draught beer will be assured. Line cleaning is a laborious and time-consuming task and an underappreciated aspect of managing a bar. Poor hygienic practices will result in the build-up of mature or established biofilms, which will be progressively harder to remove, containing – over time – an increasingly diverse range of microorganisms.

Draught beer quality was assessed using the forcing method reported by Mallett *et al.* (2018). This enabled beer quality to be related to the microbiome for each of the samples. Quality varied for all samples, with variability between accounts and styles (Table III-2). Accounts V6 (SL3), N14 (ST1), and D13 (KA1) represented the poorest quality beer of each style, which was, respectively, poor (C), and B (acceptable). Consequently, these each presented the most diverse microbiomes of all the samples, with a mix of bacteria - obligate anaerobes (SL3), pathogens (ST1 & KA1) – together with wild yeasts.

3.4.2. Methodology

Beer samples purchased from three accounts were analysed using Oxford Nanopores' next generation sequencing tool, MinION. The platform enabled the rapid identification of microorganisms present from forced samples collected from the on-trade without plating out on agar plates and isolation microbial colonies. The challenge of conventional microbiology is the selective of media and the non-culturability of

some microorganisms. Although conventional microbiology has been the bedrock of brewing science it inevitably underestimates the detail of environmental microflora. Indeed, much of our knowledge of brewing microbiology is derived from research conducted in the mid to late 20th Century (Rainbow, 1952, Hemmons, 1954, Hemmons, 1955, Ault, 1965, Fowell, 1965). Hence, these culture methods focus on identifying these microorganisms such as *Lactobacillus* sp., *Acetobacter* sp. and wild *Saccharomyces* and non-*Saccharomyces* sp.). However, over time cleaning processes have improved and microorganisms have been significantly reduced across the brewing process (Vriesekoop *et al.*, 2012). With regard to dispense, this has become more sophisticated but remains susceptible to variable hygiene management. The introduction of 'Smart-dispense' systems where draught beer is refrigerated from keg to tap will invite new challenges and impact on microflora (Jevons and Quain, 2021). Finally, the number of beer styles is growing, with novel raw materials, low or no alcohol, and the growing craft market which use artificial flavourings post-fermentation. Different styles of beer will support different microflora, and this will be driven by differences in raw material processing, residual sugars, organic acid concentrations, dispense method, and hygiene management. By applying next-generation sequencing, invaluable insight into the subtle differences in microflora between beer styles can be elucidated without the problems associated with conventional microbiology. Consequently, a range of microorganisms previously not described in beer have been identified in this work (Figure III-2-5) alongside potential human pathogens.

3.4.3. Yeast identification lacked the expected diversity

Yeasts were identified using the ITS1 and ITS4 primers, commonly used to identify yeasts, but importantly the same primers used in Chapter II (White *et al.*, 1990). Using

conventional microbiology and Sanger sequencing methods, a wider range of yeast species were identified. However, in this work yeast identification was often limited to a one or two species despite significant abundance in each sample. For example, account D2 exhibited over 80% *Pichia* sp., yet only *P. kudriavzevii* was identified throughout all the samples analysed, despite our prior work and the literature reporting *P. fermentans, P. membranifaciens,* and *P. manshurica* (Caputo *et al.*, 2012, Zhang *et al.*, 2017, Sohlberg *et al.*, 2021). Moreover, *Brettanomyces* sp. key beer spoilers are known to be *B. bruxellensis* and *B. anomalus* and are cited in the literature (Gilliland, 1961), and these are supported by our previous work in Chapter II. Here, surprisingly only *B. nanus* was identified despite prior preconceptions. The MinION approach is subject to a higher error rate than other next-generation sequencing platforms (Loit *et al.*, 2019), which is why low abundance reads were removed from analysis in this work. However, it would seem yeast identification using the current methodology does not offer the same clarity when compared to bacteria. Shinohara *et al.* (2021) did manage to use the MinION platform for identification of yeasts, however, extra workflow and sequencing processing was required which was not available here.

3.4.4. Pathogens in draught beer

Seven potential pathogenic genera were identified in beer at the abundance threshold used in this work (>1%). The 'party line' has long been that beer does not support the growth of pathogens due to the antimicrobial elements of beer, in particular the low pH, limited nutrients and content of ethanol. Further, competition from microorganisms better adapted to the conditions of beer such as wild yeasts together with lactic and acetic acid bacteria creates a competitive environment for nutrients necessary for proliferation. However, in this work it has been shown human pathogens can be found in draught beer, with *Escherichia* sp., *Staphylococcus* sp. *Bacillus* sp.,

Cutibacterium sp., *Delfitia* sp. *Klebsiella* sp., and *Corynebacterium* sp. identified, with sufficient confidence due to the 99% identification filter applied to the data. This is despite competition from (presumably) better adapted beer spoilage microorganisms such as *Lactobacillus* sp., *Saccharomyces* sp. etc. Further, this is the first description of *Cutibacterium* sp., *Delfitia* sp., and *Corynebacterium* sp. genera to be found in beer.

It is important to note that it cannot be confirmed whether these beers supported the growth or simply survival of these pathogenic bacteria. However, each were over >1% abundance in their respective samples. Pathogen survival in beer is not well-defined, the combination of low pH and %ABV are considered to be inhibitory to most human pathogens (Menz *et al.*, 2011). It is likely that the presence of pathogens in a beer is related to the hygiene of the account and contamination from bar staff rather than a consequence of beer style. As previously discussed, the poorest quality beers for SL3, ST1, and KA1 each had pathogens identified (Figures III-2-4), however, for SC1 *S. aureus* was identified but this was graded as A/excellent (Figure III-5).

Poor hygienic practices will support the increasing build-up of biofilms of dispense lines. Biofilms are the primary mode of growth of microorganisms. Microorganisms in a biofilm are highly conserved and protected by an exopolysaccharide matrix that protects from the environmental stresses (for more detail; Section 1.13.1). Potential human pathogens have been found in beer in a number of studies (Menz *et al.*, 2011, Yu *et al.*, 2019, Kim *et al.*, 2014, Haakensen and Ziola, 2008, Holzapfel and Wood, 2014, Thomas *et al.*, 2021). At lower temperatures, *E. coli* and *Salmonella* have been shown to be able to survive over 30 days at 4^{o}C in beer (Menz *et al.*, 2011). Commonly beers are dispensed between 1-8^{o}C in the UK, this would provide ample opportunity

for pathogens to survive and to be consumed. Work in 2019 found *Staphylococcus xylosus* exhibited spoilage potential of craft beer, capable of proliferating in low pH, high ethanol, and low temperature conditions (Yu *et al.*, 2019). Kim *et al.* (2014) similarly challenges this preconception of pathogens are not supported in beer. Both *E. coli* and *Bacillus cereus* were capable of long-term survival in alcoholic beverages, with *B. cereus* spores capable or surviving for extended periods (Kim *et al.*, 2014). This is further supported in other work, where a range of *Bacillus* sp. and *S. epidermidis* were found to possess the hop-resistance gene *horA* (Haakensen and Ziola, 2008). From this work is not possible to understand the significance under the conditions of dispense, however, it is clear due to the abundance and the literature these were viable microbes, and therefore possess the capability to infect consumers.

A diverse range of *Klebsiella* sp. was identified in one sample of stout (Figure III-2; N14); *K. pneumoniae, K. quasipneumoniae, K. aerogenes, K. oxytoca, K. variicola,* and *K. michiganensis* (Table III-7). *K. pneumoniae* is associated with a range of potential infections including pneumonia, however this is not the first report in beer, with both *K. pneumoniae* and *K. oxytoca* being isolated from beer (Van Vuuren and Toerien, 1981). Interestingly, *K. oxytoca* can produce 4-vinylguiaicol from ferulic acid, similar to *Brettanomyces* sp. (Ashtavinayak and Elizabeth, 2016). This is the first known report that has identified this wide range of species of *Klebsiella* in beer sourced from the on-trade.

Corynebacterium sp. were present in a sample of keg ale, accounting for approximately 2% of the total reads (Figure III-4; D13). *C. segmentosum* was the most abundant isolate found of the genera, 67% of the 43 species identified, which has

previously never been described before in beer – and possibly the only example of this genus to be found in abundance in beer. *Corynebacterium* sp. are described as opportunistic pathogen, but largely are commensal with the host (Collins *et al.*, 2004). As a side note, *C. diphtheriae* was identified in the sample, albeit very low concentrations. However, this pathogen causes diphtheria, which can lead to a variety of serious issues such as paralysis and heart failure (Murphy, 1996).

3.4.4.1. Pathogen-biofilms in draught dispense

Biofilms in dispense systems have been discussed at length in Chapter IV and (Jevons and Quain, 2021). Biofilms – rather than planktonic microorganisms - are known to be the primary growth mode for microorganisms (Aparna and Yadav, 2008). Biofilms elaborate a exopolysaccharide matrix, which protects microorganisms from environmental stress, chemicals, and antibiotics (Peterson *et al.*, 2015). Importantly, biofilm structures are capable of housing an almost undefinable number of genera and species; oral biofilms (plaque) have been noted as some of the most 'complex and diverse ecosystems… of more than 600 taxa' (Aruni *et al.*, 2015). Using culture-dependent methods, over 100 bacteria and 20 wild yeasts have been identified from biofilms sourced from draught beer dispense tubing (Bose *et al.*, 2021). Bose *et al.* (2021) further note the application of next-generation sequencing would prove insightful to identifying the nonculturable microbiome. Although biofilms were not directly analysed in the present work due to financial and time restrictions, a number of species were identified that were not previously identified in beer, using a culture-independent method, which are likely to be sourced from biofilms.

The relationship between *Pichia* sp. and *Saccharomyces* sp. in biofilm is discussed in section 3.4.7. However, these are common spoilers in beer. It was previously

suggested in section 3.4.4 that pathogens will be in biofilms. *Bacillus cereus* was the most abundant species of the *Bacillus* genera identified. Their biofilm potency is well-documented, and are commonplace in the food industry (Majed *et al.*, 2016). Its presence in beer is not new, as strains of *B. cereus* have been shown to possess hop-resistance genes and could be a beer spoiler (Wang *et al.*, 2017). However, it is more likely much of the activity is sourced in biofilms. Amongst other activity in the biofilm, importantly *B. cereus* are capable of excrete highly resistance spores (Majed *et al.*, 2016). This has been shown to be an effective method of survival in beer, where *B. cereus* concentrations rapidly depleted in beer, whereby the associated spores remained constant, irrespective of temperature (5-22^{o}C) (Kim *et al.*, 2014). The impact on the consumer cannot be predicted from this work, however, it has been reported toxins produced from this species are predominantly of the 'diarrhoeagenic type' when analysed in fermented beverages (Kim *et al.*, 2020).

Staphylococcus aureus biofilms are well understood, however, they are more associated with medical devices and lead to increased mortality (Moormeier and Bayles, 2017). Much of this is due to the genetic resistance to antibiotic treatment (Rowe *et al.*, 2021). In this work, *S. aureus* was found in both keg and cask ale. *S. aureus* has been shown to be capable of surviving up to 28 days in multiple beers at 5^{o}C, however at an increased temperature (22^{o}C), *S. aureus* viability rapidly reduced in less than 24 hours, but growth was still evident at 14 days (Kim *et al.*, 2014). *S. aureus* contamination is more commonly associated with traditionally fermented beverages (Atter *et al.*, 2014, Lues *et al.*, 2011). The work by Atter *et al.* (2014) noted the all *Lactobacilli* strains exhibited antimicrobial activity versus both *S. aureus* and *E. coli* during production of a traditional Ghanaian beer, burukutu. It is likely *S. aureus*

activity is contained to biofilms, whereas *Lactobacillus* sp. take precedence in the planktonic mode.

The presence of pathogenic bacteria in draught beer is a concern and extends the conversation from beer quality to food safety. Pathogens in other everyday food products are commonplace, examples exist for dairy produce (Oliver *et al.*, 2005), meat (Bhaisare *et al.*, 2014), and soft drinks (Sheth *et al.*, 1988). 'Presence' is distinct from 'infective dose' which will vary with consumer. Great care will be required on the dissemination of this work to avoid alarmist speculation. The identification of pathogens in draught beer is most likely a consequence of human interaction with surfaces (tap nozzles and sparklers, keg couplers). 'Environmental' *E. coli* was found in beer and nozzle soaking solutions (carbonated water) in numerous investigations between 1996-1999 (Quain, unpublished) and was the stimulus (which was not identified) for a study on approaches to removal the microbial risk from soaking tap nozzles (Quain, 2016). Here, growth of bacteria is possible where nozzles are soaked in 'beery' water with sugars from soft drinks dispensers. Elsewhere, the growth of pathogens in beer is unlikely (high %ABV, low pH), and it is anticipated that contaminating microorganisms do not grow in the dispense system and survive for an indeterminate time. Although anecdotal, there are numerous reports over time of an unexpected 'dicky tummy' from consuming modest amounts of draught beer.

3.4.5. Obligate anaerobes in lager

Identification of *Pectinatus* sp. as the primary spoiler in this account (Figure III-2; V6) is noteworthy. *Pectinatus* sp. *Megasphaera* sp., *Megamonas* sp. and *Selenomonas* sp. are Gram-negative bacteria that produce a range of off-flavours in beer (Bittner *et al.*, 2016). The production of sulphur compounds (rotten egg aroma) is a notable trait of

these genera. Dispense conditions for keg beers provide an anaerobic environment, as the beer is pushed through the beer line after an influx of CO_2 (together with nitrogen aka ‘mixed gas) into the keg. These conditions create an environment for obligate anaerobic bacteria. *P. frisingensis* (49%), *Megamonas funiformis* and *M. hypermegale* (3.5%), *Megasphaera stantonii, M. elsdenii* (3%), and *S. ruminantius* and *S. sputigena* (3.9%) were all found in V6 SL3. *Selenomonas* sp. are not widely discussed as beer spoilage bacteria, but their presence in this work suggests potential beer spoilage capability at the on-trade. *Selenomonas latifex* was identified by Schleifer (1990), but there are no reports of either *S. ruminantius* or *S. sputigena* in draught beer.

Pectinatus was first described in beer in 1980 (Lee *et al.*, 1980). The bacterium is capable of producing hydrogen sulphide and turbidity in packaged beer (Lee *et al.*, 1980). *P. frisingensis* was the most abundant species identified in SL3 from account V6. *P. frisingensis* and *P. cerevisiiphilus* have both been described in beer, however the former was found to be the ‘prevailing species’ of *Pectinatus* beer spoilage (Tholozan *et al.*, 1997). It is cited as being resistant to oxygen, alcohol, and capable of growth at a wider range of pH (pH 3.7-6) (Flahaut *et al.*, 2000, Tholozan *et al.*, 1994). In breweries, *Pectinatus* and *Megapshaera* biofilms have been described in aerobic environments, implying poor hygiene has promoted highly established biofilms (Paradh *et al.*, 2011b). *Megamonas* sp. was the second most abundant genera of the obligate anaerobes, and is the most closely related to *Pectinatus* (Juvonen, 2015). Commonly, *Megasphaera cerevisiae, M. paucivorans,* and *M. sueciensis* are most associated with beer spoilage (Juvonen, 2015). Here, to the best of our knowledge, we have identified *Megapshaera stantonii* and *M. elsenii* for the first time from beer. The former was first described in 2018 (Maki and Looft, 2018) and was

isolated from a ‘healthy chicken’! Importantly, this was the most abundant species identified, accounting for 95% of sequenced reads in this genus.

The identification of obligate anaerobes using next-generation sequencing tools has proven to be a powerful tool. Previous work in Chapter II failed to identify any obligate anaerobic bacteria, despite using standard plating methods. By avoiding innately selective media and using the beer as the media to incubate strict anaerobes have been able to be identified and quantified in relation to other microorganisms present in the beer.

3.4.6. Lactic acid bacteria are less significant in ales

Lactobacillus sp. are commonly cited as the most prevalent beer spoiler (Suzuki, 2011, Tsuchiya *et al.*, 1993). This is driven by their ability to proliferate in both aerobic and anaerobic environments alongside their hop-resistance and ethanol tolerance (Ponomarova *et al.*, 2017). However, much of this literature has been focused on the globally predominant lager-style beer. Our previous work identified *Lactobacillus* sp. (*Levilactobacillus*) as more prevalent in lager and less-so in ale styles (Chapter II). Four species of *Lactobacillus* were identified in lager from account V6: *L. brevis, L. koreensis L. suatsaii* and *L. zymae*. Only in one sample of stout (ST1) from N14. were lactic acid bacteria identified (*Pediococcus* sp. and *Secundilactobacillus* sp.) above the 1% abundance threshold.

Ale and lager environments differ in several ways, but in particular the sugar profiles of these beers are different. Ale typically contains much higher concentrations of fermentables and dextrin than lager, which is classically a very attenuated beer. For ale styles, it is common for beers to be ‘primed’ post-fermentation to add sweetness

to the beer. Priming is the addition of fermentable sugars to the beer. Therefore, in lager it is necessary for beer-spoilage microorganisms to possess a diverse carbohydrate metabolism. In the presence of low carbohydrates or a low pH, pyruvate is used for the production of acetoin which supports cellular homeostasis (Holzapfel and Wood, 2014). Further, as primary carbohydrates deplete, LAB species can use amino acids to further produce ATP to resist acid stress (Geissler *et al.*, 2016). Due to the attenuated style of lagers, spoilage organisms must be capable of using a variety of carbon sources in beer to survive. Thus, LAB species prevalence in lager style beers will be driven to the attenuated nature of beer coupled with their diverse carbohydrate metabolism. This insight also explains the increased prevalence of yeasts in ale style beers.

3.4.7. *Pichia* and *Saccharomyces* -key ale spoilers

Pichia sp. and *Saccharomyces* sp. were found to be abundant across all ale styles investigated. Both yeasts have previously been described in beer by Lois Hemmons, who conducted an 'exploratory survey' of the wild yeasts found in ales in London (Hemmons, 1954, Hemmons, 1955) in 1954 and 1955. *Pichia* sp. require oxygen for optimal growth (Hemmons, 1954), but are capable of growing under anaerobic conditions (Macrae, 1964). Biofilm formation in dispense lines is a known problem, where ales-style beers are known to be potent biofilm formers (Jevons and Quain, 2021). *Pichia anomala,* sourced from a brewery, has been found to be a primary biofilm former, after which *Saccharomyces* sp. later colonises a pre-formed biofilm (Timke *et al.*, 2008b). *Pichia kudriavzevii* was the only identified *Pichia* sp. and was not previously identified in Chapter II. *P. kudriavzevii* has been previously isolated from sorghum beer (N'guessan *et al.*, 2011), rice beer (Ghosh *et al.*, 2019), and Brazilian craft beer (Dos Santos *et al.*, 2022) together with being identified using next-

generation sequencing, isolated from a brewery (Sohlberg *et al.*, 2021). More commonly, *P. manshurica* (Zhang *et al.*, 2017), *P. membranifaciens* (Pham *et al.*, 2011), and *P. fermentans* (Caputo *et al.*, 2012) are the most referred to beer spoilers form this genus. The relationship between *Saccharomyces* sp. and *Pichia* sp. in biofilm formation supports the findings in this work with the yeasts known to promote biofilm formation (Jevons and Quain, 2021).

Saccharomyces sp. were a ubiquitous spoiler in this work. With the exception of one account (N5; 0.61%), the genus was identified in all accounts above the 1% threshold. *Saccharomyces* sp. in beer have been cited widely (Richards, 1968, Wiles, 1949, Wiles, 1950), and are associated with the production of phenolic off flavours (Coghe *et al.*, 2004). However, the yeast species data lacks sufficent clarity. In this work, using the MinION platform, only *S. cerevisiae* was identified, which is unexpected considering the prevalence throughout the sampling. For SC1 samples, there was a high abundance of *S. cerevisiae,* but neither *S. bayanus* or *S. uvarum* were found as in Chapter II. Brewing yeast are integral to the conditioning of cask ales, and therefore were expected to be abundant. Each sample exhibited *S. cerevisiae* as the most dominant species. Moreover, in keg ale KA1, *S. cerevisiae* was the most abundant in all samples. The dominance of *S. cerevisiae* could reflect residual brewing yeast, wild strains, or the lack of discrimination in the microflora database.

3.4.8. *Brettanomyces sp.* ubiquitous spoilers or not?

Initial conclusions in Chapter II using culture-based microbiology showed that *Brettanomyces* sp. were ubiquitous irrespective of style or dispense method. With the MinION approach *Brettanomyces* sp. are also isolated from ales and lagers (Figure I-2-5), but their abundance is much reduced *in situ*. Indeed, the yeasts *Saccharomyces*

and *Pichia* sp. were more dominant in this work and – using this platform – are the primary beer spoilers of ale style beers. In lager there was less consistency of spoilers and increased diversity, *Brettanomyces* dominated the microflora of V8, *Saccharomyces* sp. of V13 and *Pectinatus* sp. of V6. But the supposed ubiquity of *Brettanomyces* sp. was not revealed using a culture-independent methodology.

3.4.9. *Eremothecium* sp. are found in a range of styles and may contribute to sunstruck flavours and staleness in draught beer

Eremothecium gossypii was identified in at least one account for each of the four styles of beer. *E. gossypii* is filamentous fungi and is part of the *Saccharomycetaceae* family (Ashby and Nowell, 1926), used in the commercial production of riboflavin (Tanner Jr *et al.*, 1949). This genus has not previously been described in beer but its repeated occurrence in this work suggests warranted further discussion. It is noteworthy that optimal spore production by *E. ashybii* uses beer wort as the key ingredient in the media (齋藤賢道 and 箕浦久兵衛, 1949, Nordström, 1969). The impact of this microorganism on draught beer quality is not obvious but there are interesting considerations that can be reflected upon. *E. gossypii* has been found to secrete invertase, which hydrolyses sucrose into assimilable glucose and fructose (monosaccharides) (Aguiar *et al.*, 2014). Sucrose is not a common sugar in beer as it is readily assimilated, but in other work it has been found maltose can be an effective carbon source alongside glucose for the production riboflavin (Tanner Jr *et al.*, 1949). As noted above, riboflavin can be produced by *E. gossypii* and in beer riboflavin in beer is associated with ‘sunstruck’ aroma (Kuroiwa *et al.*, 1963) and the reactive oxygen species responsible for a stale flavour (Duyvis *et al.*, 2002). Riboflavin is photosensitive, where, in response to light, the sunstruck flavour in beer is formed. Riboflavin is sourced from yeast, malt, and hops (Duyvis *et al.*, 2002). However, the

isolation of *E. gossypii* in a range of beers from the on-trade, coupled with the commercial production of riboflavin, it can be postulated *E. gossypii* is associated with the flavour instability of beer at the on-trade, sunstruck flavours, and staleness.

3.4.10. Draught beer dispense hygiene

Beer spoilage microorganisms have long been known to be a complex mix of wild yeasts and bacteria. Previous work on draught beer microbiology used culture-based methods and introduced bias into the identified microflora. Using a culture-independent method we have identified the *in-situ* relative abundances in four beer styles, sampled from three accounts. Common beer spoilers have been identified, alongside new pathogens, and new wild yeasts. However, their survival and impact on beer cannot be defined without much further study. It is suggested that the viable but non-culturable (VBNC) microorganisms will be sourced in biofilms or spores. This raises fundamental questions regarding dispense hygiene and what factors must be considered important.

Current line cleaning processes employ a simplistic and primarily static process and there is no measurement post cleaning of effectiveness. It is clear different styles of beer are promoting different microflora, are harbouring pathogens and VBNC bacteria and yeasts. Hence, it is essential accounts employ best hygienic practices, ensuring both beer quality and the safety of the product. This will become increasingly important as accounts across the UK start serving low and no alcoholic beverages using conventional dispense lines.

3.5. Conclusions

Consideration of draught beer spoilage has previously been restricted to a few beer spoilage microorganisms including *Lactobacillus* sp., *Acetobacter* sp., *Brettanomyces* sp., *Saccharomyces* sp., and *Pichia* sp. Much of this knowledge was derived from work conducted many decades ago and was limited by the technology of the time. As dispense methods evolve, beer styles and brand numbers increase, and the NABLAB market exponentially increases, there are likely to be new and important beer spoilage microorganisms. Here in this work, using culture-independent methods, we have (i) described some new microorganisms isolated for the first time in beer, (ii) identified a range of pathogens and (iii) finally discussed how spoilage differs depending on beer style and dispense method.

The application of next-generation sequencing has been shown to be a powerful tool for identifying new beer spoilage microorganisms. But has further highlighted that disparity between conventional microbiology and the *in-situ* microbiome. Although this work cannot evaluate the impact of these microorganisms, nor their ability to grow or survive, their presence is an important reminder of the importance of best practice to assure hygiene of beer in the on-trade.

Chapter IV: Draught beer hygiene: use of microplates to assess biofilm formation, growth, and removal

Foreword

Work in this Chapter was completed during 2019 to the year end and was prepared for publication during 2020. The aim of this work was to develop a method capable of quantifying draught beer dispense sourced biofilms and the environmental factors influencing growth.

This work was published on the 19th January 2021 in Journal of the Institute of Brewing and Distilling.

I was responsible for the production of all data in this work and lead author under the supervision of Dr David Quain

Abstract

Draught beer quality is assured by the management of microbial biofilm in dispense lines through regular and effective line cleaning with alkaline detergent. Here, a method is described which enables biofilm formation, growth, and removal to be assessed in 96 well polyvinyl chloride microplates. Draught beer (and cider) microflora formed reproducible biofilms in their 'parent' beer after incubation at 15°C for seven days. Biofilm formation by four draught beer styles – keg lager, ale, stout, and cask ale - was assessed and was enhanced by periodic replenishment with fresh beer. The rate of biofilm formation by microflora from keg beers decreased with increasing temperature whereas with cask ale it increased. Oxygen enhanced biofilm formation with microflora from cask ale but not keg. Simulation of line cleaning in microplates with a proprietary alkaline solution failed to kill all microflora and the microorganisms regrew in all four beer styles. Further, the line cleaning process was increasingly ineffective with older biofilms. It is suggested that the method reported here will help focus attention on the efficacy of line cleaning, in particular, the role of mechanical action, which contributes little to the standard manual line cleaning process in the UK. This and other investigations will hopefully contribute to the ultimate intention of improving and assuring draught beer quality.

4.1. Introduction

In 1912, Seton (Seton, 1912) reported that with cask beer 'it is a lamentable fact that the original good quality of draught beer in the brewery becomes deteriorated through incorrect handling in the public-house. Similarly, 70 or so years later, Harper et al. (Harper *et al.*, 1980) noted 'although keg beer was free of infection on delivery, it soon became contaminated by bacteria and yeasts that grow in the dispense lines'. Today, although the technology underpinning beer dispense has undoubtedly evolved, regrettably draught beer quality remains inconsistent. Although physical factors – temperature, gas pressure/mix and dispense speed – can contribute, poor hygiene and consequent spoilage microorganisms are major considerations in compromising the quality of draught beer. Although there have been relatively few publications on draught beer microflora, it is accepted that Gram-positive lactic acid bacteria, Gram-negative acetic acid bacteria and wild yeasts predominate (Quain, 2015). Similarly, measurement of the microbiological loading of beer post dispense has received little attention, either directly (Boulton and Quain, 2013, Hough *et al.*, 1976, Storgårds, 1997), indirectly via ATP (Storgårds and Haikara, 1996) or using forcing (Mallett and Quain, 2019).

As with most aqueous environments, the microorganisms in draught beer are free floating or 'planktonic'. However, as in any liquid system, the microbial community in beer dispense is a mix of the planktonic and 'sessile' microorganisms attached as a biofilm to surfaces. Biofilms are how most microorganisms exist in the real world and - as biofilms are a major interest for industry (corrosion damage) and medicine (healthcare) - research has grown exponentially since 2000. Biofilms (Flemming *et al.*, 2016) are three dimensional structures which are highly organised, slow growing,

multicellular communities of bacteria and/or yeast that form at solid-liquid interfaces. Biofilms communicate through chemical signalling or ‘quorum sensing’ which coordinates gene expression. Although predominately water, the biofilm’s ‘cement are diverse hydrated polysaccharides secreted by microflora in the biofilm’ (Sutherland, 2001). It is important to note ‘that biofilms may develop in an enormous number of environments, and that the structural intricacies of any single biofilm formed under any specific set of parameters may well be unique to that single environment and microflora’ (Sutherland, 2001).

In brewing, biofilms have been described as ‘the real enemy of process and product hygiene’ (Quain, 1999) and has been reviewed (Mamvura *et al.*, 2011, Quain and Storgårds, 2009). Much of the focus on biofilms in brewing has been in packaging, particularly bottling (Quain and Storgårds, 2009). With draught beer, the existence of microbial growth or biofilm on the internal surface of dispense tubing and equipment has long been recognised by line cleaning bar staff and dispense service technicians. It is this microbial attachment that regular line cleaning with proprietary sodium (or potassium) hydroxide-based solutions is focussed on removing. However, line cleaning is an unsuccessful process as the biofilm regrows on dispense surfaces which must then be recleaned. In the UK, the recommended line cleaning frequency is every seven days (BBPA, 2020) but elsewhere the frequency varies by market reflecting system complexity together with cellar and dispense temperature (Quain, 2015).

Attachment of microorganisms to draught beer dispense tubing was reported in the 1980’s by researchers from British School of Malting and Brewing at the University of Birmingham (Casson, 1985, Harper, 1981, Harper *et al.*, 1980). Whilst predating

the use of ‘biofilm’ as a descriptor, these publications used electron microscopy to demonstrate the attachment of draught beer microorganisms to different types of dispense line tubing. Further, Casson (Casson, 1985) reported the presence of an ‘extracellular polymer’ and suggested that this would provide protection for the biofilm. Subsequent work (Thomas and Whitham, 1997) built on this, exploring factors impacting on the attachment to different line materials and evaluating the efficacy of line cleaning. The potential scale of biofilms in dispense systems is large (Casson, 1985). The surface area of a 6mm (internal diameter), 25 metre dispense line is 471 cm^2 or the equivalent of seven and a half A4 sheets of paper. More recently, biofilms have been grown in draught beer test rigs to evaluate the impact of cleaning on the loading of planktonic and sessile organisms using ozonated water (Fielding *et al.*, 2007), enzymes (Walker *et al.*, 2007) or line cleaning solution (Quain, 2015).

Quantification of biofilm attached to draught beer tubing has been either via washing, sonication and plate counts (Thomas and Whitham, 1997), by vortex, swabbing and plate counts (Fielding *et al.*, 2007, Quain, 2015) or by vortex, swabbing and measurement of absorbance at 660 nm (Mallett & Quain, unpublished).

Although hitherto not used with draught beer, biofilm studies commonly use microplates (Azeredo *et al.*, 2017). Microorganisms are grown and biofilms formed in the wells of the microplate and after a period of incubation the wells are emptied, washed (to remove planktonic cells) and the biofilm stained with crystal violet which is quantified by measurement of absorbance. Whilst, the simplicity is attractive, the microplate method can be compromised by evaporation, cell sedimentation and loss of loosely attached biofilm on washing. Consequently, the microplate method has been

repositioned as a screening tool to reflect experimental variation through well heterogeneity, coupled with variable approaches to washing and staining (Kragh *et al.*, 2019). To address these issues of reproducibility, minimum information guidelines for methods to assess biofilms have been published (Allkja *et al.*, 2020).

Microplates have been used to screen biofilm formation by brewing microorganisms including yeast (Timke *et al.*, 2008a), bacteria (Maifreni *et al.*, 2015) and lactic acid bacteria (Riedl *et al.*, 2019a, Wang *et al.*, 2020). These studies used pure cultures of brewery organisms at 26-30ºC incubated for one (Riedl *et al.*, 2019a, Timke *et al.*, 2008a), two (Maifreni *et al.*, 2015) or four days (Wang *et al.*, 2020). Biofilms were grown in rich media (Tryptic Soy, MRS) although Riedl et al. (Riedl *et al.*, 2019a) also successfully used beer (wheat, lager, pilsner as is and diluted 50%).

Here, biofilm formation was assessed using a consortium of microorganisms from forced samples of draught beer inoculated into microplate wells containing the parent beer. The microplates were covered with an anaerobic seal and incubated at 15ºC for seven days with replenishment with fresh beer after three days. Biofilm was quantified by staining with crystal violet. A diagrammatic overview of the method is presented in Figure IV-1.

4.2. Materials and Methods

The microplate experiments conformed to the minimum information guideline for spectrophotometric and fluorometric methods to assess biofilm formation in microplates (Allkja *et al.*, 2020).

Microplates (polyvinyl chloride and polystyrene) were obtained from Corning. Polystyrene plates (clear flat bottom polystyrene, non-treated, sterile, product #3370) were supplied sterile whereas the polyvinyl chloride (PVC) microplates (clear flat bottom PVC, non-treated, non-sterile, product #2595) were sterilised before use by soaking with 95% (v/v) ethanol, draining and air drying in a laminar flow cabinet. Anaerobic plate covers (sterile sealing film clear non-pierceable SealPlate sheets) was from Alpha Laboratories, Hampshire, UK. Aerobic plate covers (sterile Axygen Rayon breathable film 50µm) were from Scientific Laboratory Supplies, Nottingham, UK. Pipeline Original line cleaner was generously supplied by Chemisphere UK. Pipeline contains sodium carbonate, potassium hydroxide, sodium hypochlorite and potassium permanganate. The line cleaner solution is purple but becomes green when in contact with contamination in a dirty beer line. Crystal violet solution (PL7000) was obtained from Pro-Lab Diagnostics. The cycloheximide solution (0.1%, w/v) was from Sigma-Aldrich.

4.2.1. Draught beers and cider

Between May and November 2019, various draught beers were sampled in sterile 250mL Duran containers on eight occasions from a local Nottinghamshire public house. The account (L7) was used in previous work (Mallet *et al.* 2018) and is a village gastro pub with beer turnover of *ca.* 350 hL per year. Initial experiments were performed with two standard lagers (SL6, 10 – both 4% abv), standard keg ale (KA6, 3.5% abv), premium lager (PL3, 5% abv), two standard cask ales (SC1, 4.2% abv and SC4, 4.5% abv), premium keg ale (PKA1, 4.5% abv), stouts (ST1, 4.4% abv) and a keg cider (C2, 4.8% abv).

More detailed experiments focussed on four draught beer styles: lager (PL3), stout (ST1), keg ale (PKA1) and cask ale (SC1). In experiments where microplate wells were replenished, the beer was aseptically added from 'commercially sterile' bottles or cans of the same brand.

4.2.2. Forcing

The microbiological quality of draught beer *ex* dispense was determined – in triplicate - using the forcing method as described by Mallet et al (Mallett *et al.*, 2018). Freshly dispensed draught beer (25 mL) in 30 mL universal tubes was incubated at 30^{o}C for 96 hours. Absorbance at 660nm was determined at 0 and 96 h using a Jenway 7315 spectrophotometer. Cycloheximide (4mg/L) was added to cask beer before forcing to inhibit the growth of primary yeast.

4.2.3. Biofilm formation

Biofilm formation was assessed using flat bottom PVC 96 (12 x 8) well microplates. Samples of beer were either draught beer samples or preferably, forced samples to assure consistency of the inoculum. The total volume of each well was 200μL. With forced beer, the absorbance was adjusted to $\Delta A_{660} = 0.2$ with sterile water and 20μL added to 180 μL beer (same brand *ex* can or bottle). Where draught beer samples were used directly, this was shortly (30 minutes) post dispense. Similarly, beer samples post forcing were added within two hours of the completion of the forcing method. Fresh beers were sampled for all the experiments reported here.

Where possible to minimise edge effects, the outer rows (A1:A12, A1:H1, A12:H12 and H1:H12) of microplates were not used. Plates were sealed with an anaerobic cover and incubated at 15^{o}C under static conditions in a non-humidified incubator for a total

of 7 days. Plates were carefully inverted on a paper towel after 3 days and the wells aseptically replenished (200μL) with the parent brand of beer from a pasteurised can or bottle.

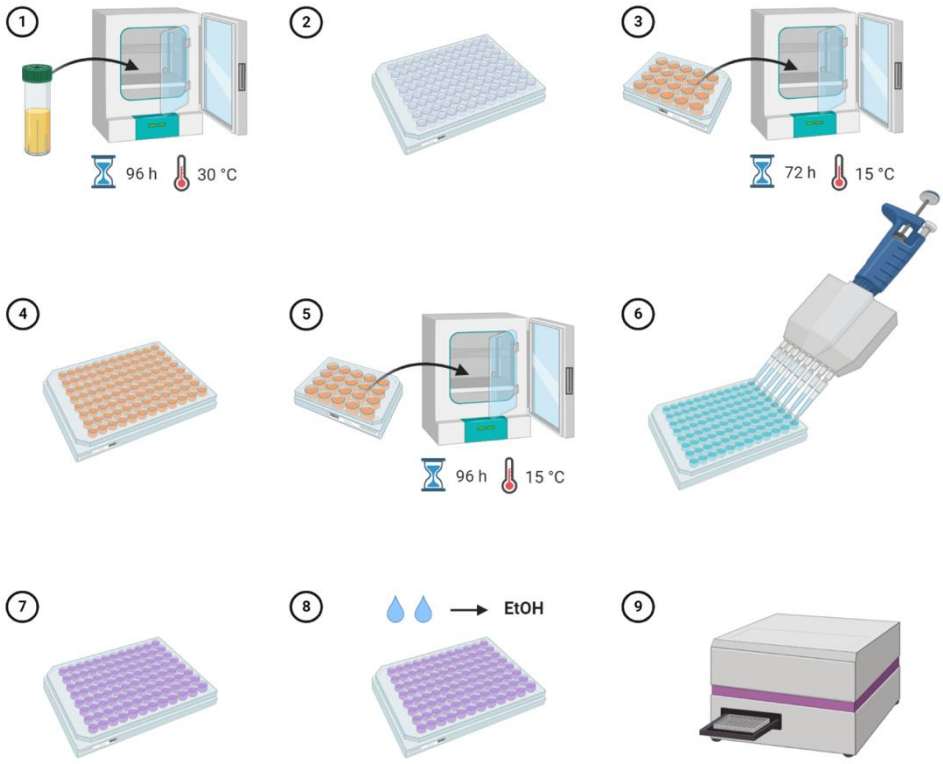

Figure IV-1: Method for quantification of biofilm formation for microorganisms from draught beer. Figure created with BioRender.com (https://www.biorender.com/).

1. Beer samples from trade, forced for 4 days and diluted with sterile distilled water to A_{660} = 0.2.
2. Sterilise PVC microplate with 95% (v/v) ethanol and allow to air dry in a laminar flow.
3. Inoculate samples (20µL) into parent beer (180µL) and incubate for 3 days at 15ºC.
4. Carefully remove media and replenish with parent beer.
5. Incubate the samples for 4 days at 15ºC.
6. Remove media by inverting microplate on a paper towel and wash wells with water at a 45º angle.
7. Add 0.4% (v/v) crystal violet to wells and stand for 45 minutes.
8. Invert microplate on a paper towel crystal and wash wells with water twice, add 150 µL 95% ethanol and stand for 45 min.
9. Recover the ethanol + crystal violet and transfer into a fresh 96 well plate and read absorbance at 570nm.

4.2.4. Biofilm assessment

The plate was inverted on a paper towel to remove planktonic and loosely attached cells. The wells were washed with distilled water (200μL) using a Gilson P200 micropipette inserted slowly at a 45° angle and avoiding touching the sides and bottom of the wells. After inverting again on paper towel, the plates were left to air dry for 15 min at room temperature (20°C). The biofilm was stained with crystal violet (200 μL, 0.4% v/v, A_{590} = 0.765) for 45 min at room temperature, under static conditions. The wells were washed and inverted twice with 200 μL of distilled water using a Gilson P200 micropipette and left to air-dry for 15 min at room temperature. The crystal violet was recovered by the addition (per well) of 150 μL of 95% v/v ethanol for 45 min at room temperature without shaking. The eluted stain was mixed by pipetting up and down 4 times and the recovered crystal violet transferred to a fresh 96 well plate. The absorbance was measured at 570 nm using an automated Tecan plate reader (Infinite® 200 PRO) with MagellanTM data analysis software.

In experiments with line cleaning solution, non-specific binding of crystal violet to wells was subtracted from the results presented in Figures IV-9 and 10.

4.2.5. Experiments

A number of experiments were performed using the above method. Table III-1 reports the details of experiments on reproducibility, nutrient replenishment, temperature, and availability of oxygen. The table reports the experimental detail prior to the assessment of biofilm.

4.2.6. Statistics

GraphPad's Prism v8.3.0 software (GraphPad, San Diego, CA, USA was used to determine one-way ANOVA, trendlines and t-tests at 95% confidence interval.

4.2.7. Method development - material

Microplates used in biofilm studies are typically made from polystyrene. Samples of draught beer (SL6, SL10, KA6, PL3, SC1, SC4, PKA1 and ST1) and cider (C2) were assessed in triplicate for biofilm formation over 7 days in microplates made from polystyrene and polyvinyl chloride. Plates were carefully inverted on paper towel after 3 days and the wells were replenished (200μL) with appropriate brand of beer on day 4.

4.2.8. Biofilm formation and line cleaning

Draught beer samples - lager (PL3), stout (ST1), keg ale (PKA1) and cask ale (SC1) – were added in sextuplicate to seven microplates (24 wells per plate) and incubated at 15°C, with anaerobic covers. Biofilm was grown for 3, 6, 9, 12, 15, 18 and 21 days with brand specific replenishment every four days. At each timepoint, the plate was inverted onto a paper towel, and sterile distilled water added to each well and allowed to stand for five minutes. The plate was inverted and Pipeline Original line cleaning solution (2.5% v/v, 200 μL) added to the wells. After standing for 30 minutes at room temperature (20°C), the microplate was inverted to remove line cleaner and washed with sterile distilled water (200 μL) for five minutes. The microplate was inverted on paper towel, and the same brand of beer (ex can or bottle) added to the wells (200 μL) and incubated statically at 15^{o}C. After 3 days, the plate was inverted, drained and the wells replenished with fresh beer (200 μL). After incubation for a further 4 days, the planktonic and sessile microorganisms were mixed by pipetting up and down 4 times

and microbial growth measured at A_{660} using a Tecan plate reader against control wells containing beer of the same brand.

4.2.9. Line cleaning and time and biofilm regrowth

Individual draught beer samples - lager (PL3), stout (ST1), keg ale (PKA1) and cask ale (SC1) – were added to two microplates with 12 wells arranged in four rows by style in two blocks. Plates were incubated at 15°C, with anaerobic covers and biofilm was grown for 7 days with brand specific beer replenishment after 4 days. The plate was inverted, and sterile distilled water added to each well and allowed to stand for five minutes. The plate was inverted and Pipeline Original line cleaning solution (2.5% v/v, 200 μL) added to the wells. Cleaning was assessed every five minutes up to 40 minutes with wells A1:D3 (5 minutes), E1:H3 (10 min), A4:D6 (15 mins) etc. Line cleaning solution was carefully removed from each group of wells by pipette. Washing with sterile distilled water was either for five minutes (microplate 1) or twice for 2.5 minutes (microplate 2). As with the line cleaning solution, the water wash was removed from each well by pipette. After cleaning/washing, the appropriate beer (from bottle or can) was added to the wells (200 μL) and incubated statically at 15^{o}C. After 3 days, the plate was inverted, drained and the wells replenished with the brand specific fresh beer (200 μL). After incubation for a further 4 days, the planktonic and sessile microorganisms were mixed by pipetting up and down 4 times and microbial growth measured at A_{660} using a Tecan plate reader against control wells containing beer of the same brand.

4.2.9.1. (i) Line cleaning

Biofilms are established following the standard ‘dispense biofilm formation assay’ protocol previously described but is ceased prior to crystal violet staining. After the 7-

day incubation period the plate is inverted, and residual beer is washed using sterile RO water once. Line cleaning solution is added for 30 mins at room temperature, after the line cleaning solution is inverted and excess washed away with water. 200µL of the parent beer was added to the wells and plate is incubated again according to the 'dispense biofilm formation assay'. For quantification, total microflora is calculated opposed to just biofilms using crystal violet. Microbes in each well are suspended into the media through pipetting up and down, media is then transferred to a clean, sterile, polystyrene 96-well plate and measured in a TECAN at $A_{660.}$ The ΔA_{660} is calculated by subtracting the absorbance of a degassed parent beer.

4.2.10. 'Sinner circle' investigations

For each experiment, minor alterations to the method protocol are conducted to investigate influence on biofilm formation, line cleaning efficacy and biofilm recalcitrance in using the standard assay methodology as a control. Below describe the alterations to each method during the experimentation:

4.2.10.1. (i) Temperature

When the line cleaning solution is added to the microplate, the plate was incubated at a range of temperatures from 4ºC to 55ºC for 30 minutes. Plates were also incubated at temperatures between 7.5ºC to 30ºC, for 7 days, cleaned for 30 minutes and then incubated again at the original temperature for 7 days. For both experiments, total A660nm was used to measured regrowth.

4.2.10.2. (ii) Mechanical action

Similarly, during the line cleaning phase plates were placed in a shaking incubator between 50 and 300 rpm.

Table IV-1: Details of individual experiments up to the assessment of biofilm

Experiment	Beer (cider C2)	inoculum	Samples	Microplates	replenished	Time (days)	Temperature (ºC)
Material	SL6, SL10, KA6, PL3, SC1, SC4, PKA1, ST1, C2	draught	200μL x 3	2 plates 27 wells/plate polyvinyl chloride (C) v polystyrene (T)	After 3 days with 200μL parent beer	7	15
Cleaning	PL3, ST1, PKA1, SC1	draught	200μL x 6	7 plates 24 wells/plate anaerobic	Every 3 days with 200μL parent beer	21 (7 cycles of 3 days)	15
	At each timepoint, the plate was inverted, and sterile distilled water added to each well. After five minutes, the plate was inverted and Pipeline alkaline cleaning solution (2.5% v/v, 200 μL) added. After standing for 30 minutes at 20°C, the microplate was inverted and sterile distilled water (200 μL) added to each well. After five minutes, the microplate was inverted on paper towel, and parent beer added to the wells (200 μL) and incubated statically at 15ºC. After 3 days, the plate was inverted, drained and the wells replenished with fresh beer (200 μL). After incubation for a further 4 days, the planktonic and sessile microorganisms were mixed by pipetting up and down 4 times and microbial growth measured at A_{660} using a Tecan plate reader against control wells containing beer of the same brand.						
Cleaning and regrowth	PL3, ST1, PKA1, SC1	draught	200μL x 3	2 plates 96 wells/plate anaerobic	After 3 days with 200μL parent beer	7	15
	2 x 12 wells of each beer (A-D and E-H). After 7 days incubation (replenishment at 3 days) the plates was inverted, and sterile distilled water added to each well. After five minutes, the plate was inverted and Pipeline alkaline cleaning solution (2.5% v/v, 200 μL) added. Cleaning was assessed every five minutes up to 40 minutes with wells A1:D3 (5 minutes), E1:H3 (10 min), A4:D6 (15 mins) etc. At each timepoint, line cleaning solution was carefully removed from each group of wells by pipette. Sterile distilled water was added to groups of wells for five minutes (microplate 1) or twice for 2.5 minutes (microplate 2) and removed by pipette. The appropriate beer was added to the wells (200 μL) and incubated statically at 15ºC. After 3 days, the plate was inverted, drained and the wells replenished with the brand specific fresh beer (200 μL). After incubation for a further 4 days, the planktonic and sessile microorganisms were mixed by pipetting up and down 4 times and microbial growth measured at A_{660} using a Tecan plate reader against control wells containing beer of the same brand.						

4.3. Results and Discussion

4.3.1. Draught beer microflora

Since 1950, the few studies of microflora in draught beer have reported the predominant presence of bacteria (*Lactobacillus, Acetobacter, Gluconobacter*) and wild yeasts (*Saccharomyces, Pichia, Brettanomyces*) (reviewed in (Quain, 2015). The use of microflora from draught beer – rather than pure single cultures of beer spoilage microorganisms – enables the assessment of biofilm formation in microplates that is more representative of draught beer systems. Additionally, a heterogeneous microbial population may facilitate metabolite cross-feeding mutualisms between species, as seen between yeast (*Saccharomyces cerevisiae*) and bacteria (*Lactobacillus plantarum* and *Lactococcus lactis*) (Elias and Banin, 2012).

A recent study (Jevons & Quain, in review; Chapter II) has sought to understand whether beer style and account impact the microflora of draught beer microflora. Using culture-based recovery of microorganisms, four beer styles – lager, stout, keg ale and cask ale – were sampled on two occasions from five different accounts per style. The 40 samples were forced and plated (after appropriate dilution) on aerobic (WLN) and anaerobic (Raka Ray) media. PCR culture-based microflora from five colonies per plate were obtained after DNA extraction, amplification, sequencing and BLAST identification. In all 386 colonies were identified, with 19 different microorganisms found in stout, 17 in lager, with 12 in keg and cask ale. *Brettanomyces bruxellensis B. anomalus* and *Acetobacter fabarum* were ubiquitous in all four styles representing 48-50% (ale, stout, keg ale) and 66% (cask ale) of the detected colonies. Yeasts predominated in keg ale (82%), stout (58%) and cask ale (55%) but not in lager

(42%). Notably, *Rhodotorula mucilaginosa* represented 25% of the identified colonies in keg ale and 12% in stout but was not found in cask ale or lager. Stout was the most diverse in yeasts with species of *Saccharomyces* (x3) *Brettanomyces* (x2), *Pichia* (x2), *Rhodotorula* and *Candida*. Lactic acid bacteria were found in lager (21% of the recovered colonies), with low abundance in stout (6%), cask ale (1%) and absent in keg ale.

Related work (Quain & Jevons, in preparation) assessed the spoilage capability of the 40 draught beers described above. Spoilage of 10 different lager brands was assessed by inoculating under controlled conditions (Mallett and Quain, 2019) with microflora from the forced samples of beers. Despite the 20 locations, unknown cleaning history and time gap between sampling, there was a respectable linear relationship (R^2 = 0.5525) between the spoilage capability of the sample pairs of each beer style. This, coupled with the above data, suggests that the microflora is determined by beer style and is consistent between and within accounts. Accordingly, if the capability for beer spoilage is broadly consistent, it is reasonable to conclude that biofilm formation with a controlled inoculum will also be comparable.

4.3.2. Microplates

Microplates have been widely used in the spectrophotometric assessment of biofilms in medical, environmental and industrial research (Azeredo *et al.*, 2017). Here, the microplate approach was adapted to make it more applicable to the assessment of biofilm formation by draught beer microorganisms. Using the framework of the minimum information guidelines for methods to assess biofilms (Allkja *et al.*, 2020), Table IV-2 compares the diverse protocols with that in this work. Whilst ‘assessment’ is not customised, conditions of ‘biofilm growth’ are tailored to better reflect the

environment of microorganisms in draught beer. Whilst many biofilm studies use monocultures (Ponomarova *et al.*, 2017), mixed species biofilms dominate in nature (Mclean *et al.*, 2004). Accordingly, this work uses the mixed microflora present in draught beer which is amplified post dispense by forcing for four days at 30^{o}C. The inoculum (A_{660} = 0.02) was lower than is typically used (A_{660} = 0.1-1) (Table IV-2). Further, the incubation conditions were modified to be anaerobic with the temperature reduced to 15^{o}C, the time extended to seven days and (brand specific) beer used for the growth media. In recognition that beer is a minimal medium, this was replenished with fresh beer (again brand specific) after three days. Assessment of the formation of draught beer microbial biofilm using crystal violet is shown visually in Figure IV-2.

Table IV-2: Applications of 'minimum information guidelines' for the microplate method used for biofilm screening in this work

	Allkja *et al.* (2020)	**This work**	**Explanation**
Inoculum preparation			
Microorganisms	Pure culture to environmental	Environmental	Spoilage microflora from draught beer
Media	Minimal to complex rich media	Beer (minimal media)	Beer post dispense with indigenous microorganisms
Incubation time	≤ 24 h	4 days	Forcing method (Mallett *et al.* 2018)
Incubation temperature	25-37ºC	30ºC	Forcing method (Mallett *et al.* 2018)
Inoculum	A_{600} 0.1-1	A_{660} 0.02	Based on challenge testing method (Mallett *et al.* 2019)
Biofilm growth			
Media	Minimal to complex rich media	Beer (minimal media)	Different brands, styles
Aeration/shaking	Aerobic	Static, anaerobic	Reflects dispense conditions
Microplate material	Polystyrene	Polyvinyl chloride	PVC is/has been used and better reflects the common material, MDP
Incubation time	2-48 h	7 days	Reflecting the slower microbial growth rate (low temperature, minimal media) and recommended UK frequency of line cleaning
Incubation temperature	25-37ºC	15ºC	Similar to cellar temperatures (12-14ºC)
Nutrition supplementation	N/A	4 days	Biofilms *in situ* are replenished with beer on dispense
Biofilm assessment			
Washing agent	water, saline, phosphate buffered saline	water	
Washing steps	1-3	1	
Crystal violet concentration	0.01 - 2.3%	0.4%	
Staining time	1-45 min	45 min	
Solubilisation agent	30-33% v/v acetic acid, 95% v/v ethanol	95% v/v ethanol	
Absorbance wavelength (nm)	540-595	570	

* example microorganisms - *Staphylococcus aureus* spp, *Pseudomonas aeruginosa* spp, *Candida albicans*

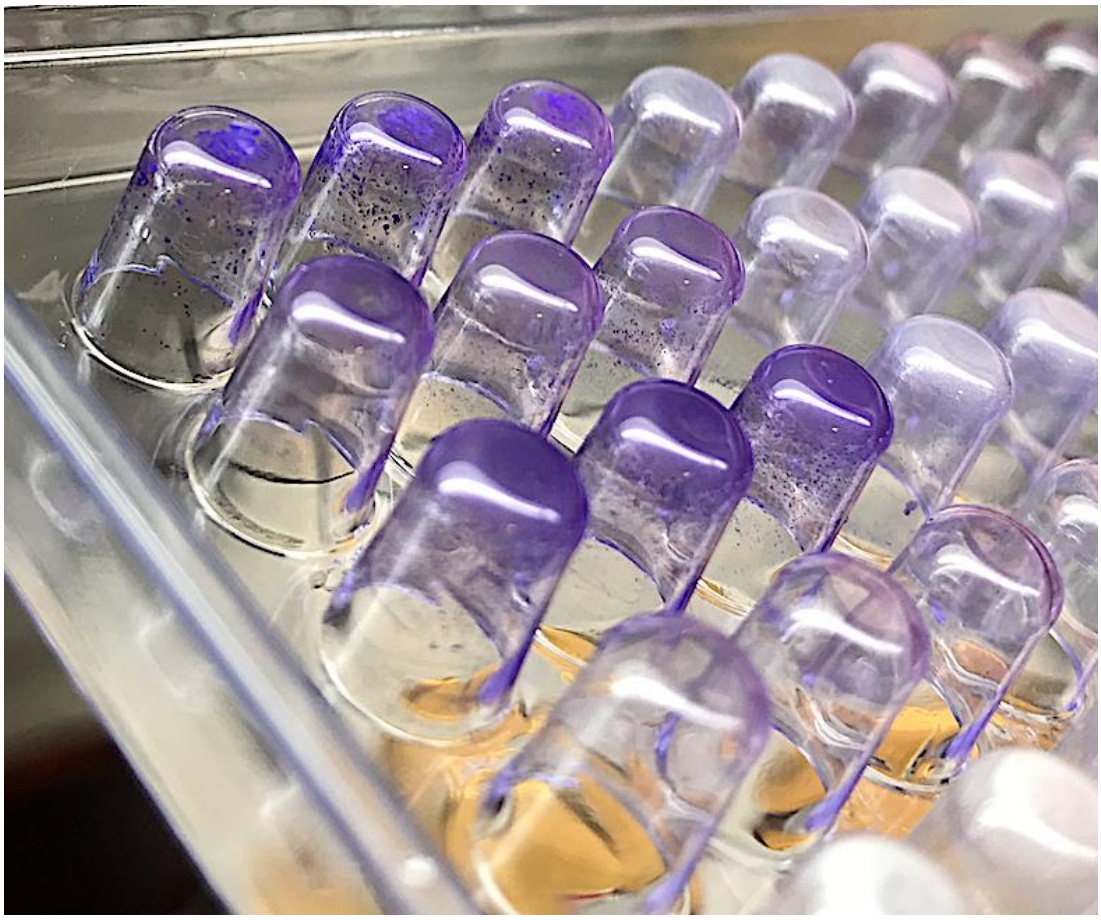

Figure IV-2: Crystal violet stained biofilms in microplate wells. In triplicate from (top to bottom) in lager (PL3), stout (ST1), keg ale (PKA1) and cask ale (SC1).

4.3.3. Growth of biofilms

The work focussed on four brands/styles - lager (PL3), stout (ST1), keg ale (PKA1) and cask ale (SC1) (Figures IV-3, IV-4, IV-5, IV-6, IV-9, IV-10). Biofilm formation by four additional beer brands and a cider were also evaluated (Figures IV-7 and 8). All tested beers and cider produced quantifiable biofilm. In these experiments, with nine draught products, neither beer style nor ABV (range 3.5-5%) had any apparent impact on biofilm formation. It is noteworthy that appreciable biofilm formation was demonstrated with cider microflora. With a lower pH and added sulphur dioxide, cider has a reputation in the trade for being more microbiologically robust. In some accounts, this results in the line cleaning of cider brands being less regular than for beers. This work suggests that line cleaning of ciders should be at the same frequency as beers.

4.3.4. Reproducibility

As noted above, the use of microplates to screen biofilm formation has its limitations. Accordingly, the reproducibility of biofilm formation was assessed directly on draught samples for each beer style in 48 wells in four microplates (Figure IV-3). A one-way ANOVA of biofilm – measured as A_{570} values – showed no significant difference (p>0.05) within each beer style. The mean value (± sem, n = 48) for each style was: lager 0.083 ± 0.013, ale 0.140 ± 0.026, cask ale 0.080 ± 0.011 with stout creating the most biofilm with an A_{570} value of 0.165 ± 0.030. The variation (sem) was consistent at 16.5 ± 1.6%, irrespective of beer style or degree of biofilm formation.

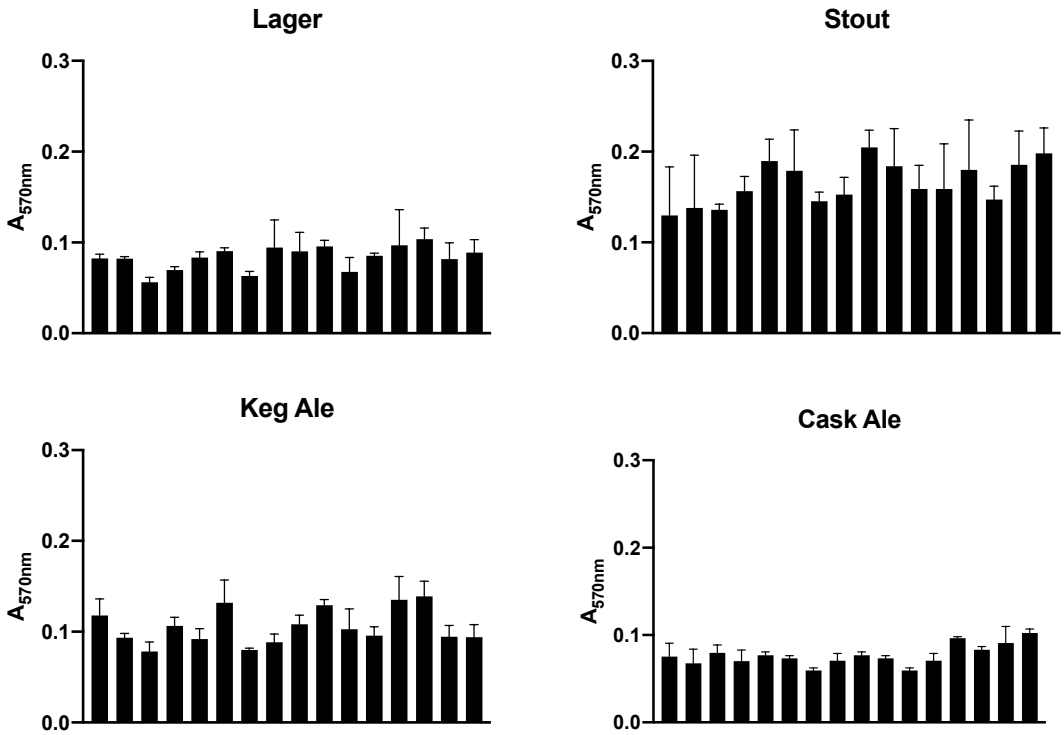

Figure IV-3: Reproducibility of the biofilm screening assay across 16 groups of three for lager (PL3), stout (ST1), keg ale (PKA1) and cask ale (SC1).

4.3.5. Nutrient replenishment

The effect of nutrient replenishment on biofilm formation was assessed in lager (PL3), stout (ST1), keg ale (PKA1) and cask ale (SC1) (Figure IV-4). Nutrients were added via addition of the parent beer. A similar approach was used by Maifreni *et al.* (2015) with bacterial biofilms of beer spoilage bacteria where the rich nutrient broth was refreshed every two days over a seven day period. In addition to nutrient replenishment, there is the possibility of oxygen pick up. Here, replenishment was made every three days over a period of 21 days. Biofilm was also determined in parallel in control plates without nutrient replenishment.

Nutrient replenishment increased biofilm formation in all four beer styles suggesting nutrient limitation. The difference between incremental biofilm formation between 3 and 21 days between the control and microplates with replenishment was greatest with keg ale (Δ A_{570} = 0.2077) compared to cask ale (0.0979), lager (0.0790) and stout (0.0625). The rate (Δ A_{570}/day) of biofilm formation for the four beer styles was greater with replenishment (0.010 $\pm$ 0.003) compared to the control (0.003 $\pm$ 0.002). The increase in rate was higher with keg ale (Δ A_{570}/day, 0.014 v 0.003) than with cask ale (0.008 v 0.002) and stout (0.06 v 0.001). The increase in rate of biofilm formation in lager was smaller as the rate in the control (Δ A_{570}/day 0.007) was markedly higher than in the other beers which minimised the impact of replenishment (0.010). Replenishment was significantly different (p<0.05) to the control for stout, keg ale, cask ale but not for lager (p>0.05), suggesting that nutrient limitation in lager PL3 was less of an issue than for the other three styles.

This approach demonstrates the enhancement of biofilm formation by the regular replenishment of fresh beer. However, it cannot replicate the variable environment in a dispense line which reflects the unique trading pattern of the account. The biofilm will experience peaks and troughs in exposure to fresh beer which will depend of the frequency and duration of dispense. This will vary by tap/line within the account, together with factors including day of the week, time of the day, season, number of taps, consumer demographic, brand popularity etc. Low or no throughput (e.g., out of hours) would be anticipated to support biofilm attachment. Overlaid on this is the impact of flow through beer dispense. Conrad and Poling-Skutvik (2018) have identified three ways in which shear flow can impact on bacterial biofilms ranging from enhancement, through to structural and mechanical change to removal of cells, clusters and biofilms. It is assumed that these responses occur in draught beer biofilms and contribute to the planktonic loading of microorganisms in dispensed beer. Shear flow will vary with the dispense line internal diameter coupled with dispense speed which broadly ranges from delivery of a pint in 12 to 22 seconds and a corresponding flow rate of 2.8-1.6 L/min.

As is well recognised, beer is an inhospitable environment to microorganisms with compositional hurdles that together create a cumulative antimicrobial effect (Dysvik *et al.*, 2020a). One of these, the depleted nutrient status of beer, impacts on the vulnerability of different beers to spoilage (Quain & Jevons, in preparation). Accordingly, replenishment may be more effective in those beers with comparatively more nutrients (amino acids, sugars etc) than others. This may explain why replenishment was most effective in keg ale (Figure IV-4), a style which, in an extensive trade audit (Mallett and Quain, 2019), was found to have poorer quality than

draught lager. In this work, of 149 samples of standard lager, 44% were in the ‘excellent’ quality band compared with 16% of 88 samples of keg ale (Mallett and Quain, 2019).

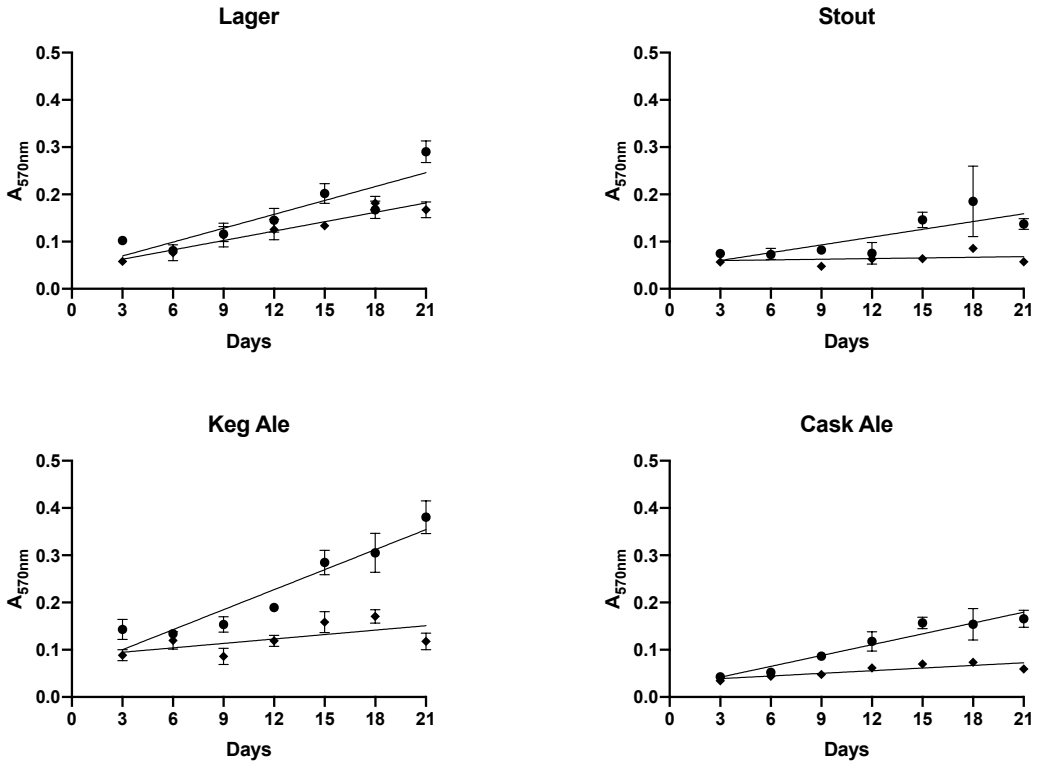

Figure IV-4: Impact of nutrient replenishment on biofilm formation in lager (PL3), stout (ST1), keg ale (PKA1) and cask ale (SC1) where (■) control and (●) replenished.

4.3.6. Impact of temperature on biofilm formation

An initial experiment was performed with eight draught beers and one cider sampled weekly over a three-week period. Biofilm formation over seven days was compared with microplates incubated at 15 and 30ºC. Across the nine draught brands, there was no statistical difference (p>0.05) in biofilm formation at the two temperatures with an average A_{570} at 15ºC of 0.118 ± 0.035 and 0.109 ± 0.035 at 30ºC. However, the inoculum was 'as is' and not adjusted. Accordingly, the extent of biofilm formation was dependent on the microbial loading which over a three-week period is likely to have fluctuated.

A second more detailed experiment considered the impact of temperature at 7.5, 15, 20, 25 and 30°C on biofilm formation with a controlled inoculum from the four beer styles with nutrient replenishment every three days. Figure IV-5 shows biofilm formation by lager (PL3), stout (ST1), keg ale (PKA1) and cask ale (SC1) over 21 days. Between 7.5 and 30°C, biofilm formation was consistently greatest with keg ale. However, between 7.5 and 15ºC, biofilm formation was significantly higher (p<0.05) at 7.5ºC for lager and stout but not for keg ale. Conversely, for cask ale, biofilm formation was significantly higher (p<0.05) at 15 ºC compared to 7.5ºC and 20ºC compared to 15ºC.

The rate of biofilm formation (ΔA_{570}/day) for each beer style at the five temperatures is reported in Figure IV-6. However, the rate of biofilm formation declined with temperature, notably with lager PL3 (gradient = - 0.0008) but also directionally with keg ale (PKA1) (- 0.0004) and stout (ST1) (- 0.0002). Conversely, the microflora in

cask ale (SC1) responded to increasing temperature by increasing the rate (gradient = + 0.0003) of biofilm attachment.

Biofilm formation by microorganisms is typically assessed at 25-37ºC (Table IV-2), reflecting the experimental context (environment, microorganism) together with the convenience of accelerating microbial growth and attachment. Such a temperature range was inappropriate for draught beer microflora as the specifications for dispense temperature in the UK range from 2-6ºC (extra cold category) through 4-6ºC (lager), 4-8ºC (stout), 6-12ºC (ale) to 11-14ºC (cask). These are indicative temperatures for beer dispense as 'in glass' temperature can vary with cooling capacity (in both the cellar and the bar), line location in the chilled python, volume throughput, retailer temperature specification and technical dispense issues.

The impact of temperature on biofilm formation has had a 'mixed press' with an array of publications reporting an increase, decrease or no change. This reflects the diversity of reports together with the experimental complexity of microflora (single v mixed, laboratory v environmental) and media (laboratory v environmental, minimal, rich, with or without replenishment etc). In this work, it is notable that – with the keg beer styles – biofilm attachment broadly declines with increasing temperature. This is consistent with the selection by each beer style of environmental microflora that are adapted to exploit the beer and associated dispense conditions. Of the four styles (Figure IV-6), the dispense temperature of lager is coldest and biofilm formation is the most compromised by increasing temperature. Although the data is less convincing, biofilm formation by keg ale and, to a lesser extent, stout microflora declines with increasing temperature. Conversely, biofilm formation by cask beer

microflora increases with increasing temperature. This is in keeping with cask beer storage and dispense temperatures (11-14^{o}C), which would suggest the selection of microorganisms that form biofilm at warmer temperatures. These results suggest that – together with beer composition - dispense temperature is a factor that determines the environmental microflora to spoil beer.

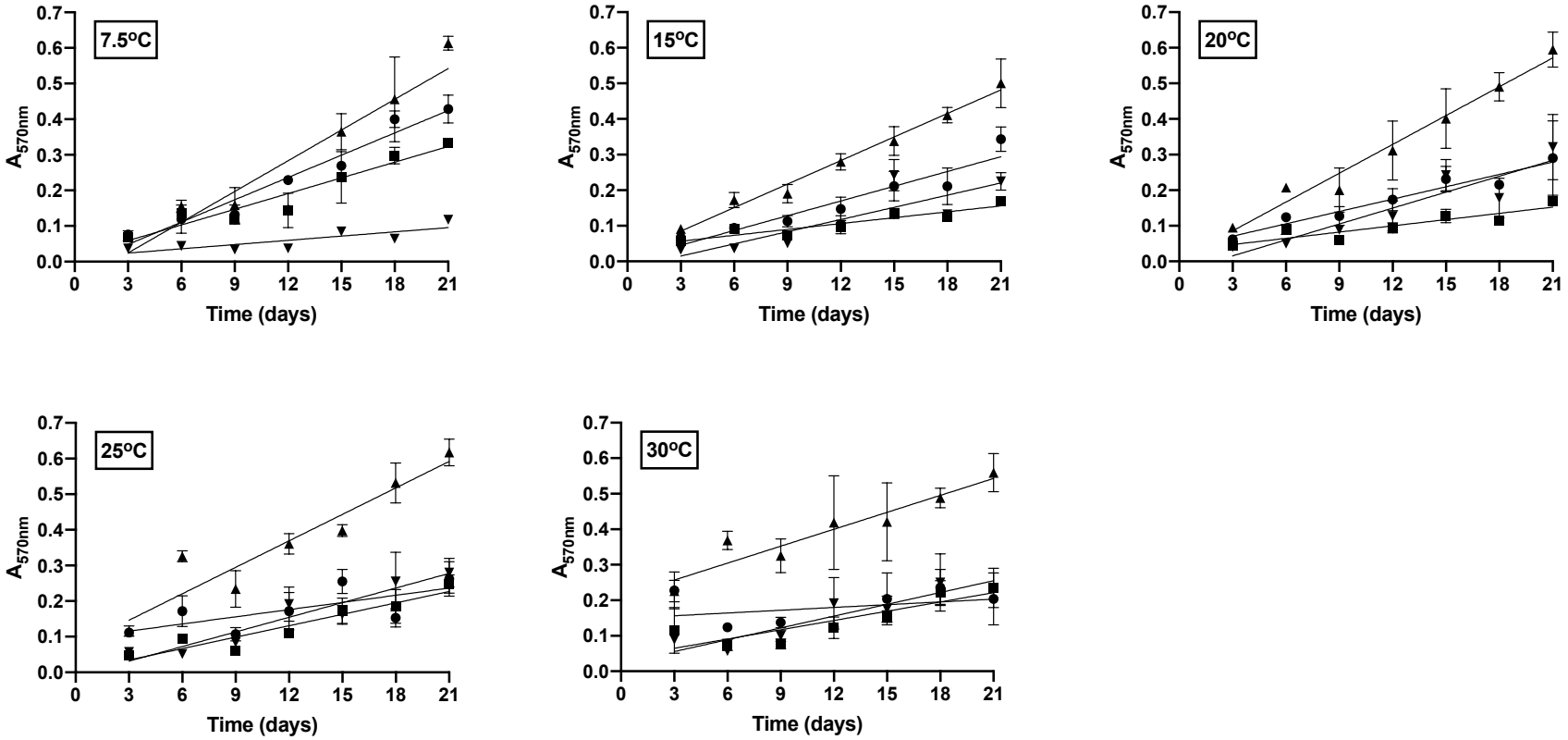

Figure IV-5**:** Impact of time and temperature on biofilm formation were quantified in lager (PL3) (–●–), stout (ST1) (–■–), keg ale (PKA1) (–▲–) and cask ale (SC1) (–▼–) across 5 temperatures.

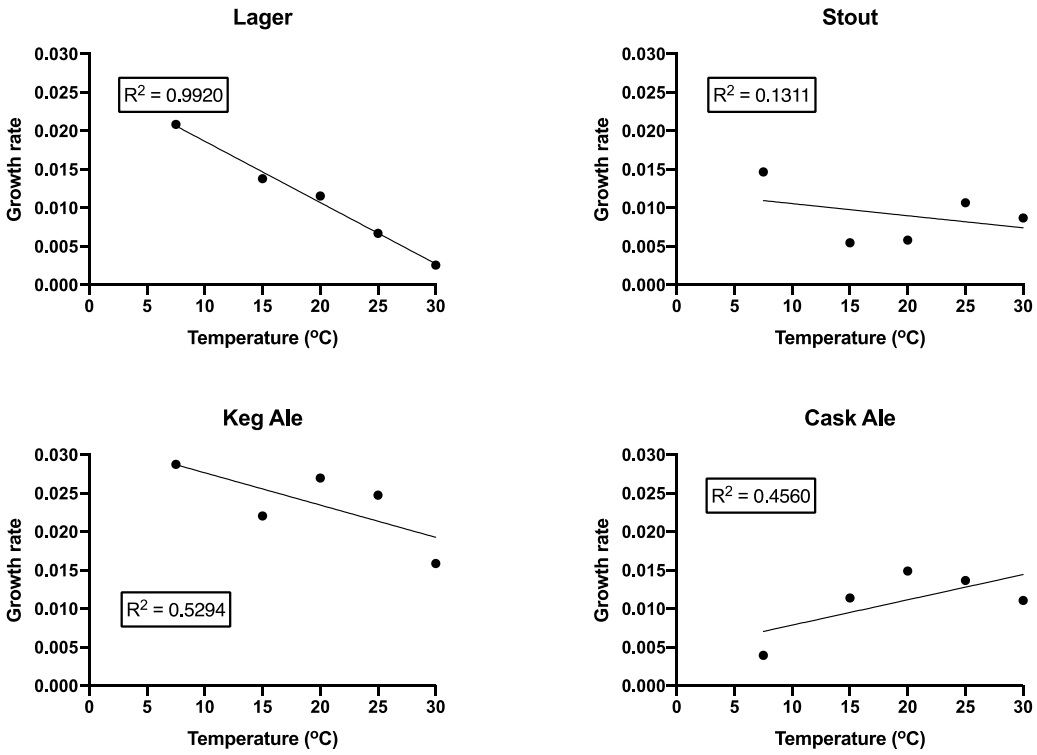

Figure IV-6: Growth rate was plotted from the gradient of the slope for style and temperature in Figure IV-5.

4.3.7. Impact of oxygen

For kegged beers and cider (SL6, SL10, KA6, PL3, PKA1, ST1 and C2), there was no significant difference between aerobic and anaerobic conditions ($p>0.05$) (Figure IV-7). However, the biofilm formed by the microflora from two cask ales (SC1, SC4) was significantly different ($p<0.05$) greater aerobically than anaerobically.

Cask beer or 'real ale' is a traditional beer style in the UK (O'neill, 2010) and is markedly different to keg beer in being unpasteurised with a secondary fermentation in cask. Typically, on dispense, the beer is pulled (rather than pushed by a top pressure gas) using a hand pull (or 'beer engine'). On tapping, the shelf life of cask beer should be no more than two days reflecting the deleterious impact on beer quality of microbiologically unclean air being drawn into the cask. Accordingly, as noted above, aerobic bacteria such *Acetobacter* and *Gluconobacter* are the predominant microflora in cask beer (Boulton and Quain, 2013). This would explain the significant increase in biofilm formation in the two cask beers when grown with greater access to oxygen. Conversely, to assure flavour stability, keg beers are packaged to minimise oxygen ingress (Bamforth, 2020) to less than 0.025 mg/L (https://www.hach.com/appnote-measure-DO-brewery). Although gas permeation (oxygen in, carbon dioxide out) is recognised in different grades of dispense tubing (https://www.micromatic.com/5-16-inch-id-barriermaster-flavourlock-tubing-549BF), it is insufficient to select for a more aerobic microflora. Accordingly, as reported in Figure IV-7, the microflora in the biofilm of draught keg products (lager, ale, stout and cider) was not significantly enhanced by the availability of oxygen.

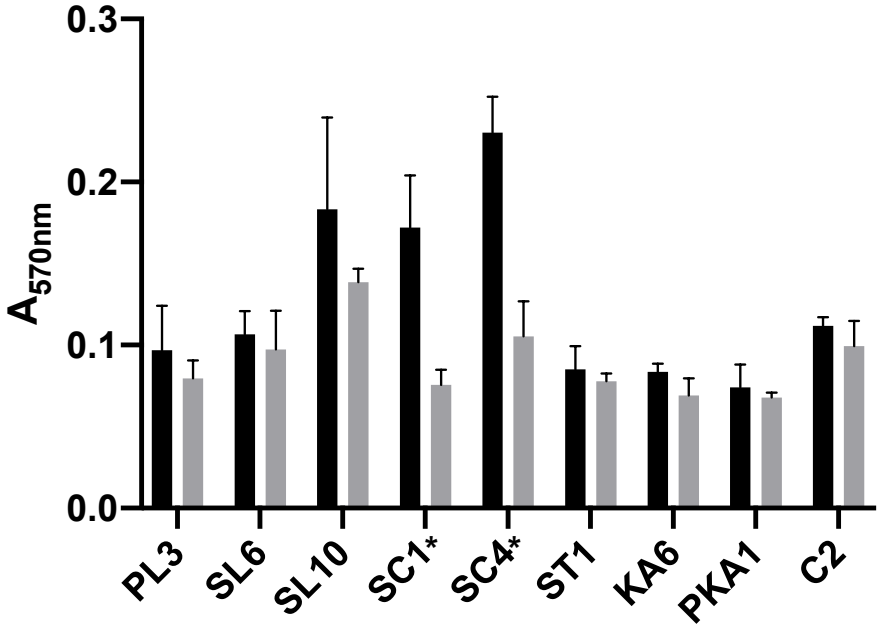

Figure IV-7: Biofilm formation was quantified under (■) aerobic and (■) anaerobic conditions at 15°C (n=3, ± SD). *Significant difference between aerobic and anaerobic biofilm formation.

4.3.8. Microplate material

Typically, polystyrene microplates are used in biofilm assessment. Here, the attachment of draught beer microflora to polystyrene was compared to polyvinyl chloride (PVC, vinyl). Although, no significant difference (p>0.05) in biofilm formation was found (Figure IV-8), it was directionally greater in seven (of the nine) products when grown in polystyrene plates.

Although counselled by choice of test microflora and experimental conditions, it is a telling observation that 'there is hardly any material that does not allow biofilm formation' (Meyer, 2003). Whilst glass and stainless steel are more resilient, plastics are more susceptible to biofilm attachment reflecting, in part, the leaching of nutrients (Rogers *et al.*, 1994). Beer dispense tubing ranges from medium density polythene (MDP, polyethylene) to more premium nylon lined MDP which can be further co-extruded with a barrier layer to minimise loss of carbon dioxide or ingress of oxygen.

Microplates are predominately made of polystyrene which is not used in beer dispense. Polyvinyl chloride tubing is used for the delivery of dispense gases but – apart from short runs as restrictor tubing - has been superseded by MDP. It is noteworthy that the Brewers Association (Association, 2017) in the USA recommend that 'vinyl tubing should be replaced every one to two years, because it is relatively porous and susceptible to bacterial and flavour contamination'. Despite this reservation, PVC microplates were used in this work as a more relevant material for dispense studies than polystyrene.

In addition, to the choice of material, the state of the tubing surface is an important variable in biofilm attachment. Dispense lines are used for many years and the internal surface becomes damaged - over time – by repeated cycles of line cleaning. Other variables include the line composition/barrier layer, line-cleaning frequency and efficacy together with the number of protracted high strength ‘bottoming out’ cleans. Accordingly, distressing the surface of the microplate wells by varying degrees of abrasion and assessing biofilm attachment by draught beer microflora would be of interest. Conversely, applying a slippery, anti-adhesive surface to the well with assessment of biofilm attachment would potentially reveal opportunities for the development of dispense tubing, which is less supportive of biofilm formation.

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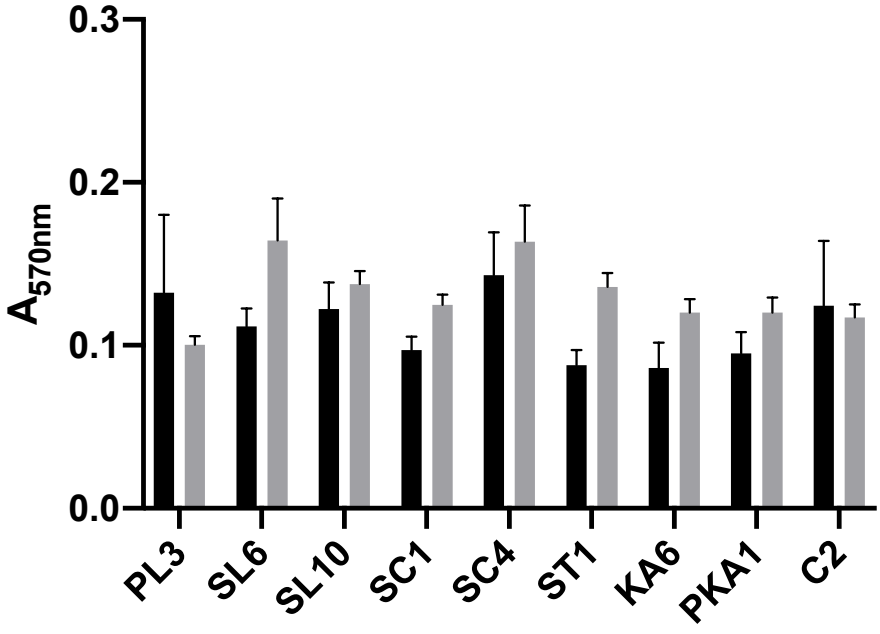

Figure IV-8: Biofilm formation was quantified with two materials, (■) polyvinyl chloride (PVC) and (■) polystyrene (PS) (n=3, ± SD).

4.3.9. Line cleaning

Biofilms from draught beer of lager (PL3), stout (ST1), keg ale (PKA1) and cask ale (SC1) were grown on microplates for between 3 and 21 days. Biofilms were subject to small scale, static line cleaning with Pipeline Original (2.5%, v/v) for 30 minutes and then washed with water. After this process, parent beer was added to wells and replenished after three days. After a total incubation time of seven days, the combined growth of resuspended sessile and planktonic cells was determined at A_{660}.

The line cleaning process was unsuccessful as regrowth of the microflora was demonstrated – to varying degrees - with each beer style (Figure IV-9). The extent of microbial regrowth varied, being greater with lager (PL3) and cask ale (SC1) but less convincing with stout (ST1) and keg ale (PKA1). This suggests that soaking a biofilm with proprietary line cleaning solution neither penetrates nor kills all the microflora embedded in the biofilms. Further, there was evidence, notably with the lager and cask ale, that the line cleaning process was increasingly ineffective with older biofilms. Whilst a model, experimental system, this insight is relevant to the on-trade, where the recommended frequency of line cleaning (every seven days) (BBPA) is extended in practice to every two or three weeks. This is of significance, as insight from a UK retailer (Quain, 2007) shows that account profitability (as volume growth) is demonstrable (+ 2%) with weekly line cleaning, break even at two weeks but negative (-2%) with cleaning between two to four weeks and increasingly negative thereafter.

Line cleaning of draught beer lines in the UK is predominately a passive process with limited mechanic action. Line cleaning, other than a pull through at halfway, is a static process. Conversely, in the USA, the recommended best practice is a pumped

recirculation of the line cleaning solution for at least 15 minutes during cleaning (BBPA, 2017). The two approaches to line cleaning would be anticipated to achieve different outcomes, analogous to the impact of soaking soiled crockery compared to using a dish washer.

Generically, effective cleaning in the food industry and beyond, is achieved through a combination of temperature, chemical action, time, and mechanical action. These four parameters are known as the Sinner's circle after the chemical engineer who first identified their interaction in 1959 (Basso *et al.*, 2017). The model suggests that although all four parameters are required for cleaning to take place, one can be reduced (in this case, mechanical action) and this can be compensated by changes in the three other parameters (time, temperature and chemical action/detergent strength). Accordingly, the absence of any significant mechanical action in UK line cleaning would be expected to compromise its efficacy as the microbial biofilm will be subject to little or no shear and will not be removed from the line surface. Although the caustic detergent may kill some of the microorganisms in the biofilm, sufficient viable microflora will remain in the line and regrow when beer dispense resumes.

This method of screening biofilm formation enables a structured approach to the evaluation of line cleaning and, in particular, the elements of the Sinner's circle. Indeed, it would be of interest to assess whether time, temperature or detergent strength can compensate for the limited mechanical action of UK line cleaning. However, any outcomes would be tempered by the practical constraints of increasing time and temperature together with increasing chemical damage to the internal surface of the line. More directly, the impact of mechanical action on the removal of draught

beer biofilms could be addressed by lateral or rotary agitation of microplates for different time intervals.

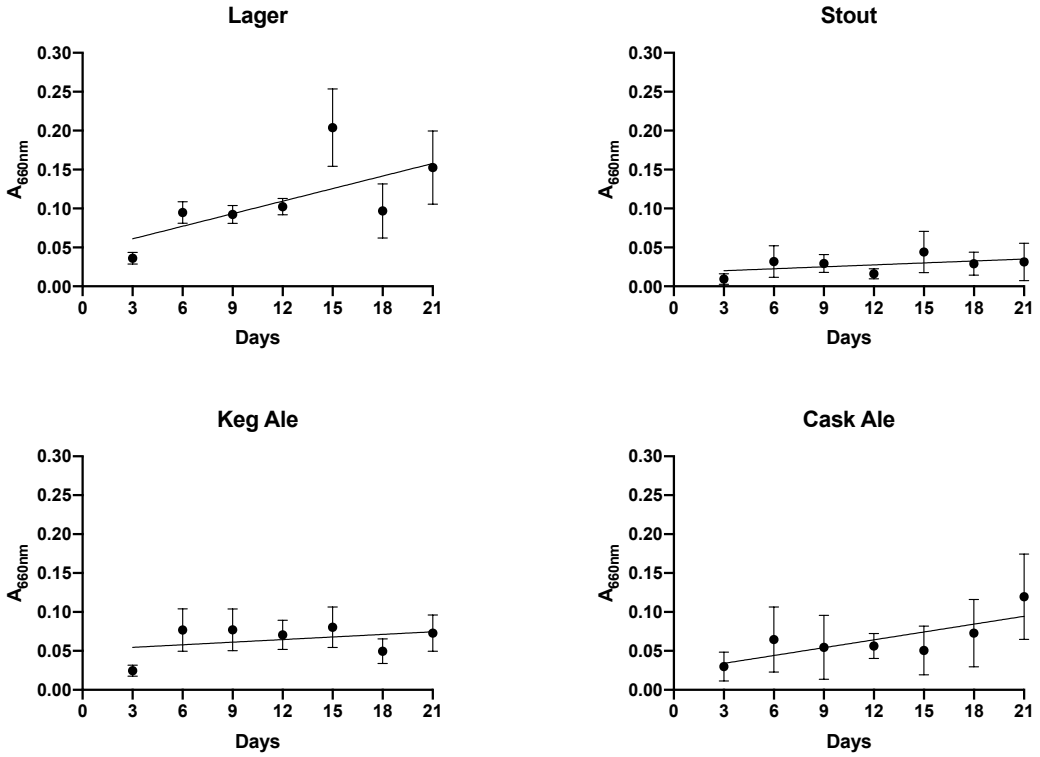

Figure IV-9: Regrowth of biofilm after 30 minutes line cleaning was quantified every 3 days, with replenishment, over 21-day period (n=3, ± SD). Total regrowth of planktonic and sessile microflora (A_{660}) was quantified as a measure of line cleaner efficacy.

4.3.10. Line cleaning time and biofilm regrowth

In a further experiment, the exposure to Pipeline line cleaning solution was flexed between five and forty minutes with seven-day old biofilms with PL3, ST1, PKA1 and SC1 (Figure IV-10). Further, the post cleaning water wash was delivered as a single five-minute wash or as two 2.5-minute washes, although there was no significant difference in outcome ($p>0.05$). However, it is noteworthy that in all four beer styles there was a peak in resuspended sessile and planktonic cells after 35-40 minutes of exposure to line cleaning solution.

Line cleaning was equally effective whether exposure was as little as five minutes or as long as thirty minutes. Curiously, regrowth was enhanced with exposure to line cleaning solution for 35 or 40 minutes. This response occurred in all four beer styles and irrespective of a single or double washing with water (Figure IV-10). Biofilms are highly heterogeneous in terms of genotype, phenotype and physiology with individual microbial species not compartmentalised in layers (Stewart and Franklin, 2008). Similar to results reported in Figure IV-9, exposure to line cleaning solution did not completely kill the biofilm and microorganisms regrew. This is of interest as the microflora (genera, mix) in the biofilms would be different in each of the four beer styles, possibly reflecting different extracellular polysaccharides and biofilm structure. However, the response was consistent across the four styles and, it is suggested, that treatment with line cleaning solution for 35-40 minutes exposed part of the biofilm with the capability - on replenishment – for enhanced microbial growth.

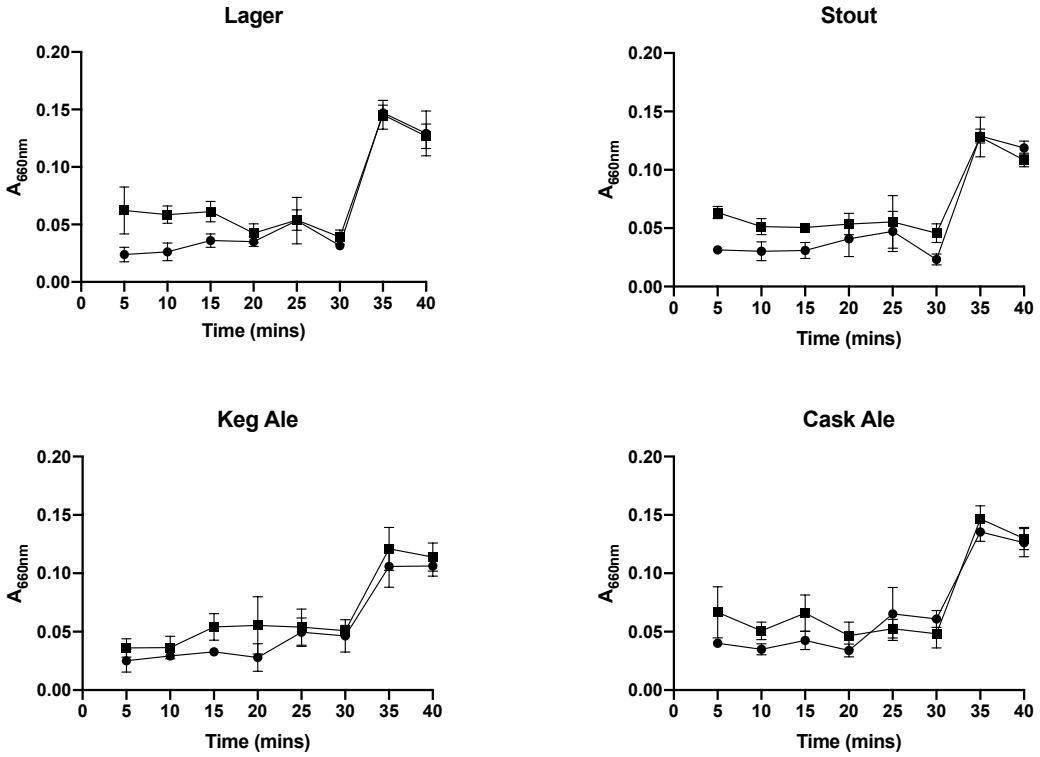

Figure IV-10: Effectiveness of line cleaning versus established biofilms 5 – 40 minutes (n=3, ± SD) with washing once (–●–) or twice (–■–) post cleaning. Total regrowth of planktonic and sessile microflora (A_{660}) was quantified as a measure of line cleaner efficacy.

4.3.11. Impact of temperature and mechanical action on line cleaning effectiveness

Vlkova *et al.* (2008) explain for a sanitation regime to be deemed effective 3 key conditions must be met, '1) efficient sanitation agent (lowest concentration, minimum time) 2) low demand on energy and work 3) least damage to the environment and surfaces.'. The 'Sinner Circle' dictates four factors are essential for efficient sanitation; chemical agent, time, temperature and mechanical power (Wirtanen and Salo, 2003). Most industries are applying these fundamental practices to ensure an effective sanitation regime. However, during beer dispense two factors are not considered, temperature or mechanical action. A standard line clean at a retailer will rinse the line with water, soak in caustic statically for up to 30 minutes and chase with water again until the sanitation agent is completely removed. All of which is conducted at dispense temperatures (<15^{o}C), to compare the temperature for closed dairy systems is between 75^{o}C and 80^{o}C (Vlkova *et al.*, 2008).

Figure IV-11 applies increased the temperature of the line cleaning caustic to between 7.5^{o}C to 55^{o}C. At lower temperatures there is a negligible impact on cleaning, with variable response on regrowth. Thus, implying low temperatures are less effective at biofilm removal. However, at 55^{o}C there is <0.02 change in OD_{660} after 7-days of incubation. Although the practicality of this in the trade cannot be assessed, it highlights the potential of introducing temperature into standard line cleaning practice. Furthermore, introduction of mechanical irrespective of style was found to significantly improve line cleaning effectiveness (Figure IV-12). Lager, stout, and keg ale experience >50% reduction in microorganism recovery, whereas cask ale was slightly higher but still significantly reduced (p<0.05).

The lack of mechanical action and heat would in other industries render current dispense line-cleaning practices ineffective (Mallett and Quain, 2019). Largely this has already been proven by the high rate of spoilt beer being served. Typically, a beer line cleaning agent is over pH 12 and is extremely potent. Excessive use can significantly damage lines and shorten the lifespan of the system. Consequently it has been noted that rougher surface can enhance biofilm formation, Teughels *et al.* (2006) demonstrated a rougher surface retains more biofilm when measuring thickness, area and colony forming units. Here we have demonstrated the impact of introducing these elements to standard line cleaning practices using an *in vitro* methodology, however, the practicality of these elements at the on-trade and collectively cannot be evaluated.

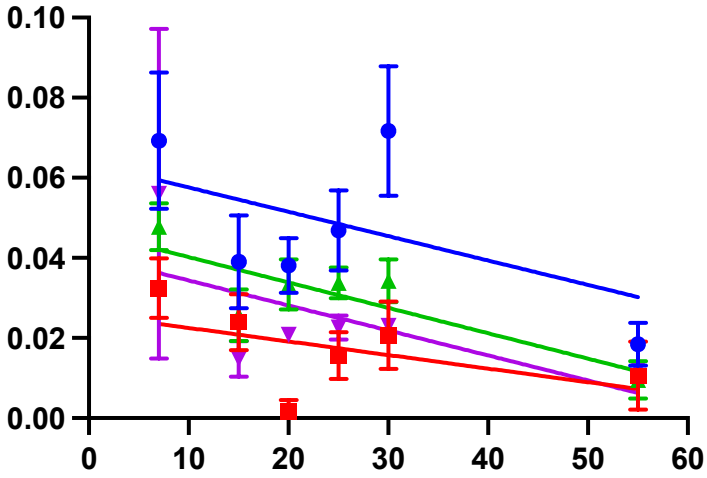

Figure IV-11: Effectiveness of line cleaning solution at a range of temperatures between 7.5°C and 55°C (n=6, ± SD). The impact of increased temperatures was assessed in four beer styles SL3 (blue), ST1 (red), PKA1 (green), and SC1 (purple). Increased line cleaning temperature did not have an impact between 7.5°C to 30°C. At 55°C microorganism recovery is significantly reduced (p<0.05).

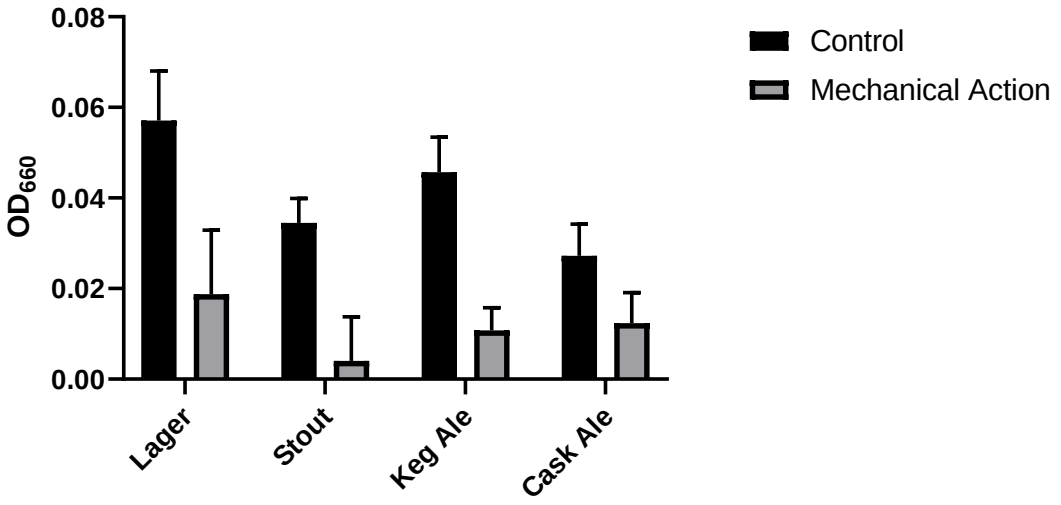

Figure IV-12: Impact of introducing mechanical action into line cleaning. For each sample there is a significant reduction in microorganism recovery versus a static control after a 7-day post-line clean incubation period (p>0.05) (n=6. ± SD)

4.4. Conclusion

The method reported here will enable biofilms formed by draught beer microflora to be assessed in a convenient and cost-effective way. In addition to evaluating the impact of the parameters of the Sinner's circle, the method will allow comparison of line cleaning solutions, surface finish, biofilm age together with the sessile and planktonic microbiome. The method will help focus attention on the efficacy of line cleaning with the ultimate intention of improving draught beer quality in the UK and elsewhere.

Chapter V: Identifying key components of beers influencing susceptibility to beer spoilage microorganisms

5.1 Introduction

The draught beer market has been in decline since the mid-1970s, with poor quality one of many factors that are responsible (Mallett and Quain, 2019). In response to this, international brewers such as Heineken (and others) are proactively managing the hygiene of dispense lines in their public houses by introducing 'Smart dispense' together with technicians to clean the lines every six weeks. The smart dispense system refrigerates the line from keg to tap, which reduces growth of spoilage microorganisms, enabling a reduction in frequency of cleaning, and, accordingly, reducing wastage. However, by introducing limiting but not fatal conditions will apply a new selective pressure, and encourage the prevalence of other, better-adapted microorganisms. The relationship and temperature for biofilm growth has already been revealed, identifying how lager sourced biofilms optimally mature at low temperatures, whereas cask ale perform best at room temperature (Jevons and Quain, 2021).

Standard beers will contain 4-5 % ABV, low pH, low nutrition, low oxygen together with anti-microbial hop bitter acids. These parameters render beer a relatively robust beverage, and safe from the growth of pathogens. Despite these inhospitable conditions, numerous beer spoilage microorganisms persist. This is an example of the innate adaptability of microorganisms, which in the most extreme circumstances allow bacteria and yeast to survive. Beer is not an extreme environment, and still contains sufficient nutrition to support spoilage. A beer in small pack (can or bottle), is described as 'commercially sterile' with low levels of microorganisms (< 1 cfu/L) which will not cause a spoilage 'event' over the shelf life of the product. Further, and more importantly, the taste, flavour, and aroma of the product meets the requirements

of the brewer. However, for draught beer where spoilage microorganisms are present, there can be notable differences in the organoleptic properties compared to its bottled counterpart. This may convince the consumer to either change beer brand, bar, or opt for bottled/canned beer. The microorganisms responsible for this are well adapted to the conditions of beer and the dispense method.

Research into draught beer microbiology is minimal, with much of our knowledge derived from work in the mid to late 20th Century. For example, in 1954 work by Louis Hemmons conducted a survey of wild yeasts in cask beers in the on-trade, finding a wide range of wild yeasts including *Candida* and *Pichia* sp. (Hemmons, 1954, Hemmons, 1955). Other work by Wiles (1949) and Ault (1965) are examples of literature on draught beer microbiology. Wiles (1950) identified a range of wild yeasts in beer, included a range of *Candida* sp. and *Pichia* sp. in agreement with Hemmons (1954) and Hemmons (1955). Work by Ault (1965) references the susceptibility of cask ales to acetic acid bacteria, post-conditioning. More recently, there have been an increasing number of publications related to draught beer microbiology investigating the quality and spoilage of beer, (Mallett and Quain, 2019, Mallett *et al.*, 2018, Quain, 2007, Quain, 2015), and biofilms in dispense lines (Jevons and Quain, 2021). It is clear that draught beer is a complex mix of yeast and bacteria, and the style of the beer or even brand, alongside hygiene management, can have a profound impact on the spoilability and microbiome of a beer. The style, brand and dispense method of beer (lager, stout, keg ale, cask ale etc.) determine the genus/species that will dominate the spoilage microflora. Bacteria (*Lactobacillus* sp. (Suzuki, 2011), *Acetobacter* sp. (Garofalo *et al.*, 2015), *Gluconobacter* sp. (Sakamoto and Konings, 2003)) and yeast (*Brettanomyces* sp. (Gilliland, 1961), *Saccharomyces* sp. (Fleet, 1992), *Rhodotorula*

sp. (Hemmons, 1954)) predominate in spoilt beer. It has yet to be investigated what environmental niches of beer and its dispense are driving these differences. From our own prior work, differences in 'spoilability' between ten lager beers are clear, with some being more difficult to spoil than others.

Rainbow (1952) reported the more attenuated a beer is, the less potential there is for bacteria to spoil beer. Attenuation refers to the conversion of sugars into ethanol and carbon dioxide during fermentation. For a lager, there can be up to 30 g/L of total carbohydrates, of which 1 – 7 g/L are fermentables (Ferreira, 2009). Yet, lager beers, which are largely attenuated continue to spoil at significant rates, suggesting there are other nutritional elements available to beer spoilage microorganisms. Sugar is a vital carbon source; the breakdown of sugar (glycolysis) is a universal pathway across all living organisms (Fothergill-Gilmore and Michels, 1993). Through glycolysis, microorganisms are able to synthesise adenosine-5-trisphosphate (ATP), the energy 'currency' of living cells (Peretó, 2011), which is required for maintaining cellular homeostasis and cellular growth. For example, hop-resistance of LAB species is achieved through the removal of H^+ ions from the intracellular matrix to maintain the pH levels. This process is mediated by a multi-drug transporter *horA*, which is an energy-dependent process. For it to function, ATP is dephosphorylated to ADP to provide the energy to remove excess H^+ ions. This example of hop resistance for potent beer spoilers to be effective; ATP production is an essential prerequisite. This may suggest the ATP content or energy potential of a beer may correlate with its spoilability.

The fermentation of Belgian Lambic ales may offer an insight to the spoilage of beers, as there is a correlation between microorganisms responsible for Lambic fermentation and those which spoil beers. Our previous work has identified several correlations between the metagenomic analysis and cultured microflora, with those seen found in the Lambic ale literature. As described by De Roos *et al.* (2020) Lambic ales go through a series of phases where different microorganisms predominate and then are succeeded by others. This is related to changes in sugars, organic acids, pH and ethanol. De Roos *et al.* (2020) reports that early phases include growth of *Enterobacter* sp., *Pediococcus* sp., and *Acetobacter* sp., which are thought to be responsible for the reduction of malic to lactic acid. *Brettanomyces* sp. can thrive in low pH environments and can use a variety of 'exotic' carbohydrates and produce high ethanol concentrations (Passoth *et al.*, 2007). Their significance in Belgian Lambic beers is well documented (De Roos *et al.*, 2020), but typically only become significant during the maturation phase, which is driven by acidification of the medium and high ethanol levels. Ferulic acid, hydrocinnamic111 acids, and *p*-coumaric acid are known to be key substrates of *Brettanomyces* sp. spoilage or fermentation. Although not directly related to energy production, increased presence of weak acids was found to trigger the increased concentration of enzymes related to the TCA cycle (Carmona *et al.*, 2016).

Lactic acid bacteria are considered the most prevalent beer spoilage microorganism (Suzuki *et al.*, 2004, Suzuki *et al.*, 2006). The use of malic and citric acids is widespread in LAB species (De Figueroa *et al.*, 2000, Kennes *et al.*, 1991, Passos *et al.*, 2003). Heterofermentative lactic acid carbohydrate metabolism is diverse, capable of using sucrose, ribose, and oligosaccharides (Geissler *et al.*, 2016), further organic

acids (pyruvate, malate, citrate) can be used to resist acid stress via the arginine deaminase pathway, which both creates ATP and increases intracellular pH (Geissler *et al.*, 2016). In situations where the cells are energised and pyruvate has accumulated intracellularly, the cell will convert pyruvate to acetoin, which contributes to maintaining cellular homeostasis (Tsau *et al.*, 1992). In heterofermentative (hef) LAB, this is induced through depleting carbon sources, often at the end of fermentation.

During fermentation, yeast converts sugars to pyruvic acid, which is further reduced to produce ethanol and CO_2. Pyruvic acid is a central molecule in energy production, it links both glycolysis and the Krebs cycle. Pyruvic acid is known to be a suitable carbon source for microorganisms. There are over 200 organic acids in beer (Buiatti, 2009), providing sour flavour notes and acidity, which contributes to the inhibition of spoilage microorganisms, contributing to beer's robustness (Montanari *et al.*, 1999, Charalambous, 1981, De Stefano and Montanari, 1996). Those acids involved in the Kreb's cycle (pyruvic, citric, malic, succinic acid) are possibly key substrates. Energy production by LAB species has been shown by Suzuki *et al.* (2005), where pyruvate, citrate and malate were all readily assimilated resulting in significant ATP increases.

Predictive models have been produced in an attempt to predict the spoilability of a beer (Fernandez and Simpson, 1995). Fernandez and Simpson (1995) use various *Lactobacillus* strains and challenged them in a range of beers, where SO_2, hop bitter acids, polyphenol, maltotriose, free amino nitrogen and colour were quantified. Although this work was able to predict the spoilability of beer, using a single species and strain does not represent the real-world or enable the mutualisms between species and/or kingdoms. Screening methods have shown ATP as an accurate indicator to

predict low-level spoilage in soft drinks, removing the need for conventional microbiological methods (Littel and Rocco, 1986).

The vast majority of work on beer spoilage focuses on a single genera or species and then extrapolates the data to all beer spoilage microorganisms. But as beer spoilage microbiology is a complex mix of yeast and bacteria, influenced by brand, style, and dispense, there are likely numerous mutualisms. Manipulating/reducing the susceptibility to spoilage of beer spoilage rate may in the future prove an important consideration for determining a beers suitability to draught dispense. In this work, the ability of microorganisms sourced from draught beer to metabolise a range of exogenous sugars, quantify sugars in lagers and ales, track organic acid concentrations and seek to relate these insights to the spoilage of the beer.

5.2 Methods and Materials

5.2.1 Sample collection

Beer samples were collected from public houses across Merseyside, Nottinghamshire, and Leicestershire in sterile 250mL Duran bottles.

5.2.2 Forcing

The microbiological quality of draught beer *ex* dispense was determined – in triplicate - using the forcing method as described by Mallet *et al.* (2018). Beer (25 mL) in 30 mL universal tubes was incubated at 30^{o}C for 96 hours. After mixing, the absorbance at 660nm was determined at 0 and 96h using a Jenway 7315 spectrophotometer. Cycloheximide (4mg/L) was added to cask beer before forcing to inhibit the growth of primary yeast. At 0, 24, 48, 72 and 96 hours a 5mL aliquot of forced sample was

collected after inverting 3-4 times. Aliquots were stored at -80°C until they were prepared for LC-MS analysis. Samples used as inoculum source were stored at 4°C for no more than 1 week or used directly after completion.

5.2.2.1. Quality index

A 'quality index' was calculated from the sum of the individual scores for each quality band (where A = 4, B = 3, C = 2, D = 1) divided by (number of samples x 4) x 100.

$$\text{Quality index (\%)} = \frac{\Sigma\, quality\ score}{number\ of\ samples\ x\ 4} \text{ x } 100$$

If all samples are measured as excellent (quality band A), the quality index is 100% whereas if all samples are in quality band B (acceptable) the index is 75%.

5.2.3 Spoilage screen

The spoilage susceptibility of 10 commercial lager samples was determined by inoculating the microorganisms sourced from four styles of beer lager (SL3), stout (ST1), keg ale (KA1), and cask ale (SC1); and forcing them at 30°C for 96 hours. Four of the beers were 'standard lager' (SL1, 3, 5 and 6) with an abv of 4-4.1% and six beers were 'premium lager' (PL1, 2, 3, 6, 8 and 9) at 4.5-5.1% abv. The beers were produced by five global brewing companies and were packaged in either bottle or can.

To standardise the method, an aliquot of beer ex-forcing equivalent to A_{660} = 1 (e.g. 4 mL at Δ A_{660} = 0.25, 2 mL at Δ A_{660} = 0.5) was diluted with sterile water to a final volume of 5 mL. From this, 0.1 mL (A_{660} = 0.02) of brand specific spoilage

microorganisms were inoculated into the 10 brands (25 mL pasteurized beer) in duplicate, forced at 30°C for 96 h and the ΔA_{660} measured.

5.2.4 Free amino nitrogen (FAN) quantification

Free amino nitrogen (FAN) was quantified using the 'Primary Amino Nitrogen Assay Kit' (PANOPA) by Megazyme (K-PANOPA) https://www.megazyme.com/primary-amino-nitrogen-assay-kit. The commercial beers 'challenged' in section 5.2.3 were quantified for FAN.

5.2.5 LC-MS sample preparation and method

Aliquots of each time point were centrifuged at 10,000 x g for 5 mins to remove microorganisms. The supernatant was transferred to a sterile 1.5ml Eppendorf tube. 100µL of the sample (n=3) was added to 350µL of LC-MS grade methanol. Mixture was vortexed and subsequently centrifuged at 18,000 x *g* for 15 minutes at 4^{o}C. 75µL aliquots were dried in a vacuum centrifuge and stored at -80^{o}C until required for LC-MS analysis. Quality controls were prepared in the same way using pooled beer samples. Further, extraction blanks of water were also conducted. Upon analysis, samples were resuspended with 40µL of water. Data acquisition was conducted as described by Wright Muelas *et al.* (2020)

5.2.6 LC-MS data analysis

Data files (.raw) were imported into the open-source LC-MS software MzMine v2.6. Mass detection was run for each raw data file and chromatograms. Chromatograms were deconvoluted and deisotoped, isolating peaks and reducing noise. Duplicate peaks were removed, and retention times were calibrated. The feature lists containing processed peaks were identified against several databases (below). Possible identification was restricted to the 10 best matches from each database.

- Human metabolome database
- Yeast metabolome database
- Lipidmaps
- KEGG
- PubChem

5.2.7 Sugar profiles of beers using high performance anion-exchange chromatography

Four styles of beer – standard lager (SL3), stout (ST), premium keg ale (PKA1) and cask ale (SC1) were inoculated with microorganisms sourced from either SL3 or ST1 collected and forced from the on-trade. Time points of each beer during forcing were taken to track change in sugar profiles. For example, time 0 (n=3) forcings were stored at -80^{o}C until required for analysis. The time points recorded were 0 hr, 24 hr, 48 hr, 72 hr and 96 hr. Before storage, forcings were spun at 10,000 x *g* for 5 minutes, with no temperature control, to remove microorganisms with the supernatant transferred to a sterile 2mL tube.

For sugar profiling, 100µL of forced beer was suspended in 9.9 mL sodium hydroxide (10mM) prepared in ultra-distilled water. Tubes was inverted 2-3 times to ensure an equal distribution of the sample. Approximately 2mL of the solution was filtered through a 0.45µm filter into HPLC vials. The concentration of glucose, maltose and maltotriose in each sample were determined using High Performance Anion-Exchange Chromatography (HPAEC). The instrument used was a Dionex ICS 6000 instrument utilising a CarboPac PA210 column (250mm x 4mm) and guard (50mm x 4mm). The mobile phase of the HPAEC was composed of four eluents at varying ratios and were all prepared using ultrapure water obtained using a Suez Select Fusion ultrapure water

deionisation unit which had been degassed under vacuum and with a sonicating water bath. The four eluents were 1) 100% H_2O, 2) 100 mM NaOH, 3) 200 mM NaOH and 4) 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 is used with a push-partial injection mode, with 2.5 µL of sample used per injection.

The chromatography method, summarising flow rate, buffer composition and time duration are summarised in Table V-1.

Table V-1: High performance anion-exchange chromatography parameters

Time / min	Flow rate / ml min^{-1}	% Eluent 1 (H_2O)	% Eluent 2 (100 mM NaOH)	% Eluent 3 (200 mM NaOH)	% Eluent 4 (100 mM NaOH + 500 mM NaOAc)
-10.0	0.2	99	1	0	0
0.0*	0.2	99	1	0	0
8.0	0.2	99	1	0	0
13.0	0.2	0	0	70	30
19.0	0.2	0	25	0	75
22.0	0.2	0	25	0	75
22.1	0.2	0	0	100	0
27.0	0.2	0	0	100	0

*Time of sample injection

For each of the three sugars, a linear calibration was established using the following stock solutions: 2.00, 1.50, 1.00, 0.75, 0.50, 0.25, 0.10, 0.05 mg ml^{-1}. Samples were diluted at a 1:100 ratio in 10 mM NaOH (100 µL in 10 mL total volume) and agitated using a bench top vortex. Samples are then filtered through a 0.22 µm filter into a glass vial with split septum cap and placed into the autosampler of the Dionex instrument, equilibrated to 4°C. The fitting parameters of the linear calibration as well as the R^2 value, limits of detection (LOD) and quantitation (LOQ) were calculated using Excel and are summarised in Table V-2.

Table V-215: Standards and detection limits used for fermentable quantification

	Glucose	Maltose	Maltotriose
Value (m)	2.7340	6.1920	3.6670
Value (b)	0.0745	0.8806	0.1690
R^2	0.9989	0.9986	0.9968
LOD	0.0836	0.0918	0.1400
LOQ	0.2534	0.2782	0.4241

5.2.8 Sugar use by different beer style microflora

Trade samples from the same four styles of beer (as above) were taken from an account in Leicestershire. PL3, ST1, PKA1 and SC2 were force aged and used as inoculum . Forced samples were measured at OD_{660} and were appropriately diluted to 0.1 OD_{660}, these were then used to inoculate into 96-well plates at a final OD of 0.01 OD_{660}. The microorganisms were assessed for growth on a range of sugars in a modified-YPD media. YPD typically contains 1% yeast extract, 2% peptone, and 2% glucose but here the glucose was replaced with either fructose, maltose, sucrose, or maltotriose. The concentration equate to the number of glucose molecules. For example, 100mM glucose (monosaccharide), 50mM maltose (disaccharide), 33mM maltotriose (trisaccharide). See Table V-3.

Table V-3: Concentration of each sugar added to modified-YPD medium

Sugar	**Concentration (mM)**			
Glucose	100	50	25	12.5
Fructose	100	50	25	12.5
Maltose	50	25	12.5	6.25
Sucrose	50	25	12.5	6.25
Maltotriose	33	16.67	8.33	4.167

5.2.9 Spoilage susceptibility with different sugars

Beers of the 'lager spoilage screen' from section 5.2.3 were supplemented with a range of sugars at multiple concentrations (Table V-4). In 96-well plate format ten lager beers were supplemented with glucose, fructose, maltose, sucrose, or maltotriose. A forced sample of PL3 was used as inoculum, added at a final concentration of 0.01 OD_{660}. The OD_{660} of the wells was measured every 30 mins for 96 hrs. The growth rate and final OD_{660} was quantified for each beer, each sugar and concentration. These data were correlated with the sugar profiles of the beers and other antimicrobial factors in beer.

Table V-4: Concentrations of each sugar added to beer

Sugar	SL1	PL8	SL3	PL9	SL5	SL6	PL1	PL2	PL3	PL6
Glucose	10mM, 1mM, 100µM, 10µM									
Fructose	10mM, 1mM, 100µM, 10µM									
Maltose	5mM, 0.5mM, 50µM, 5µM									
Sucrose	5mM, 0.5mM, 50µM, 5µM									
Maltotriose	3.3mM, 0.33mM, 33µM, 3.3µM									

5.2.10 Pyruvic acid quantification

Pyruvic acid was quantified using the Megazyme pyruvic acid assay procedure (https://www.megazyme.com/pyruvic-acid-assay-kit) using the 'manual assay'.

5.2.11 Organic acid growth curves

The spoilage of eight beers was investigated by the addition of pyruvic, citric, and lactic acid to the following beers - PL8, SL3, PL9, PL3, ST1, PKA1, KA1, SC1, and SC2. In a total volume of 200µL, the inoculum was a forced PL3 culture added at a

final OD_{660} of 0.01. Organic acid impact on spoilage was determined by assessing impact over a logarithmic scale of concentrations; 100nM, 1µM, 10µM, 100µM, 1mM, 10mM and 100mM. Samples were incubated in a 96-well plate reader for 96 hours with absorbance measured every 30 minutes at 660nm.

5.2.12 Data analysis

All statistical analysis was conducted using GraphPad Prism v9.3.1.

5.3 Results

5.3.1 Spoilage screen comparison

Ten lagers were inoculated with microorganisms sourced from on-trade, from four different styles of beer. Each was forced (n=2) in 30 mL universal tubes, as according to the method published by Mallett *et al.* (2018). Despite the inoculum, it is clear when microorganisms were inoculated into sample PL9 microbial growth is poor, suggesting there exists an innate resistance to spoilage (Figure V-1). Whereas PL8 exhibited a susceptibility to spoilage. Average OD_{660} for PL9 (0.235) and PL8 (0.606) present an approximate 3-fold difference (Table V-3). Interestingly, microorganisms sourced from lager were less effective 'spoilers' than those from other styles. Microorganisms sourced from cask ale demonstrated potent spoilage of all brands compared to keg ale, stout, or lager. For each beer, the average OD_{660} for cask ale (0.609) was 27% higher than keg ale - the next most potent beer spoiler (Table V-5).

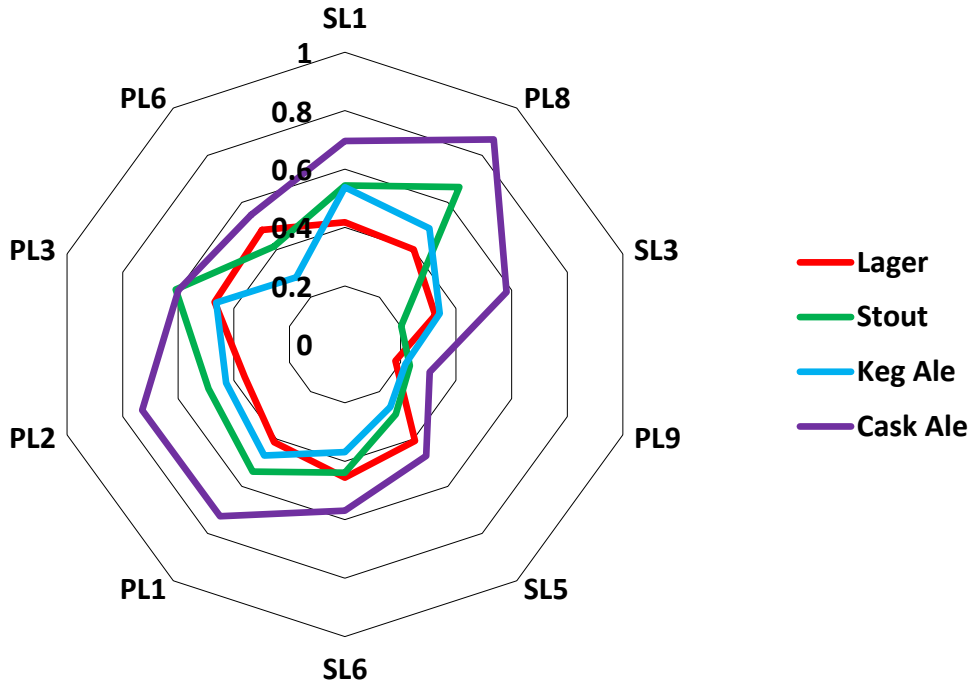

Figure V-1: Radar plot of spoilage screen beers challenged with microorganisms sourced from the on-trade from four different styles of beer. Irrespective of the source of the inoculum, PL9 demonstrated a greater robustness to spoilage, whereas PL8 was more susceptable from microorganisms from Stout and Cask Ale. Cask ale sourced microorganisms were potent spoilers of beer irrespective of the brand

Table V-5: Ten lager beers were inoculated with microorganisms sourced from on-trade samples of four different beer styles. Each was forced for 96 hrs at 30°C and optical density at 660nm was measured. [a]Most susceptible beer. [b]Least susceptible beer. [c]Most potent spoilage microorganism source

	Inoculum from				
Beer	**Lager**	**Stout**	**Keg Ale**	**Cask Ale**	**Average**
SL1	0.418	0.544	0.538	0.696	0.549
PL8	0.401	0.666	0.491	0.8671	0.606[a]
SL3	0.325	0.203	0.341	0.581	0.363
PL9	0.182	0.234	0.217	0.305	0.235[b]
SL5	0.408	0.296	0.264	0.4724	0.360
SL6	0.456	0.439	0.368	0.569	0.458
PL1	0.413	0.538	0.47	0.727	0.537
PL2	0.361	0.491	0.428	0.729	0.502
PL3	0.469	0.609	0.463	0.599	0.535
PL6	0.484	0.414	0.284	0.547	0.432
Average	0.392	0.443	0.386	0.609[c]	

5.3.2 Free amino nitrogen (FAN) v pH v OD_{660}

FAN was quantified in relation to the pH and spoilage 'response' was measured using OD_{660} after 96 hrs. PL8, PL1, PL3, PL2, and SL1 exhibited the highest concentrations of FAN (Figure V-2; Table V-6). The spoilage susceptibility disparity has previously been noted between PL9 (least spoilable) and PL8 (most spoilable) in Figure V-1. FAN during the brewing process is understood to be a reliable indicator for yeast potential growth and fermentation efficiency (Taylor and Boyd, 1986). PL8 (180 mg/L) presented the highest concentration of ten commercial lagers. PL1, PL2, PL3 and PL8 contained the highest concentrations of FAN, and this correlated with increased 'spoilability' (Figure V-2).

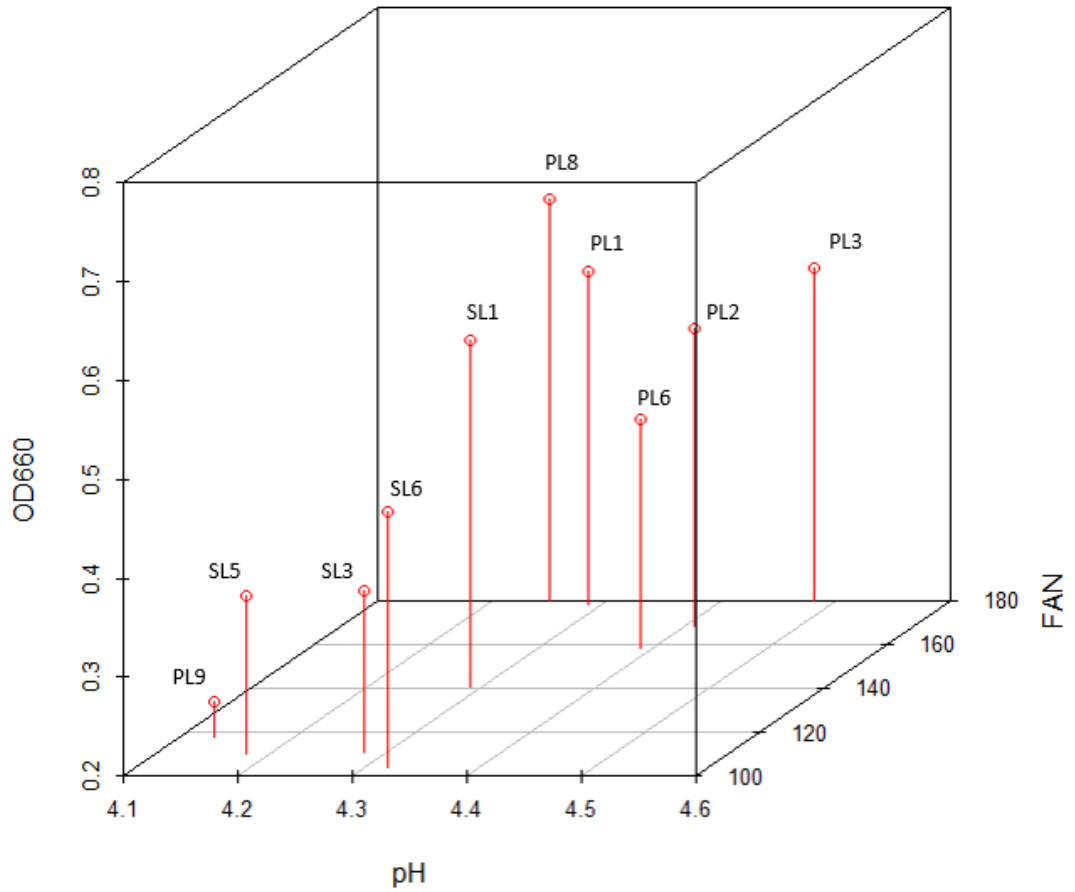

Figure V-2: 3D plot presenting the relationship between FAN (mg/L), pH and spoilage. Each beer brand was inoculated with lager-sourced microflora to a final concentration of 0.01 OD_{660}

Table V-6: Details of Figure V-2

Beer	pH	FAN (mg/L)	OD_{660}
SL1	4.29	141	0.549
PL8	4.35	180	0.606
SL3	4.28	111	0.363
PL9	4.13	118	0.235
SL5	4.18	110	0.36
SL6	4.32	104	0.458
PL1	4.29	178	0.537
PL2	4.41	168	0.502
PL3	4.48	180	0.535
PL6	4.38	158	0.432

5.3.3 Addition of sugars to spoilage screen lager beers

Various sugars were added to the ten lager beers (Table V-7). The sugars were added at a concentration to match the number of glucose molecules; for example, if 100mM of glucose was added to one experiment, the equivalent experiment for maltose would be 50mM. The addition of these sugars was to determine the response of lager sourced microorganisms to the addition of fermentable sugars. Metrics including final OD, growth rate, sugar concentrations, pH and % ABV were recorded. Further, three sugars (glucose, maltose, maltotriose) were quantified in each of the ten beers by comparing to a standard curve (Figure V-2).

PL8 contained the highest concentration of the three sugars with a total of 9.07 mg/mL (Table V-6). Maltose was over 2mg/mL higher in PL8 (6.41mg/mL) to the next closest beer (PL3) (Table V-8). After 96 hours PL8 recorded the highest OD_{660} after supplementation with glucose, fructose, and maltose (Table V-7). Further, PL8 (and SL3) exhibited the highest growth rate of the ten beers when supplemented with glucose, fructose, or maltose (Table V-7). Optimal growth was found after fructose supplementation (0.8 ± 0.031), although total growth on maltose was the highest amongst the other 9 beers (0.703 ± 0.018). PL8 has regularly been an easy beer to 'spoil' when compared to beers of the spoilage screen. Finding high residual fermentables amidst a more favourable pH and high FAN are likely key factors influencing PL8's 'spoilability'.

In contrast to PL8, PL9 has been comparatively robust to contamination during spoilage analysis. The addition of glucose had a profound effect on final OD, with an increase of 0.623 ± 0.035. The second largest impact on OD versus an untreated

control, behind PL8 (Table V-7). Comparatively, fructose was the poorest performing supplement for PL9 versus the other nine beers. Maltose had the least impact on total growth (0.416 ± 0.005). Growth rate on fructose, maltose and maltotriose was the lowest amongst all beers (Table V-8). Juxtaposed to PL8, PL9 has the lowest pH of the 10 lagers whilst containing relatively low residual fermentables 5.92mg/mL and FAN (Table V-6).

Maltose addition was the least effective supplement for four of the ten beers (PL9 0.416 ± 0.005, SL5 0.322 ± 0.062, PL1 0.441 ± 0.016, PL2 0.304 ± 0.012) (Table V-7). Maltotriose was similarly poor, albeit only for three beers, (PL8 = 0.51 ± 0.019, PL2 = 0.452 ± 0.005 and PL6 = 0.407 ± 0.024) (Table V-7). Maltose was less effective at inducing spoilage than maltotriose, suggesting maltose is a selective pressure in beers for the microbiome phenotype.

Across all beers, interestingly glucose only proved to be the optimal supplement in PL9 (Table V-4). SL3, SL5, SL6 and PL1 each achieved their highest OD_{660} when supplemented with maltotriose (Table V-7). SL3, SL5 and SL6 at 4% ABV may imply low ABV supported the breakdown of complex sugars in beer (Table V-8). However, contrary to this PL1 (5% ABV) performed optimally despite a high ethanol concentration. Inferring other undefined factors or relationships are yet to be revealed influencing sugar metabolism (Table V-7).

SL3 performed optimally when supplemented with maltotriose (0.743 ± 0.075) (Table V-7) which was the highest of the beers – an increase of 0.129 compared to when SL3 was supplemented with glucose (0.614 ± 0.023) (Table V-7). SL3 is relatively low in

residual fermentables 4.98mg/mL (Table V-9) and with a pH of 4.28 and 4% ABV may offer a favourable environment for the assimilation of complex sugar(s). SL3 across all sugars performed better when supplemented with di- and trisaccharides than with monosaccharides (Table V-8).

A ‘spoilability’ metric was created to understand whether specific factors could be used to predict the spoilage of beer (Table V-9). For the beers of the spoilage screen, each of the measured parameters (pH, growth rate, FAN (mg/L), ABV %, and total residual fermentables) were ranked 1 to 10. How each was ranked is below:

- Lowest pH = 1, Highest pH = 10
- Highest % ABV = 1, Lowest % ABV = 10
- Lowest residual fermentables = 1, Highest residual fermentables = 10
- Lowest growth rate = 1, Highest growth rate = 10
- Lowest FAN = 1, Highest FAN = 10

Each of the beers in Table V-9 were ranked between 1 and 10 according to the above rules. The sum of the ranks dividing over the number of parameters (5) was defined as the ‘spoilability score’, which provided a crude measure of whether spoilability was predictable. The lowest average score would be predicted to be the least spoilable and vice versa. PL9 and PL8 are good controls to determine the accuracy of this methodology. In section 5.3.1, Table V-3, PL8 was recorded as the beer most susceptible to spoilage irrespective of source inoculum, whereas PL9 was the least susceptible. The spoilability metric accurately predicted this outcome, with PL8 scoring 8.4 (most spoilable), whereas PL9 was the lowest with 3.6 (Figure V-3; Table

V-7). The data from Figure V-1 was averaged per brand and compared to the spoilability score (Table V-7) in Figure V-4a. The 'spoilability score' was able to reasonably predict the outcomes of an unrelated experiment. Figure V-4a highlights how the metric was capable of predicting the 'extreme' ends of the spectrum (PL8, PL9 and SL5), however, is unable to produce the required clarity for the central values, represented by an R^2 value of 0.304 (Figure V-4b).

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Interestingly SL5 scored similarly low with 4 (Table V-9). Across all sugars SL5 was found to have the second lowest average total growth OD (0.461 ± 0.055), PL9 was third (0.472 ± 0.112). Using this simple metric, SL5 and PL9 were predicted as two of the least spoilable beers present in the screening method, which was corroborated by poor growth rate despite sugar additions. However, there remains other yet to be defined parameters and relationships in this investigation influencing the spoilability of a beer and would be better achieved using a more robust methodology.

	Glucose	Maltose	Maltotriose
R squared	0.9989	0.9986	0.9968

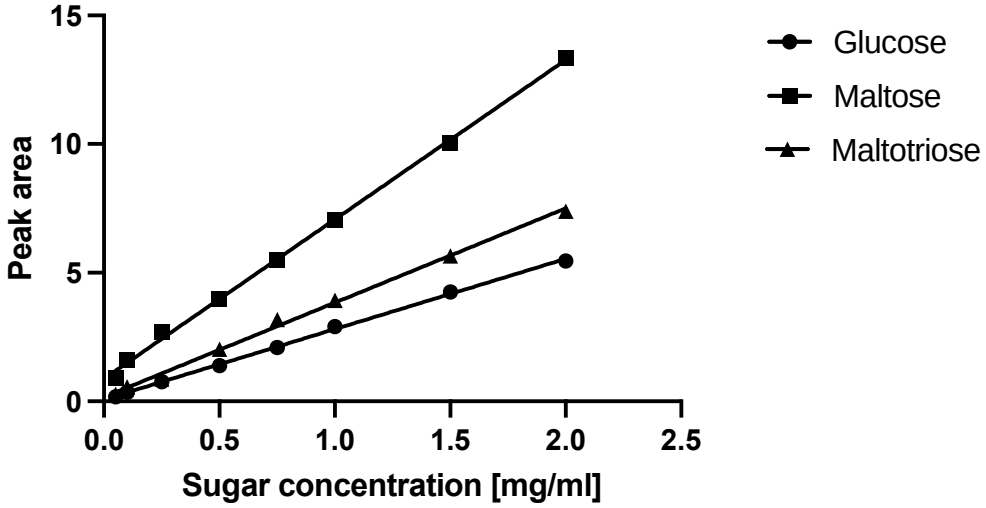

Figure V-2: Standard curve of glucose, maltose, and maltotriose (n=3)

Table V-7: Beers were supplemented with five sugars at concentrations equal to the number of glucose molecules. OD_{660} was measured after 96 hrs (n=3). Superscripts [a, b, c and d] are as follows. [a]Largest impact on growth by the sugar across all beers. [b]Lowest impact on growth by the sugar across all beers. [c]Largest impact on the individual beer across the five sugars used. [d]Lowest impact on the individual beer across the five sugars. In all cases the absorbance is after subtracting an untreated control.

	Sugar (concentration)					
Beer	**Glucose (10mM)**	**Fructose (10mM)**	**Maltose (5mM)**	**Sucrose (5mM)**	**Maltotriose (3mM)**	**Average OD_{660}**
SL1	0.543 ± 0.008[d]	0.717 ± 0.118[c]	0.614 ± 0.025	0.689 ± 0.035[a]	0.552 ± 0.043	0.623 ± 0.079
PL8	0.676 ± 0.031[a]	0.8 ±0. 031[ac]	0.703 ± 0.018[a]	0.63 ± 0.013	0.51 ± 0.019[d]	0.664 ± 0.106
SL3	0.614 ± 0.023	0.563 ± 0.062[d]	0.666 ± 0.046	0.613 ± 0.091	0.743 ± 0.075[ac]	0.64 ± 0.068
PL9	0.623 ± 0.035[c]	0.423 ± 0.002[b]	0.416 ± 0.005[d]	0.439 ± 0.04	0.46 ± 0.028	0.472 ± 0.112
SL5	0.542 ± 0.073	0.482 ± 0.049	0.322 ± 0.062[d]	0.374 ± 0.045[b]	0.587 ± 0.138[c]	0.461 ± 0.055
SL6	0.566 ± 0.023	0.495 ± 0.226	0.575 ± 0.048	0.494 ± 0.181[d]	0.622 ± 0.14[c]	0.55 ± 0.548
PL1	0.578 ± 0.089	0.525 ± 0.028	0.441 ± 0.016[d]	0.587 ± 0.025	0.611 ± 0.035[c]	0.548 ± 0.068
PL2	0.447 ± 0.043	0.459 ± 0.084	0.304 ± 0.012[bd]	0.489 ± 0.04[c]	0.426 ± 0.027	0.43 ± 0.071
PL3	0.564 ± 0.027	0.639 ± 0.073[c]	0.555 ± 0.09	0.562 ± 0.012	0.452 ± 0.005[d]	0.554 ± 0.067
PL6	0.437 ± 0.02[b]	0.444 ± 0.018	0.481 ± 0.079	0.623 ± 0.091[c]	0.407 ± 0.024[bd]	0.478 ± 0.085

Table V-8: Sugar concentrations of ten lagers. Growth rate was quantified on five sugars of increasing complexity and compared to sugar concentrations. [a]Fastest growth rate and [b]slowest growth rate on sugar across all beers

	Sugar concentration (mg/ml)			Growth rate				
Beer	**Glucose**	**Maltose**	**Maltotriose**	**Glucose**	**Fructose**	**Maltose**	**Sucrose**	**Maltotriose**
SL1	1.26	2.26	0	0.009	0.01	0.009	0.01[a]	0.009
PL8	2.22	6.41	0.44	0.01[a]	0.01[a]	0.01[a]	0.01	0.006
SL3	1.59	3.39	0	0.01	0.01	0.01	0.01	0.01[a]
PL9	2.10	3.82	0	0.007	0.006[b]	0.006	0.005[b]	0.005[b]
SL5	1.68	3.06	0	0.008	0.007	0.004[b]	0.005	0.005
SL6	2.46	4.13	0	0.007	0.007	0.008	0.007	0.009
PL1	2.27	4.12	0.035	0.008	0.007	0.006	0.008	0.009
PL2	1.69	3.29	0.028	0.006[b]	0.006	0.005	0.007	0.006
PL3	0.42	4.1	4.23	0.009	0.01	0.008	0.008	0.006
PL6	1.50	3.07	0	0.007	0.006	0.007	0.009	0.006

Table V-9: Average growth, total sugars, pH and ABV for each of the beers. A metric to measure spoilability within the ten beers was created to rank them within each other. [a]Score is measured between 1 to 10, where 1 is the least spoilable and 10 the most. By ranking each of the measured variables 1 – 10 and recording an average, a spoilability score could be calculated. [b]Average growth rate is calculated from Table I-5 for each beer across the five sugars.

Beer	**Average growth rate (± SD)[b]**	**Total quantified sugars (mg/ml)**	**FAN (mg/L)**	**pH**	**%ABV**	**Spoilability score[a] (maximum 10)** =
SL1	0.0093 (± 0.001)	3.53	141	4.29	4.1	4.6
PL8	0.0097 (± 0.002)	9.07	180	4.35	4.5	8.4
SL3	0.0097 (± 0.001)	4.98	111	4.28	4	5.6
PL9	0.0059 (± 0.001)	5.92	118	4.13	4.8	3.6
SL5	0.0059 (± 0.001)	4.74	110	4.18	4	4
SL6	0.0077 (± 0.001)	6.59	104	4.32	4	6.2
PL1	0.0077 (± 0.001)	6.39	178	4.29	5	4.6
PL2	0.0059 (± 0.001)	5	168	4.41	5.1	4.2
PL3	0.0081 (± 0.001)	8.65	180	4.48	5	6.2
PL6	0.0071 (± 0.001)	4.57	158	4.38	4.8	4.8

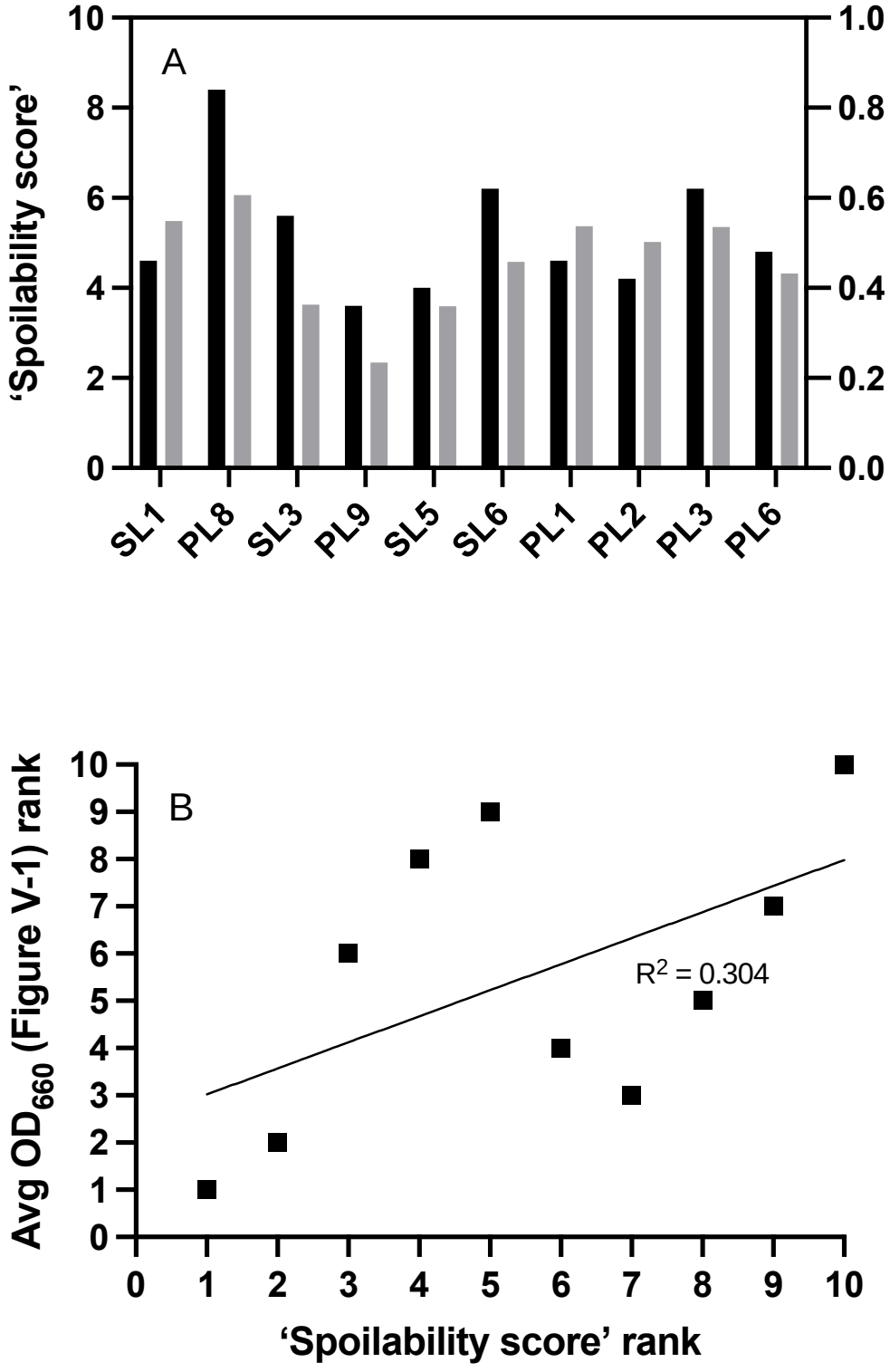

Figure V-4: (a) Spoilability score calculated using the five parameters in Table V-7 (black) and average OD_{660} for each inoculum from Figure V-1 (grey). (b) Each was ranked 1-10 with 1 = lowest OD/spoilability score and 10=highest.

5.3.4 Sugar profile changes over time during forcing

Samples of four beers (PL3, ST1, PKA1 and SC2) from a local account were forced and the concentration of glucose, maltose and maltotriose (mg/ml) monitored (Table V-10) periodically. Glucose was most abundant in PL3 (0.42 mg/ml) compared to the other beers. During forcing, glucose was extensively assimilated within 24 hours with a 76% utilised which was further reduced to 88% on day 4 (Figure V-5). The stout ST1 consumed only 28% of the glucose after 24 hours and 66% on day 4. In the premium keg ale (PKA1) and cask beer (SC2) glucose concentration remained consistent and is perhaps reflecting the release of glucose through the breakdown of dextrin.

Previously in section 5.3.2, maltose was suggested to be a difficult sugar to be assimilated, which was reinforced when maltose concentration was tracked during forcing. PL3, contained 4.1 mg/ml of maltose, a relatively high concentration compared to other lagers, which decreased by 19% after 4 days (Figure V-5, Table V-10). The remaining styles showed slight fluctuations in concentration but were not significant enough to show a trend. Maltotriose was the most complex sugar analysed. With PL3, the maltotriose concentration of 4.23 mg/ml was high and may lead to a selection of microorganisms suited to its assimilation. By day 4 maltotriose for PL3 had reduced by 16%, with slight fluctuation on day 3, suggesting some breakdown. Interestingly, PKA1 - the keg ale - often a ‘yeastier’ beverage, was able to utilise maltotriose. Over 4 days, the utilisation was linear ($R^2 = 0.95$) with near complete use of maltotriose. The initial concentration of 5.31 mg/ml maltotriose coupled with a low glucose concentration may have selected microflora capable of utilising more complex sugars.

Table V-10: Four beers inoculated with 0.05 OD of contaminants from forced 'parent' beers were analysed for glucose, maltose and maltotriose concentrations over the four days of forcing for four-days Further, the same beers were also quantified for residual fermentables prior to forcing.

Sugar (mg/ml)															
	Glucose					Maltose					Maltotriose				
Day	**0**	**1**	**2**	**3**	**4**	**0**	**1**	**2**	**3**	**4**	**0**	**1**	**2**	**3**	**4**
PL3	0.42	0.1	0.06	0.07	0.05	4.1	3.62	3.17	3.25	3.33	4.23	3.94	3.42	3.61	3.55
ST1	0.3	0.22	0.1	0.09	0.1	1.79	1.82	1.87	1.95	1.9	0.56	0.59	0.61	0.68	0.62
PKA1	0.16	0.13	0.16	0.14	0.14	1.74	1.59	2.41	2.49	2.39	5.31	3.51	2.45	1.47	1.76
SC2	0.1	0.11	0.14	0.15	0.15	1.15	0.98	1.26	1.3	1.25	1.5	1.35	1.66	1.76	1.52

Residual sugar (mg/mL)	**Lager (PL3)**	**Stout (ST1)**	**Keg Ale (PKA1)**	**Cask ale (SC2)**
Glucose	0.42	0.3	0.16	0.1
Maltose	4.1	1.79	1.74	1.15
Maltotriose	4.23	0.56	5.31	2.5

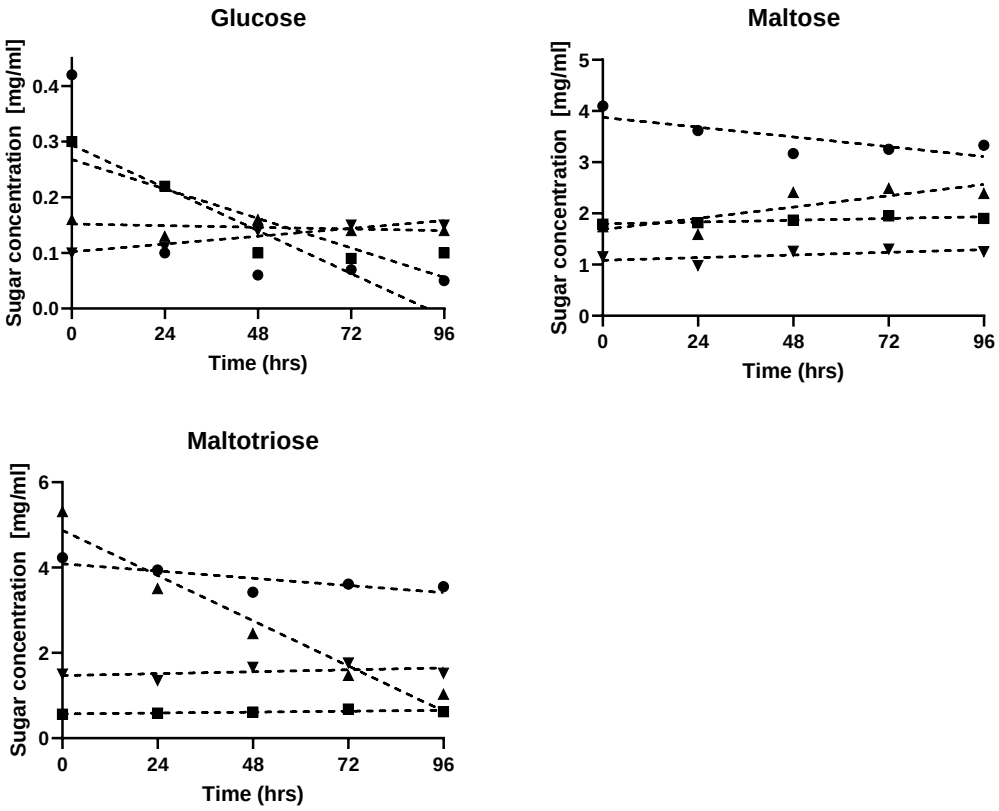

Figure V-5: Glucose, maltose, and maltotriose concentrations (mg/mL) over 96 hours in four styles of beer. PL3 (–●–), ST1 (–■–), PKA1 (–▲–) and SC2 (–▼–)

5.3.5 Sugar use by beer spoilage microorganisms

Microorganisms sourced from four beer styles, lager, stout, keg ale and cask ale were inoculated into a modified-YPD medium with glucose, fructose, maltose, sucrose, or maltotriose at a range of concentrations. Sugars were added at concentrations equal to the number of glucose molecules present to investigate the impact on microbial growth over a 96-hour period. Growth rate, length of growth phases (lag, exponential, and stationary) and final optical density were measured.

5.3.5.1 Influence of style on growth phases

Overall, microorganisms from lager exhibited the fastest rate of growth across each sugar, resulting in reduced lag phase (Figure V-6). Cask ale and stout were comparable to lager with increasing sugar complexity, but the growth rate decreased, and length of the lag phases increased. Keg ale exhibited the longest lag phase of the four styles, however, the microorganisms were less impacted by increasing sugar complexity, with the growth rate only slightly reduced. This was consistent with the microorganisms sourced from stout, keg ale and cask ale which showed no noticeable response to increased sugar complexity. However, the average lag phases of these styles (stout -23 hr, KA - 38 hr, CA - 32 hr) were higher compared to lager (6 hr). Further, but to a lesser extent, there was a difference between the average length of the exponential phase of growth with ale styles and stout (20 hr), keg ale (18 hr) and cask ale (16 hr) were 2-fold higher or more than lager (9 hr).

5.3.5.2 Growth rate

The exponential growth rate was calculated using the slope of a linear regression. Lager exhibited the clearest response to increased sugar complexity, using the growth rate (0.1107) for glucose as the reference point, a percentage change could be

calculated for the remaining sugars. Fructose (-15%), sucrose (-1.5%), maltose (-37%) and maltotriose (-49%) (Figure V-7). Maltose and maltotriose were least preferential sugars compared to glucose, fructose, and sucrose in lager. The uptake of glucose and fructose is a universal and a passive process, similarly sucrose is hydrolysed via cell-wall invertases and is also readily metabolised. Whereas maltose and maltotriose are initially transported and reduced, an ATP-dependent process, but this transport can be inhibited in the presence of glucose.

Ale samples did not exhibit this response to increasing sugar complexity. PL3 and SC3 experienced prolonged lag phases as sugar complexity increased, however, once the microflora had 'adapted' log/exponential phases exhibit no significant variation length (hrs) versus the simpler sugars. In the presence of maltose, only keg ale demonstrated no significant variance in growth rate when challenged with maltose – suggesting environment had driven selection for microorganisms adapted to maltose assimilation. Lager (-37%), stout (-63%) and cask ale (-31%) were notably impacted in the presence of maltose (Figure V-7).

5.3.5.3 Glucose

It was expected glucose would be the most rapidly assimilated irrespective of style, as it is a primary carbon source universally. However, it is possible for the more complex sugar profiles of ales, there is as much glucose being produced as is being assimilated. As no significant impact on growth rate in the presence of disaccharides and trisaccharides is revealed. Lager is the only style demonstrating a clear response to increasing sugar complexity (Figure V-6). As lager is innately a more attenuated beverage and with reduced energy producing potential due to reduced sugars and dextrin, the environment may drive the selection of microorganisms with a more

diverse carbohydrate metabolism but with the inability to hydrolyse dextrin or experience metabolic repression in due to the presence of glucose.

5.3.5.4 Fructose

Fructose is a sugar produced during the first stage of glycolysis. Therefore, it was expected there would be minimal impact versus glucose on growth rate and potentially reduce the lag and log phase. For lager and cask ale, the introduction of fructose in place of glucose had a negative impact on the growth. There is a 15% and 44% reduction in growth rate for lager and cask ale respectively (Figure V-7). Lag phase was not impacted for lager, however, there is a slight reduction for cask ale (Figure V-6). Alternatively, the introduction of fructose for both stout and keg ale, which have prior both expressed similar microbiomes (Chapter II & III), had a positive impact on growth rate (Figure V-6). An increase of 14% and 47% for stout and keg ale respectively. For keg ale, the log phase of growth is much reduced versus glucose. Overall, there was minimal impact on the lag and log phases, there are slight reductions for lager, keg ale and cask ale. Whereas the length of both phases increased minimally for stout.

5.3.5.5 Sucrose

Sucrose is a disaccharide that is hydrolysed to glucose and fructose by cell wall invertases found in *Saccharomyces* sp. amongst other yeasts and bacteria. Hence, it was presumed to have an inhibitory effect on growth rate and final OD.

For the lager style, there is no impact on growth rate, and this is slightly increased versus fructose. Suggesting microorganisms were adapted to metabolise sucrose versus glucose. Stout exhibited a 29% increase in growth rate, despite an extended lag

phase. The increased log phase, albeit slight, is expected but was not significant (Figure V-6). The notable increase of growth rate suggests a proclivity for microorganisms to rapidly assimilate sucrose and invertase production, whether intracellular, cell bound, or extracellular, were widespread amongst stout and lager microflora.

The remaining styles (keg ale and cask ale) were reduced in growth rate versus glucose (Figure V-6). There is a 32% and 76% reduction in growth rate for the keg ale and cask ale styles (Figure V-7). The lag and log phases for cask ale were much lower in length (approx. 20 hrs each) when supplemented with monosaccharides. However, the presence of sucrose approximately doubled the length of lag phase whilst the log phase did not increase.

5.3.5.6 Maltose

Maltose is the only sugar used to have a consistent negative impact on growth rate on each style. Keg ale was unaffected (2% reduction) whereas a 37%, 63% and 30% reduction was observed for lager, stout, and cask ale respectively (Figure V-7). Stout was the most affected, demonstrated by the increased lag and log phases of growth. It is suggested that maltose specific enzymes to reduce maltose is driving this negative impact. This is in line with previous findings in section 5.3.2.

5.3.5.7 Maltotriose

Maltotriose is the most complex of the five sugars investigated and was anticipated to have a negative impact on growth rate. For lager, keg ale and cask ale this was the case with respectively a 48%, 36% and 60% reduction (Figure V-7). The reduction in growth rate is presumably driven by requirement to transport and then hydrolyse the

sugar to glucose to enable glycolysis. Somewhat counter intuitively, for stout, there is an 18% increase in growth rate and there was no impact on the final OD.

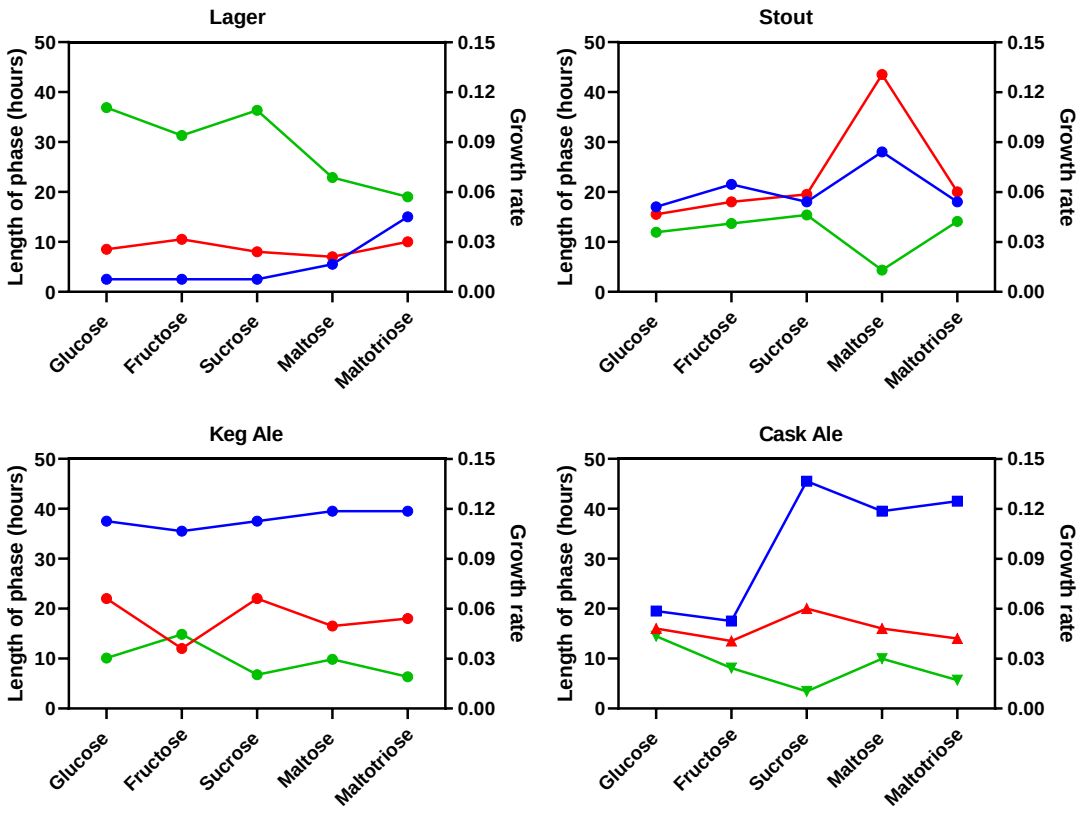

Figure V-6: On-trade beer spoilage microorganisms were challenged in a YPD-modified media versus a range of sugars. The growth rate (green), lag phase (blue), and log phase (red) were determined to assess the impact in four styles of beer.

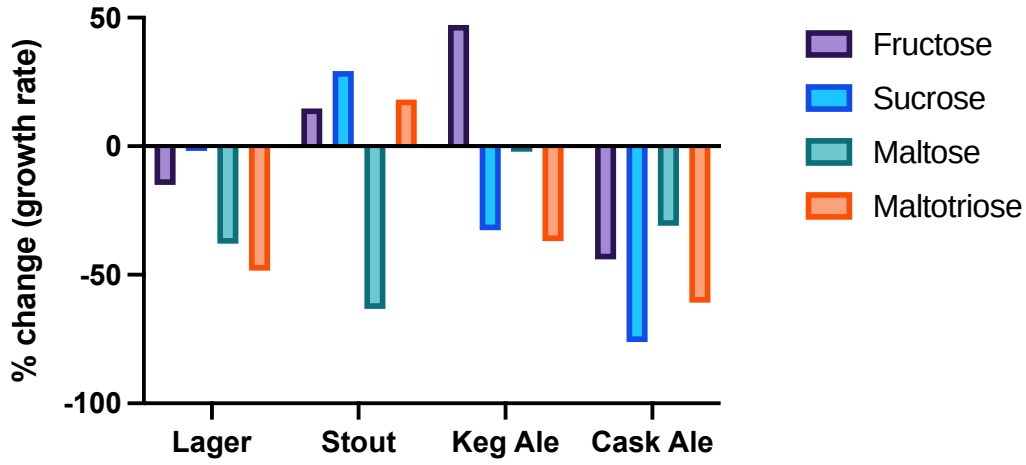

Figure V-7: Using growth rate with glucose as a reference value, the change in growth rate was calculated as a percentage to assess the impact of different sugars.

5.3.5.8 Influence of sugars on total growth

Glucose was efficiently metabolised by all styles, producing the highest OD for microflora from lager, keg ale, and cask ale (Figure V-8, Table V-11). For stout, there was a slight reduction in final OD of glucose versus fructose, although this was insignificant. The metabolism of fructose was found to have little to no impact on final OD for lager, stout, and keg ale. Cask ale reduced by almost 20% compared to glucose, suggesting fructose was not an efficient carbon source for cask ale microorganisms.

For each style there was a reduction in the use of maltose as the primary carbon source, with troughs in final OD visible for both stout and lager (Figure V-8). Maltose use did peak in comparison to sucrose and maltotriose for cask ale but remained significantly lower than the monosaccharides ($p<0.05$).

Sucrose in lager was utilised at a comparable rate to the monosaccharides and produced the similar final OD. Stout was similar, although the final OD was slightly greater (1.135 ± 0.04) (Table V-11). Keg ale and cask ale microorganisms performed poorly when challenged with sucrose as the primary carbon source. However, for keg ale the final OD was only slightly reduced in comparison to the final OD of glucose (glucose 0.76 ± 0.18, sucrose (0.5 ± 0.05), whereas cask ale was approximately 2-fold reduced compared to glucose (glucose 1.29 ± 0.04, sucrose 0.6 ±0.05).

Analysis of variance between the different sugars in the individual styles highlighted significant variance between the different sugars for lager, stout, and cask ale ($p<0.001$). However, for keg ale, there is no significant variance, suggesting sugar complexity had little impact on the spoilage microorganisms sourced from this style

of beer. Further, when analysing the variance of sugar performance in the different styles, only for maltose was there no significant difference between the means (p<0.001). Thus, maltose use was equally poor irrespective of style or source inoculum.

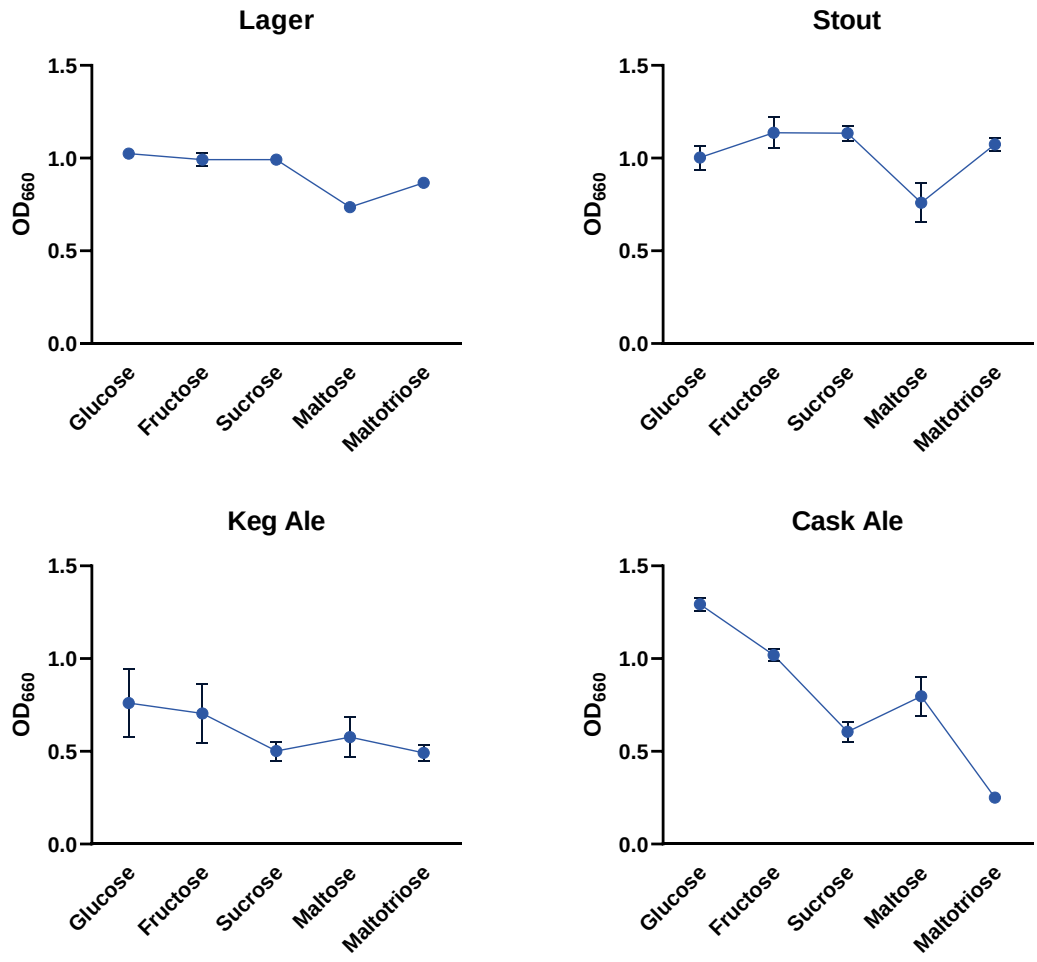

Figure V-8: Optical density (660nm) at 96 hrs was plotted for each style against sugar. Each sugar was supplemented to a final concentration of 100mM for glucose and fructose, 50mM for maltose and sucrose, and 33mM for maltotriose. The impact of maltose can be seen for lager and stout, and cask ale. Cask ale optimally performed with glucose, with reduced rates of growth on the other sugars. Keg ale overall produced the lowest OD_{660}, however, was the least affected.

Table V-11: On-trade sourced microorganisms were challenged to grow on a range of sugars. Optical density 660nm was measured after 96 hours (n=3). *Significant variance between different sugars. **Significant variance between styles.

	Style			
Sugar	**Lager***	**Stout***	**Keg ale**	**Cask ale***
Glucose**	1.025 ± 0.01	1.003 ± 0.07	0.76 ± 0.18	1.294 ± 0.04
Fructose**	0.991 ± 0.04	1.136 ± 0.085	0.704 ± 0.16	1.019 ± 0.03
Sucrose**	0.992 ± 0.01	1.135 ± 0.04	0.502 ± 0.05	0.606 ± 0.054
Maltose	0.736 ± 0.03	0.759 ± 0.107	0.577 ± 0.11	0.797 ± 0.107
Maltotriose**	0.866 ± 0.01	1.074 ± 0.04	0.491 ± 0.04	0.25 ± 0.01

5.3.6 Organic acid supplementation in beer

Organic acids were added to the four lager brands to from the 'spoilage screen' to compare the impact of adding organic acids to the beer.

5.3.6.1 Pyruvic acid

Pyruvate concentrations were quantified in a range of beers and beer (Table V-12). PL9 (26.4 mg/mL), despite being a robust beer was over 2-fold higher than PL8 (11.4 mg/mL). SC1 and KA1 were particularly high with concentrations of 37.5 mg/mL and 38.2 mg/ml, respectively. ST1 (17.7 mg/mL) and SC2 (24.0 mg/mL) contained a reduce concentration of pyruvic acid. Despite the role of pyruvate in energy production, the higher concentration in PL9 may be directed to other pathways to counteract the increased acidity of the beer.

Table V-12: Concentration of pyruvate using pyruvate Megazyme assay in a range of beers

Beer	Pyruvate concentration (mg/l)
PL8	11.4
PL9	26.4
SL3	11.8
PL3	16.2
ST1	17.7
PKA1	31.5
KA1	38.2
SC1	37.5
SC2	24.0

The addition of only 100nM to PL9 and PL3 exhibited a significant increase in total growth versus an untreated control (p<0.001) (Table V-13). For PL9 supplementation of 100nM to 10mM significantly increased total growth (Table V-13). In all four beers, the addition of 100mM pyruvic acid had a negative impact, presumably due to the increased acidity. Pyruvate supplementation at 100nm increased the final OD by 72% suggesting its addition supported the growth of spoilage microorganisms. Addition of pyruvate to SL3 had no positive impact on growth at 10mM. Addition of citrate (Table V-14) and lactate (Table V-15) had less impact. Lactate had a significantly positive impact on total growth versus an untreated control across five concentrations for PL1. There was also a small positive impact for PL8 and PL9 when treated with lactate (Table V-15)

Table V-13: Growth as OD_{660} of four beers inoculated with microorganisms sourced from an on-trade sample of PL3. Each was supplemented with a range of concentration of pyruvate into the beer. (n=3), *statistically significant difference (p<0.001)

Pyruvate concentration	PL8	SL3	PL9	PL3
100mM	0.044 ± 0.002*	0.076 ± 0.008*	0.056 ± 0.005*	0.053 ± 0.008*
10mM	0.223 ± 0.007	0.134 ± 0.045*	0.191 ± 0.037*	0.256 ± 0.069
1mM	0.255 ± 0.016	0.242 ± 0.008	0.242 ± 0.02*	0.291 ± 0.038
100µM	0.268 ± 0.037	0.236 ± 0.028	0.221 ± 0.001*	0.317 ± 0.012
10µM	0.290 ± 0.01*	0.248 ± 0.016	0.224 ± 0.028*	0.34 ± 0.027
1µM	0.276 ± 0.016	0.255 ± 0.003	0.223 ± 0.015*	0.284 ± 0.039
100nM	0.271 ± 0.004	0.263 ± 0.01	0.248 ± 0.01*	0.316 ± 0.017
Control	0.238 ± 0.022	0.221 ± 0.005	0.14 ± 0.011	0.283 ± 0.019

Table V-14: Growth as OD_{660} of four beers inoculated with microorganisms sourced from an on-trade sample of PL3. Each was supplemented with a range of concentration of citrate into the beer. (n=3), *statistically significant difference (p<0.001)

Citrate concentration	PL8	SL3	PL9	PL3
100mM	0.03 ± 0.007*	0.071 ± 0.005*	0.051 ± 0.013*	0.055 ± 0.008*
10mM	0.256 ± 0.01	0.199 ± 0.017	0.202 ± 0.008	0.299 ± 0.016
1mM	0.246 ± 0.01	0.207 ± 0.028	0.22 ± 0.009	0.307 ± 0.022
100µM	0.262 ± 0.012	0.246 ± 0.04	0.219 ± 0.024	0.304 ± 0.006
10µM	0.277 ± 0.013	0.225 ± 0.007	0.234 ± 0.03	0.297 ± 0.003
1µM	0.266 ± 0.003	0.232 ± 0.021	0.218 ± 0.037	0.285 ± 0.02
100nM	0.257 ± 0.023	0.258 ± 0.013	0.237 ± 0.015	0.299 ± 0.021
Control	0.259 ± 0.014	0.238 ± 0.022	0.199 ± 0.03	0.289 ± 0.032

Table V-15: Growth as OD_{660} of four beers inoculated with microorganisms sourced from an on-trade sample of PL3. Each was supplemented with a range of concentration of lactate into the beer. (n=3), *statistically significant difference (p<0.001)

Lactate concentration	PL8	SL3	PL9	PL3
100mM	0.103 ± 0.026*	0.075 ± 0.004*	0.071 ± 0.021*	0.146 ± 0.085*
10mM	0.261 ± 0.013	0.242 ± 0.008*	0.223 ± 0.018*	0.282 ± 0.034*
1mM	0.282 ± 0.012*	0.259 ± 0.006	0.249 ± 0.038	0.352 ± 0.004*
100µM	0.282 ± 0.014	0.255 ± 0.041	0.244 ± 0.02	0.316 ± 0.017
10µM	0.279 ± 0.009	0.27 ± 0.009	0.28 ± 0.026*	0.345 ± 0.018*
1µM	0.289 ± 0.012*	0.295 ± 0.026*	0.297 ± 0.024*	0.359 ± 0.017*
100nM	0.279 ± 0.013*	0.295 ± 0.023*	0.228 ± 0.028	0.334 ± 0.018*
Control	0.258 ± 0.015	0.268 ± 0.01	0.241 ± 0.029	0.299 ± 0.032

5.3.7 Organic acid metabolism in beer

ATP is an essential metabolite required for a multitude of cellular functions. For microorganisms to grow and spoil beer, the cell must produce ATP through the glycolytic breakdown of sugars. Part of this process includes the use of organic acids to produce ATP. Using the untargeted metabolomic analysis of the LC-MS data, the relative intensity of three organic acids were tracked during spoilage (n=3 per sample). Samples collected from the Liverpool City Centre area were subject to forcing to assess the quality of the beer prior to analysis (Table V-16).

Table V-16: Quality of beers sampled in Liverpool City Centre. Each was scored according to Mallet & Quain (2018).

Lager SL3				Stout ST1			
Account	**£/pint**	**Feb-20**	**Quality index (%)**	**Account**	**£/pint**	**Feb-20**	**Quality index (%)**
L4	£3.70	B		**M3**	£3.19	C	
L6	£2.99	C	**66.7%**	**M5**	£3.69	C	**41.6%**
L8	£3.65	B		**M7**	£3.69	D	
Average	£3.45	8		**Average**	£3.52	5	

Ale PKA1				Cask ale SC2			
Account	**£/pint**	**Feb-20**	**Quality index (%)**	**Account**	**£/pint**	**Mar-20**	**Quality index (%)**
S2	£3.70	B		**C1**	£2.25	A	**91.6%**
S8	£2.99	C	**50%**	**C10**	£2.25	B	
S10	£3.65	B		Not sampled - pandemic			
Average	£3.45	8		**Average**	£2.25	7	

Across all samples there were variable reductions in each of the three organic acids investigated, pyruvic, citric, and malic acid. Changes in pyruvic acid in the 11 samples showed the best correlation with growth after forcing ($R^2 = 0.74$) (Figure V-9A). The relative abundance was calculated by using the peak intensity at sampling and calculating a percentage change post forcing.

The lager samples exhibited quality scores of B, C and B, respectively (Table V-13), with samples L4 and L8 for showing a 58% and 30% reduction in the relative intensity of after forcing (Table V-17). For sample L6, an 82% reduction in pyruvic acid was observed which was associated with an 'unacceptable' quality score. This suggests that pyruvic acid availability may play a role in the 'potential' of a beer for spoilage. There was no obvious trend between the three lager samples and the concentration of citric or malic acid (Table V-17).

The three samples of stout were of very poor quality either 'poor' (C) or 'unacceptable' (D). Samples from M3 and M7 (Table V-17) exhibited >97% reduction in pyruvic acid intensity. ST1 from M5, a rapid decline of pyruvic acid was observed between day 0 and day 1, where there was a 52% reduction in peak intensity, this reduced further to a total of 73% reduction by day 4 (Appendix B; Table VII-1-3).

Changes in citric acid concentration were greater in the stout samples. With accounts M5 and M7, there was a 47% and 29% reduction in citric acid level although for M3 there is no change. With malic acid there was a 60%, 37% and 50% reduction in malic acid in three stout samples (Table V-17).

The quality of all three samples of keg ale was ‘poor’ (C). Interestingly, the pyruvic acid intensities in the keg ale were rapidly reduced by 96% (S2), 98% (S8) and 72% (S10) (Table V-17). No trends could be seen with either malic or citric acid, with relative intensities both increasing or decreasing in different accounts (Table V-14).

Cask ale samples had the best quality score (‘excellent’/A and ‘acceptable’/B) although only two samples were taken due to COVID-19 restraints (Table V-17). Both samples exhibited a slow rate of metabolism of pyruvic acid with at day 2, a reduction in peak intensity of 16% and 11% (Appendix B; Table VII-1-3). By day 4, the reduction increased to a 54% and 50%. The level of malic acid showed little change with a 6% reduction for sample C1 and a 5% increase in sample C10. Citric acid was reduced in both samples, with a 30% reduction of peak intensity.

From the data it is suggested that pyruvic acid plays a role in spoilage and its utilisation can be correlated with the extent of growth during the forcing process. Although less convincing, citric acid may play a similar role, whereas malic acid is little utilised (Figure V-9B-C). The reduction of pyruvic acid level versus the final OD demonstrated a correlation to final OD ($R^2 = 0.74$), implying pyruvic acid may be a marker for spoilage in beer (Figure V-9A).

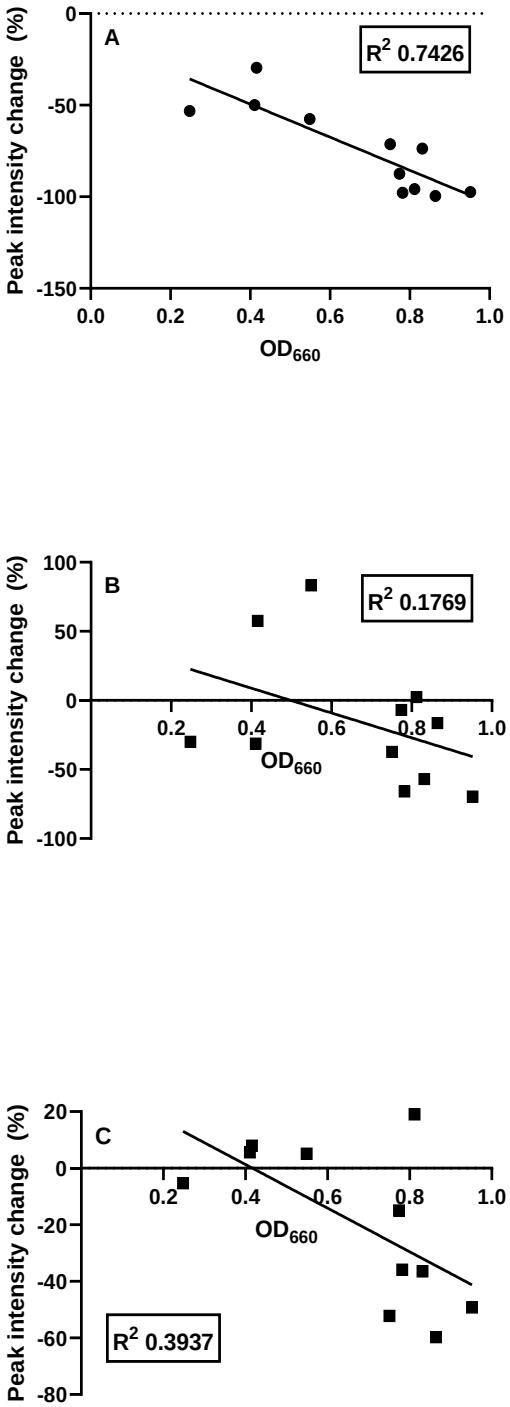

Figure V-9: Optical density (660nm) of forced samples of 96 hrs were plotted versus the relative intensity percentages of each organic acid after the same period. (A) pyruvic acid, (B) citric acid, (C) malic acid

Table V-17: Pyruvate, citrate, and malate peak intensity was tracked from day 0 to day 4. Using day 0 as the reference value, the percentage change was calculated compared to day 4. Four styles were sampled from accounts in Liverpool city centre. [a]Cask ale is from two accounts only due to the closure of the University of Liverpool laboratories at the beginning of the COVID-19 pandemic.

Style	Account	Pyruvate	Citrate	Malate	Quality grade
Lager	**L4**	-58%	-84%	+5%	B
	L6	-82%	-7%	-15%	C
	L8	-30%	+57%	+7%	B
Stout	**M3**	-99%	-7%	-60%	D
	M5	-74%	-47%	-37%	C
	M7	-97%	-29%	-50%	C
Keg ale	**S2**	-96%	+2%	+19%	C
	S8	-98%	-66%	-26%	C
	S10	-72%	-38%	-53%	C
Cask ale[a]	**C1**	-54%	-30%	-6%	A
	C10	-50%	-31%	+5%	B

5.4 Discussion

Draught beer is a complex mix of yeasts and bacteria driven by beer style, dispense method (Jevons and Quain, 2021). and the hygienic practices in the account. Poor quality beer is a common theme in the on-trade and can have financial implications for the retailer and brewer (Mallett and Quain, 2019). Beer contains similar antimicrobial elements; low pH, ABV 3-7 %, hop-bitter acids, low O_2 and low nutrition. Despite this, similar beers can spoil at significantly different rates (Figure V-1). Of the ten lager beers analysed, PL8 was comparably easy to spoil where PL9 was more resistant to spoilage (Figure V-1). When challenged with microorganisms sourced from the four beer styles, PL8 was consistently the easiest to spoil. Interestingly, cask ale sourced microorganisms contained the most potent beer spoilage microorganisms. The methodology used to assess is not a strict anaerobic environment, potentially supporting the high number of aerobic acetic acid bacteria commonly found a part of the cask ale microflora.

The most notable difference between the two beers is pH: PL8 pH 4.35 and PL9 pH 4.13 (Table V-2). However, the susceptibility of PL8 to spoilage is not easily ascribed to pH as beers with a higher pH, PL1 and PL2, did not spoil as easily (Table V-7-8), suggesting other factors influence spoilage. Sugars in beer provide energy for microbial growth and spoilage. Quantifying sugars in the ten lagers screened for spoilage showed that PL8 contained the highest concentration of the quantified sugars with 9.1 mg/mL (Table V-9) with PL9 much lower with 5.9 mg/mL. Moreover, contained the highest concentration of FAN (mg/L). High levels of fermentable sugars and FAN coupled with a more favourable pH is driving the heightened spoilage susceptibility of PL8.

Each of the beers were supplemented with a range of sugars, at various concentrations, with spoilage (optical density at 660nm) and growth rate quantified. Microorganisms in lager PL8 exhibited the highest growth rate when supplemented with glucose, fructose, and maltose. Overall, maltose was the least preferred carbon source for microorganisms from draught beer, which will be discussed further later. PL8 was significantly higher in maltose than the other beers at 6.4 mg/mL, with 2mg/mL more than the next closest beer, accounting for over two-thirds of the sugars in PL8 (Table V-8). Maltose supplementation in PL8 had little-to-no effect on total growth or growth rate (Table V-7-8). After 96 hrs the final OD_{660} with maltose supplementation (0.703 ± 0.018) was greater than when supplemented with glucose (0.676 ± 0.031). There was no significant difference between these values, suggesting the favourable conditions in PL8 promoted the use of maltose, and, possibly, the high initial concentration of maltose applying selective pressure on the microflora.

The pH of PL9 (4.13) is the lowest of the ten beers investigated. After supplementation fructose, sucrose, and maltotriose, the growth rate of microflora was the lowest in PL9 (Table V-8). When the growth rate of each of the five sugars used to supplement was averaged, PL9 exhibited the lowest growth rate, although marginally lower than PL2 (Table V-8). Indeed, PL2 exhibited a similar robustness to spoilage as PL9. Although similar, the average OD_{660} of PL2 after 96 hrs of growth was the lowest of the ten beers (0.43 ± 0.071), with PL9 had the third lowest (0.472 ± 0.112) (Table V-7). After PL9 was supplemented with glucose, it had the second largest impact on spoilability of the nine beers (0.623 ± 0.035), with PL8 the highest (0.676 ± 0.031). The importance of glucose to spoilage is not a novel insight, yet it demonstrates how increasing the concentration of simple sugars can have a profound impact on a beer

priorly deemed robust. This insight provided inspiration for the analysis of the 'fermentable' sugars in beer as a possible metric to explore a beers' spoilability.

This thinking was developed using the data collected through Tables V5-8. The average growth rate, pH, ABV %, FAN (mg/L, and total sugars (mg/mL) were ranked for the ten analysed beers from 1 to 10. The sum of these scores were averaged and ranked (Table V-9), creating a 'spoilability value'. In support of the approach, both SL5 and PL9 were the least spoilable scoring the lowest value of 3.6 and 4.2 (out of 10), respectively. Conversely, PL8 scored the highest with 8.4. By combining these analyses, this simple metric was able to predict the least and most spoilable beers. There existed a lack of clarity in the central data points suggesting there exists undefined relationships between spoilage and the antimicrobial elements. In Figure V-4b this was expressed via a low R^2 value. This should promote future to aim to create a more robust version of this spoilability metric to predict a beers vulnerability.

5.4.1 Maltotriose supplementation in standard lagers positively impacted growth rate

Maltotriose is a trisaccharide and is one of the three main fermentable sugars in wort, alongside glucose and maltose (Lei *et al.*, 2016). The growth of microflora in lagers SL3, SL5, SL6, and PL1 was greatest after forcing the addition of maltotriose. A similar result has been reported with *S. cerevisiae*, where maltotriose improved final cell densities versus glucose and maltose (Zastrow *et al.*, 2000). The transport of maltotriose in *S. cerevisiae* is facilitated by maltose permease, with each maltotriose molecule providing more energy to support cellular growth (Zastrow *et al.*, 2000). Interestingly, the growth rate of SL5 (0.005) supplemented with maltotriose was lower

than SL3 (0.01), SL6 (0.009, and PL1 (0.009) (Table V-8) – hinting at an innate robustness yet undefined.

SL3 exhibited the fastest growth rate of all beers when supplemented with maltotriose (0.01) (Table V-8). Each of the standard lagers was 4% ABV, the lowest ABV of the lagers analysed. However, PL1 similarly peaked with maltotriose addition, despite being 5% ABV. Furthermore, its spoilability metric recorded this beer to be the joint-fourth most robust beer analysed (3.75) (Table V-9). The lower % ABV of the standard lagers may support the metabolism of maltotriose. During fermentation it has been reported that maltotriose uptake is reduced at high ethanol concentrations (Zheng *et al.*, 1994), and this may suggest that a lower %ABV increases maltotriose uptake by spoilage yeasts. This is further supported by the other premium lagers, whereby maltotriose was the least optimal sugar for PL3 (5.0 %), PL6 (4.8 %), and PL8 (4.5 %). For PL2 (5.1 %) maltotriose was used preferentially than maltose, which will be discussed later.

5.4.2 Sucrose was readily assimilated in lager, stout, and keg ale

Sucrose is a disaccharide which is broken down by invertase into glucose and fructose (Kulshrestha *et al.*, 2013), *Saccharomyces* sp. are the primary source of invertase (Vitolo, 2021), containing intracellular and extracellular invertases, along with invertases on the cell wall (Vitolo, 2021, Margetić and Vujčić, 2017, Wang and Li, 2013). Invertase is actively secreted by brewing yeasts during fermentation (Enevoldsen, 1981). Between pH 3.5-4.5 invertase can function effectively, but preferentially at pH 4.5 (Kulshrestha *et al.*, 2013). When the microorganisms sourced from stout and lager were challenged with a YPD-modified medium with sucrose as the primary carbon source, the growth was on par with the performance on

monosaccharides (Figure V-6-7). Further, growth of keg ale sourced microflora slightly reduced with sucrose addition (Figure V-6-7).

Brettanomyces sp. has been identified to be a key beer spoiler in this work (Chapter II) and by others (Gilliland, 1961, Fleet, 1992, Hemmons, 1955) and it has been reported that the yeast has the necessary genes to utilise sucrose as a primary carbon source (Roach and Borneman, 2020). Furthermore, *Pichia anomala* is able to produce invertase which is almost exclusively bound to the cell wall and would rely on passive interactions with sucrose prior to hydrolysis to glucose or fructose (Rodriguez *et al.*, 1995). In the presence of hexoses, catabolite repression impacts external invertases (Vitolo, 2021). In Chapters II and III, *Saccharomyces* sp., *Brettanomyces* sp., and *Pichia* sp. are reported ubiquitous spoilage microorganisms of beer, and their presence will have contributed to hydrolysing sucrose to glucose and fructose, both of which can assimilated by a wider range of microorganisms. However, invertases are also produced by *Lactobacillus* sp. but are reported to be intracellular and therefore sucrose hydrolysis would benefit the bacterium and not the community (Awad *et al.*, 2013). It would be of interest in future work to understand whether supplementation of sucrose influenced the microbiome.

5.4.3 Maltose is not readily assimilated by draught beer microorganisms

Maltose is a disaccharide that predominates in wort (Gjertsen, 1953), and is readily fermented by brewing yeast. However, maltose utilisation is unusual in non-*Saccharomyces* wild yeasts. An article published in 1961 by RB Gilliland, on *Brettanomyces* sp., comments that “It seems strange that so many strains unable to ferment maltose should be found in beer…” (Gilliland, 1961). Indeed, *Brettanomyces anomalus* was named due to its inability to ferment maltose in wort by Custers (1940).

Given this, it is perhaps not surprising that in the work reported here, maltose was a poorly used sugar by spoilage microorganisms. Maltose was assimilated at a slower rate and produced the least growth (OD_{660}) with PL9, SL5, PL1, and PL2 compared to the other four sugars (Table V-7-8).

The three main fermentables of wort were quantified in four styles of beer, and the concentrations were tracked over a 4-day period of forcing. Maltose was not used in any of the four styles investigated (Figure V-5). Table V-8 presents the final OD_{660} of the microflora sourced from same four styles of beer versus various sugars in a modified YPD-medium. Poor use of maltose by spoilage microorganisms, irrespective of style source, could be due to the lack of maltose permease, which required to transport maltose into the cell (Medintz *et al.*, 1996) where alpha-glucosidase converts maltose to glucose (GonçAlves *et al.*, 2000). The same mechanism is used for maltotriose, and like for maltose is an ATP-dependent process, whereas uptake of fructose and glucose is passive (Vriesekoop *et al.*, 2012). Further, glucose can repress maltose permease gene transcription and inactive the mechanism (Medintz *et al.*, 1996). This work did not reveal maltose to be much higher in ales than in lager style beers (Table V-10), further earlier work (Chapter II & III) revealed an increased abundance of *Saccharomyces* sp. and *Pichia* sp. implying dextrin may be an important factor

In *S. cerevisiae,* maltose and maltotriose metabolism is regulated by glucose repression and catabolite inactivation, in the absence of glucose maltose metabolism is rapidly regenerated (Novak *et al.*, 2004). This may help explain the increased presence of *Saccharomyces* sp. in ale style beers, and why cask ale microflora

increased performance on maltose versus sucrose or maltotriose (Figure V-5, V-8; Table V-8). As glucose levels reduced, maltose will become the primary carbon source, as there is no repression of the maltose permease transporter and therefore maltose was able to be transported and hydrolysed by α-glucosidase in the cytoplasm of the brewing yeast (Novak *et al.*, 2004). Moreover, increased dextrin concentrations that may be broken down release glucose by wild yeasts, may act to further support the dominance of wild yeasts in ales, which will be discussed further in section 5.4.4.

Lager is a largely attenuated beer, which influences the microbiome, selecting for microorganisms with a more versatile carbohydrate metabolism. The concentration of maltose was unexpectedly high in the lager beers analysed in Table V-10. Despite maltose being poorly used, the presence of maltose may be a key in the abundance of *Lactobacilli* in lagers. Under fermentative conditions using maltose as the carbon source, *Lactobacillus* sp. have been shown to excrete glucose (Stolz *et al.*, 1993). Although performance overall was reduced when maltose was used as a supplement, it is suggested that LAB species will support wild yeasts incapable of using maltose (Stolz *et al.*, 1993). However, this may instead be a survival mechanism with the release of glucose repressing maltose use by microbial competitors (Stolz *et al.*, 1993, Viana *et al.*, 2000, Stolz *et al.*, 1995). Maltose phosphorylase is an enzyme present in *Lactobacillus* sp., which phosphorylates maltose to glucose and β-D-glucose-1-phosphate (Egloff *et al.*, 2001). This process does not require energy, enabling the efflux of glucose by *Lactobacillus* sp. (Stolz *et al.*, 1993). The breakdown of maltose for *Lactobacillus* sp. occurs intracellularly and is transferred by a few possible mechanisms. One which occurs via a maltose/H^+ symporter, thus maltose may be a possible mechanism of acid stress resistance (Neubauer *et al.*, 1994). Although it

cannot be confirmed in this work, the presence of maltose in beer may act to promote *Lactobacillus* sp. proliferation. Whereas dextrin in ales will promote the proliferations of yeasts (*S. diastaticus*) capable of amyloglucosidase production, which will be discussed in the following section (5.4.4).

Alternatively, it is possible CO_2 may also play an inhibitory role in maltose uptake. As discussed by Vanbeneden *et al.* (2006), CO_2 in bottled beer repressed maltose utilisation in bottle and, in some cases, prevented the consumption of the sugar by some yeast strains. Accordingly, it was concluded that maltose is an inappropriate sugar for bottle conditioning (Vanbeneden *et al.*, 2006).

Draught beer is carbonated with carbon dioxide and the gas is used to move beer from the keg through the dispense line. This could explain the depressed utilisation of maltose during beer spoilage and would similarly support the increased performance of cask ale sourced microflora using maltose (Figure V-6; Table V-8). Despite this, other work has shown beer sourced LAB to prefer maltose over glucose, however, these experiments were conducted using synthetic media and a more favourable pH, not consistent with the challenges faced in a beer (Wood and Rainbow, 1961). However, the depression of yeast maltose metabolism may contribute to the increased presence of *Lactobacilli* sp. in dispense.

5.4.4 Glucose from dextrin hydrolysis may repress maltose transport and influence ale microbiome

Unpublished data has shown how ale style beers contain notably more dextrin compared to the analysed lagers, but this is also supported by Buiatti (2009) citing lagers as containing 10-20g/L of dextrin, to ales 10-40g/L. Dextrin is broken down by

amyloglucosidase (glucoamylase) (AMG), encoded by the genes *DEX* and *STA*, which diastatic variants of *Saccharomyces* encode (Perry and Meaden, 1988). According to Ragot *et al.* (1989) approximately 20% of dextrin will contain 20 or more glucose molecules. AMG is an extracellular enzyme, and is used to hydrolyse dextrin into fermentable sugars, exploited in brewing as a commercially sourced exogenous enzyme to produce 'light beer' (Owades and Koch, 1989). Work reported in Chapter II and III revealed *Saccharomyces* sp. as a ubiquitous spoiler. Chapter II was later supplemented later to target *S. cerevisiae* var. *diastaticus*, identifying the variant in each style of beer investigated. *S. cerevisiae* var. *diastaticus* can be isolated readily from spoilt beer and has been discussed in detail (Suiker and Wösten, 2022). Abbet (2020) reported diastatic *S. cerevisiae* variants possess strong spoilage potential at temperatures as low as 8ºC. *S. diastaticus* have been sourced in biofilms, with PCR identifying *S. diastaticus* in nearly half of biofilms sourced from a breweries (Suiker *et al.*, 2021). However, *S. diastaticus* was only reported to be present in mixed-genera biofilms, suggesting it is not a primary coloniser, found repeatedly alongside *Pichia* or *Candida* sp. (Timke *et al.*, 2008b). This same work was previously discussed in Chapter III, whereby *Pichia anomala* is quoted as a primary biofilm coloniser, after which *Saccharomyces* sp. would join as a secondary coloniser. The presence of *S. cerevisiae* or *S. diastaticus* in biofilms would translate to a dispense system, coupled with the reported spoilage potency at low temperatures, suggests diastatic yeasts may play an important role in draught beer spoilage and the release of glucose into the extracellular matrix; thus, repressing maltose transport, and selecting for a yeast-dominate microbiome.

5.4.5 Keg ale microflora is not impacted by sugar complexity

The microflora from the four styles of beer was challenged to grow in a modified-YPD medium, where the glucose either remained or was replaced with fructose, sucrose, maltose, or maltotriose (Figure V-8; Table V-11). This continued the theme of the reduced capability of beer spoilage microorganisms to assimilate maltose as a primary carbon source. For microflora from lager and stout there was notable reduction in the growth with maltose, suggesting the microbiome had limited capacity to transport and/or metabolise maltose.

One-way ANOVA analysis showed there was a significant variance in sugar use by the microflora in lager, stout, and cask ale. Only microorganisms from keg ale were not significantly influenced by increasing sugar complexity (Table V-11). Whilst glucose in stout and lager were used rapidly and maltotriose in keg ale too (Figure V-5). Each of these beers were quantified for sugars in their bottled/canned counter parts (Table V-10). Interestingly, microorganisms in keg ale showed little preference for using glucose (Figure V-8; Table V-11). Hemmons (1955) has previously reported a range of wild yeasts in beer including *Candida* sp., *Pichia* sp., and *Saccharomyces* sp. Hemmons reported all strains of *Saccharomyces* sp. were able to utilise glucose, fructose, sucrose, and maltose in wort, with utilisation reducing from maltotriose to maltotetrose. The wild yeasts (*Candida, Pichia, Kloeckera* sp.), however, failed to use maltose, maltotriose, or maltotetrose in wort (Hemmons, 1955). Therefore, it is suggested that these yeasts are secondary spoilers, reliant on the presence of primary spoilers such as *Brettanomyces* sp. and *Saccharomyces* sp. to hydrolyse dextrin, releasing glucose into the medium.

Keg ale style beers tend to have a ‘yeastier’ microbiome, driven by the complexity of the ale sugar profile. Previous work in Chapter II & III showed the abundance of *Saccharomyces* sp., *Brettanomyces* sp., and *Pichia* sp. in ale style beers. *Saccharomyces* sp. are one of the most prevalent spoilers in keg ale style beers, with their ability to reduce complex sugars. One publication states *Brettanomyces* sp., and *Saccharomyces diastaticus* ‘vigorously attacked’ maltotetrose and maltotriose (Phillips, 1955). *B. bruxellensis* was found to completely assimilate these sugars, with monosaccharides still quantifiable in the media (Phillips, 1955). The ability of *Brettanomyces* sp. and *Saccharomyces* sp. to utilise these and more complex sugars has been shown in other work (Menoncin and Bonatto, 2019, Willaert, 2007b, Zheng *et al.*, 1994). The utilisation of malto-oligosaccharides has been found in the production of Lambic ales, where significant reduction was recorded in the early stages by *S. kudriavzevii*, and later by *B. bruxellensis* (De Roos *et al.*, 2020). Work by Suzuki *et al.* (2005) with LAB species where maltotriose was the sole carbon source in the presence of hop compounds. The slow rate of energy production by the increased sugar complexity will reduce acid stress resistance due to depleting intracellular ATP. This may explain why LAB species have reduced significance in ale style beers. The presence of yeasts in keg ale microflora has been reported in previous Chapters, the tendency of *Saccharomyce*s sp. and diastatic variants to readily assimilate maltose, maltotriose, maltotetrose, and dextrin may be a necessary precursor to spoilage by a wider range of yeasts reported in beer and brewery-source biofilm colonisers.

5.4.6 Pyruvate: a spoilage potential indicator

Pyruvate is a central metabolite in metabolism linking glycolysis to the tricarboxylic acid cycle. During brewery fermentation, the fate of pyruvate is predominately the

formation of ethanol and CO_2, with some being used in synthetic reactions or excreted into the beer. Therefore, its concentration is typically not high in the final product, work by Klopper *et al.* (1986) identified a pilsner type beer with concentrations ranging between 19-95 mg/L.

In this work it was postulated that - due to its position linking major energy producing pathways - pyruvate could be an indicator of spoilage potential. Synthesis of ATP is an essential prerequisite to beer spoilage potential, for example hop-resistance in LAB species is mediated by the multi-drug transported, *horA*, requires ATP to function. Without pyruvate, intracellular pH would dissipate impairing essential enzymatic reactions within the cell, disrupting cellular homeostasis.

Pyruvate as a carbon source may provide an alternative route to spoilage as sugar concentrations deplete. Initial work aimed to identify whether adding pyruvate to a beer had an impact on beer spoilage. Earlier work demonstrated differences in growth rate of beers, showing how there are differences in growth and growth rate when challenged of lagers PL8 and PL9 were inoculated with spoilage microflora. As PL9 was less spoilable, this work suggested a combination of pH and ABV may be important in tine susceptibility of beer to spoilage. Addition of pyruvate (100nM) to PL9 increased total growth (OD_{660} at 96 hrs) by nearly 2-fold. However, with lagers PL8, PL3, and SL3 the addition of pyruvic acid had no significant impact on growth. At the highest concentration of pyruvic acid addition (100mM), growth was significantly reduced presumably due to the increased acidity. However, for lager PL9, despite the increased acidity of increasing addition (1µM, 10 µM, 100 µM, 1mM, 10mM) spoilage increased at all concentrations. Acidity is a key inhibitor for spoilage

microorganisms, and therefore maintaining cellular homeostasis for optimal energy production is essential. Lactic acid bacteria are cited as the most prevalent spoiler for lager beers and across fermentation (Suzuki, 2015, Suzuki *et al.*, 2004, Suzuki *et al.*, 2006). This reflects their ability to resist the combined stress hop iso-alpha acids and low pH. Indeed, it is noteworthy that *Lactobacillus plantarum* is able to maintain intracellular pH by converting pyruvate to acetoin (Tsau *et al.*, 1992). This may offer insight as to how - despite the lower pH of lager PL9 together with the addition of pyruvate, its addition promoted spoilage. The addition of pyruvate may have two impacts, firstly the production of ATP with excess converted to acetoin to minimise the increased acidity (Tsau *et al.*, 1992). Pyruvate is a central molecule for LAB spoilage, and the junction point for many metabolic pathways (Liu, 2003).

Pyruvates importance as a central compound for ATP synthesis could act to promote increased spoilage. But pyruvate concentrations will differ depending on the beer style and brand, as this will be related to how attenuated the beer is post-fermentation. The initial hypothesis therefore presumed beers which are more attenuated (e.g., lagers) would exhibit an increased tendency to use pyruvate and other organic acids – for ATP synthesis. Using LC-MS, pyruvate was tracked during forced ageing with growth measured after 96 hours. The relative intensity from day 0 was used as a reference value to calculate the percentage change of pyruvate, citrate, and malate after 96 hrs. Although, citrate and malate showed little correlation (R^2 0.17 and 0.39, respectively), pyruvate demonstrated a relationship between spoilage and final OD (R^2 0.74). The scope of this work cannot determine the exact function of pyruvate after removal from the medium. However, from the LC-MS data it was possible to plot the key pathways identified based on number of reference molecules matched to the pathway(s). The

‘superpathway of glycolysis, pyruvate dehydrogenase, TCA, and glyoxylate bypass’ was the highest ranked pathway for all samples of keg ale and lager and a top pathway for stout and cask ale. Although not surprising, this supports the initial assumption that pyruvate is important in draught beer spoilage. Keg ale exhibited the greatest removal of pyruvate over the 96 hours, with pyruvate intensity reduced 96%, 98% and 72% for accounts 1, 2, and 3. This is contrary to the initial hypothesis, despite its metabolism by microorganisms in keg ale, it was expected this would be more significant in a more attenuated style such as lager. Despite this, lager still exhibited a high reduction of pyruvate, 58%, 82% and 30%, which correlated with growth. Although the increased complexity of carbohydrates in ale, pyruvate may be a starter carbon source whilst malto-oligosaccharides are reduced to monosaccharides.

The role of pyruvic acid as a spoilage indicator has been demonstrated and its role maybe be multipurpose. Its potential to regulate intracellular pH by being converted to acetoin plus its energy potential, suggests pyruvate may be a central metabolite for predicting a the spoilability of beer.

Chapter VI: Discussion and concluding remarks

6.1 Overview

Beer is arguably the nations' favourite alcoholic beverage. However, in pubs, bars and restaurants ('on-trade') draught beer is in decline. This reflects many issues including increasing cost, tax, changing consumer habits, and beer of inconsistent quality. Since the mid-1970s draught beer sales have been in near linear decline, paralleled with a gradual increase in small pack sales. Whilst a myriad of factors has impacted on consumer behaviour, one which remains within in the control of the retailer is quality. Poor quality beer has long been a problem nicely articulated by Seton (1912) in 1912, who discusses how beer served at the on-trade is not of the flavour or aromas intended by the brewer, nor is it handled with the same care as during manufacture. Anecdotal evidence suggests consumers who experience poor quality beer will suffer in silence, replace the beer, change brand, or change location. These negative responses will over time undermine the reputation of the account ('they serve bad beer') or the brand ('gone downhill, better from the can'). This is recognised as a problem by the brewing industry, with brand owners/brewers seeking solutions that seek to improve quality through end-to-end refrigeration and third-party cleaning of dispense lines.

Historically, draught beer spoilage research is rather one-dimensional, habitually publications will cite the same common spoilers irrespective of style, dispense method, or hygiene. Although the common beer spoilers are still commonplace (*Lactobacillus* sp., *Brettanomyces* sp., *Saccharomyces* sp. e.g.), there are likely to be beer spoilage microorganisms that have not been previously recognised. Moreover, their impact and abundance will be influenced by the brand, styles, and dispense method. The work on the identification of draught beer spoilage microorganisms in

the 20th Century, although quintessential, were either limited in scope (limited to a single style) or by the technology available at the time. Further, the 21st Century has seen the rise of diverse and non-traditional beers (‘craft”) supplemented with flavour extracts, dispense innovations, and introduction of no and low alcoholic beverages (NABLAB) on draught.

The primary aim of this work was to reveal the complexity of beer spoilage microflora by employing culture based microbiological methods and compare this to using a next-generation sequencing platform. Further to use these insights to highlight the differences between beer styles influencing the microbiome. Chapters II and III identified a host of microorganisms from four beer styles sourced from a range of on-trade accounts. Chapter II demonstrated how conventional culturing methods restricted the microbiome to those commonly reported in beer. Moreover, the work failed to identify known obligate anaerobic beer spoilers such as *Pectinatus* sp. and *Megasphaera* sp. due to their slow growth on the media used. This demonstrated the key limitations of conventional plate-based microbiology in terms of incubation time, and the selectivity of the media. Chapter III built on this work by revisiting the same accounts and using next-generation sequencing platform from Oxford Nanopore which was successful in identifying a range of beer spoilage microorganisms, including novel beer spoilage microorganisms and a number of human pathogens.

Biofilm growth is the primary mode of growth for microorganisms in the ‘real world’, enabling the conservation of a diversity of microorganisms, and providing protection and nutrition in challenging environments like beer. Chapter IV investigated the role of biofilms in dispense systems by developing a method to investigate the role of

dispense parameters on biofilm formation and microbiome selection. This work successfully designed a simple, cost-effective, and reproducible method for quantifying biofilms for microflora from draught beer (Jevons and Quain, 2021).

The final Chapter of this work sought to answer a complex question, why do beers of comparable antimicrobial composition spoil at significantly different rates irrespective of the source of the microbial inoculum? Moreover, what were the environmental niches driving microflora selection? Throughout Chapters I-III, it had become apparent that lager style beers promoted a greater presence of bacterial spoilers, whereas ale styles promoted a broader range and increased abundance of wild yeasts. Accordingly, a range of theories were explored, including sugar profiles, the 'energy potential' of a beer, and organic acids. This work sought to scratch the surface of this fundamental question in the hope of defining beer composition that is less 'spoilable' and which selective pressures dictate microflora phenotype.

6.2 Conventional microbiology vs next generation sequencing

6.2.1 The problems associated with conventional microbiology

Conventional (brewing) microbiology is where a given sample is plated on a solid agar medium designed to promote the growth of specific contaminant that may be present. In the case of the brewing industry, this process uses four industry standard plates which target a range of wild yeasts and bacteria:

- Aerobic - Lysine agar (Fowell, 1965)
- Aerobic - Lin's Copper Media Agar (LCMA) (alias: copper sulphate agar) (Taylor and Marsh, 1984)

- Aerobic - WL Nutrient agar (WLN) (Green and Gray, 1950, Greenspan, 1965)
- Anaerobic - Raka-ray (RR) (Saha *et al.*, 1975)

These selective media target desired microorganisms, but inevitably miss other microorganisms that are unable to grow. Chapters II and III highlight the disparity between the culturable and non-culturable microbiomes. Chapter II used WLN and RR plates, designed to recover a range of aerobic wild yeasts and anaerobic spoilage bacteria specific found in beer. Four beer styles were sampled from five accounts (20 in all), on two occasions (four to six weeks apart) and analysed using culture-based microbiology. Pleasingly, there was unexpected consistency between the yeast and bacterial microflora in the accounts, despite the numerous uncontrolled and unknown variables including the absence of information regarding hygiene management and line cleaning.

The resultant microbiomes identified numerous flaws with the methodology. Firstly, across all styles of beer acetic acid bacteria (AAB) were dominant, 14 different species of acetic acid bacteria were isolated from the genera *Acetobacter* and *Gluconobacter*. Acetic acid bacteria are strict aerobic bacteria and are therefore largely removed from the brewing process where oxygen is actively minimised to assure the stability of beer flavour during its shelf life. Similarly, in dispense systems, the use of CO_2 (occasionally together with nitrogen) for the dispense of keg beer provides an anaerobic or oxygen-restricted environment, which does not support the growth or survival AAB. The presence of these bacteria in draught lager, stout, and keg ale whilst unexpected has been previously reported (Harper *et al.*, 1980). The dispense process although innately anaerobic (except for cask ale), would be anticipated to support an

abundance of lactic acid bacteria and obligate anaerobes in lager, stout, and keg ale. Although this is the case in lagers, AAB dominated stout and keg ale. It is suggested that air/oxygen is more available than predicted through gas diffusion through dispense tubing and at the keg and, more likely, the tap. The introduction of oxygen using conventional microbiology does not replicate the dispense conditions experienced by spoilage microorganisms *in situ* and therefore does not accurately represent the true abundances of the isolated species. Consequently, this enabled AAB to take precedent in an oxygen rich environment and misrepresent its true abundance *in situ*.

Raka-Ray (RR) agar was used to target lactic acid bacteria. Lactic acid bacteria (LAB) were isolated in low abundance compared to *Acetobacter* sp. and *Brettanomyces* sp., which are described in Chapter II as the ubiquitous spoilers of the culturable microbiome. LAB sp. are regularly cited as potent beer spoilage microorganisms, however these studies typically focus on a single species or strain of LAB (*Lactobacillus brevis*) and are described as primary beer spoilers irrespective of style (usually lager) or dispense method (Suzuki, 2015, Suzuki *et al.*, 2004, Tsuchiya *et al.*, 1993). Here, LAB were most prominent in lager, in agreement with the literature. However, the presence of LAB species in the culturable microflora was not nearly as abundant as predicted. Growth of microorganisms under anaerobic conditions on Raka Ray requires seven days, with the obligate anaerobes *Pectinatus* sp. or *Megasphaera* sp. requiring upwards of two-weeks. Accordingly, a major issue with spread plating is that of ‘time’, where the method favours those microorganisms which can grow faster.

6.2.2 MinION next-generation sequencing: a powerful tool for beer-spoilage microbiology

The Oxford Nanopore Technologies (ONT) MinION sequencing platform provides a (reasonably) cost-effective, portable, and rapid sequencing tool for a variety of research applications. Microbiomes can be rapidly sequenced with little equipment required. By directly extracting DNA from the environment of interest, amplifying the required targets, and sequencing, mitigated against the limitations of culture based microbiological methods. The phenomenon of non-culturability is discussed throughout this thesis, whereby viable cells *in situ* are not culturable on the selected medias. Further, the problems of changing environmental parameters such as oxygen availability, pH, and nutrition will have an impact on the identifiable microflora.

ONT MinION platform was used to sequence DNA extracted microflora from forced samples of draught beer. The accounts sampled in Chapter II were revisited during the pandemic and subject to microbiome analysis. The work produced a number of interesting insights, some were not previously reported in beer. A range of potential pathogens were identified in high abundance from seven genera: *Escherichia* sp., *Staphylococcus* sp. *Bacillus* sp., *Cutibacterium* sp., *Delfitia* sp., *Klebsiella* sp., and *Corynebacterium* sp. Although unlikely to cause significant issues, there was a diverse range of species identified including those associated with more serious infections such as *Bacillus cereus* and *Corynebacterium diphtheria*, although there is a more likely outcome these species are the non-pathogenic strains. Increased diversity of microflora also related to poor quality keg beer. The identification of *B. cereus* is in agreement with work by Dr James Mallett in his PhD thesis, where he discussed how the biofilm microflora contained both *B. cereus* and *B. thuringiensis* from draught beer

samples. It is an interesting observation to isolate planktonic *Bacillus* sp. from draught beer, as it was suggested that the location of pathogens will be in biofilms (Chapter III). As previously discussed, *B. cereus* and *S. aureus* are prominent biofilm formers, and as known pathogens, are capable of causing infections ranging from mild to fatal.

The obligate anaerobes - *Pectinatus* sp., *Megasphaera* sp., *Megamonas* sp. and *Selenomonas* sp. - were all identified during this study. Lager style beer exhibited the broadest range bacterial genera across all the styles; however, no lager account presented any pathogens – the driving element(s) behind this are not defined in this work but may prove an interesting avenue for future work. Using the conventional methods employed in Chapter II it would not be possible to identify obligate anaerobes, despite their beer-spoilage capability being well-documented (Sakamoto and Konings, 2003), as they were not specifically targeted. *Pectinatus* sp. and *Megasphaera* sp. cause numerous problems in beer production although have not previously been isolated in draught beer, most commonly through the production of turbidity and hydrogen sulphide (rotten egg aroma) (Lee *et al.*, 1980). In Chapter II it was suggested the dispense conditions for keg beers should support the presence obligate anaerobes. Using a culture-independent method successfully managed to identify a number of obligate anaerobes in lager, however, less so in ales. Juxtaposed to this, the significance of acetic acid bacteria was greatly reduced using culture-independent methods. As previously discussed, the dispense for lagers, stouts, and keg ales should have reduced AAB concentrations. Although AAB were still present their ubiquity was not, it is clear selective media, and the reintroduction of abundant oxygen was influential in their significance for the conclusions of Chapter II. AAB remained significant in cask ales due to the dispense method, alongside *Saccharomyces*

cerevisiae, residual brewing yeast required for conditioning of cask ale prior to dispense. These outcomes validate the methodology used by aligning with the expected microbiome driven by the dispense parameters.

6.2.2.1 Culture dependent *Brettanomyces* sp. ubiquity is replaced by *Pichia* sp. and *Saccharomyces* sp. *in-situ*

Ale style beers throughout this work were rich in wild yeasts. *Candida* sp., *Pichia* sp., *Saccharomyces* sp., *Brettanomyces* sp., and *Rhodotorula* sp. were isolated using conventional microbiology, with stout (ST1) proving to be a 'generalist' media supporting a broad range of wild yeasts (Chapter II). Many more yeasts were identified using ONT MinION for analysis of the draught beer microbiome. The theme that ales were 'yeastier' than the other styles continued in Chapter III, but the profile of the yeasts altered. Using the culture dependent approach, *Brettanomyces* sp. was a 'ubiquitous' spoiler in Chapter II, but with the MinION platform the yeast was less prominent. Although present in a number of samples, its significance is much reduced, with *Pichia* sp. and *Saccharomyces* sp. dominating. *Pichia* sp. were more dominant in stout beers; however, *Saccharomyces* sp. were key spoilers irrespective of style, location, or account hygiene.

Brettanomyces sp. were most dominant in cask ales when using culture-based methods. Which is counter intuitive, as cask beer innately contains brewing yeast for secondary fermentation *in situ*. Thus, the role of selective media (targeting wild yeasts), would be a significant selective pressure on the microbiome mix. Without the use of selective media, *Saccharomyces* sp. dominated the forced microbiome, accounting for 37-69%. Whilst *Brettanomyces* sp. was still present, the significance

was reduced to 2-10%. This observation demonstrates a disparity between the *in-situ* microbiome versus the culture-based approach.

6.3 Biofilms are ubiquitous, resistant, and problematic in dispense

The plethora of microorganisms identified in draught beer was outlined in Chapters II and III. The latter culture independent work revealed a fuller picture of draught beer microbiology, including several pathogens (*B. cereus, C. diphtheria* etc.), new beer spoilers (*Eremothecium gossypii*), and the ubiquity of *Pichia* and *Saccharomyces* yeasts in beer spoilage. Although pathogens are unlikely to proliferate in beer because of the hostile pH, their presence raises some fundamental questions about hygiene. The presence of pathogens is anticipated to come from human handling of surfaces (nozzles, keg couplers) that are in contact with beer dispense and become consolidated in microbial communities in biofilm. Line cleaning is essential to controlling biofilms, with regular and effective cleaning managing (but not removing) biofilm. Poor or irregular cleaning will lead to more mature biofilms which are harder to reduce. The following work focused on designing a method to quantify biofilms from dispense-sourced microorganisms, investigating the impact of style on biofilm growth rate, the impact of dispense conditions on biofilm formation, and the effectiveness of line cleaning versus biofilms.

6.3.1 Dispense parameters dictate biofilm growth rate, microflora, and lifecycle

Dispense conditions vary depending on the style of beer, notably the serving temperature which ranges from 2-6^{o}C (lager), 4-8^{o}C (stout), 6-12^{o}C (ales) and 11-14^{o}C (cask ale). Oxygen availability varies too, the dispense of results in the ingress of air promoting the growth of contaminating acetic acid bacteria. Keg beers are under a CO_2 top pressure and are dispensed with CO_2 (occasionally blended with nitrogen as

'mixed' gas) broadly creating oxygen-limited environment. The key parameters in developing the microplate method were:

- Time
- Temperature
- Nutrition
- Oxygen availability

For best-practice, line cleans are recommended on a weekly basis which was adopted as a unit time in the biofilm method. Although beer is dispensed at a range of temperatures, beer in kegs and casks is stored in 'cellars' in the UK between 12^{o}C-15^{o}C. Accordingly, and unusually, growth of biofilm in microplates were incubated at 15^{o}C. Nutrition is an important consideration as microorganisms in draught beer experience a pulsed flow of 'nutrition' as beer is dispensed (during opening hours). Accordingly, during the biofilm assay regular replenishment of beer was made in order to replicate dispense conditions. Finally, access to oxygen was restricted, using an anaerobic seal to restrict oxygen availability. Together the assay proved effective at replicating dispense conditions which was validated by the results from using this method.

6.3.1.1 Impact of time and temperature on biofilm formation

Draught beer microflora was directly impacted by the dispense temperature, and at lower temperatures there was a near linear increase in biofilm formation over a three-week period. Lager beers which are dispensed at lower temperatures (1-8^{o}C) selected for microorganisms that form biofilm at lower temperatures, with biofilm formation reducing as temperature was increased. Kegged beers were found to increase total

biofilm density at lower temperatures. The biofilm lifecycle undergoes through attachment, maturation, and dispersal. Where the conditions are not favourable (e.g., low nutrition, low temperatures) biofilm dispersal can be inhibited. Dispersal can be triggered by numerous factors, such as fresh nutrition, mechanical action, and oxygen availability (Jevons and Quain, 2021). Lager biofilms were found to be capable of significant and continued growth at low temperatures. Here it is proposed at lower temperatures, biofilms do not undergo the dispersal phase and thus continue to mature. Hence, prolonged maturation will increase the difficulty of removal and require longer or harsher line cleaning which will result in damage to the surface of the line providing new niches for microorganisms to flourish.

Conversely, cask ale style beers exhibited an increased rate of biofilm formation at higher temperatures (20-25^{o}C). Cask ale is typically served between 11-14^{o}C, enabling the 'secondary' brewing yeast to produce CO_2 ('condition'). The outcomes of the assay were validated by the data which aligned with the known environmental parameters of cask ale dispense and further describing the importance of temperature on microbiome selection and biofilm formation.

6.3.1.2 Oxygen availability influenced biofilm microflora

Oxygen availability is of interest, as it is often assumed that the restriction of oxygen will prevent microbial growth, rather than enable the proliferation of other contaminants, reducing competition and creating a favourable environment. In this work we investigated the impact of oxygen availability in the assay and its impact on biofilm formation. For kegged beers there was no significant impact on biofilm formation, suggesting the selection for facultative anaerobic yeasts and bacteria. Whereas cask ales which are dispensed with the ingress of air, there was a near 2-fold

increase in biofilm formation when oxygen was reintroduced into the assay, highlighting the selective pressures dispense have on beer microflora.

6.3.2 Biofilm recalcitrance and ineffective cleaning

This work looked at the impact of cleaning on biofilm removal, by quantifying the recovery of microorganisms after one week of incubation and measuring the total growth (planktonic and sessile) by optical density. During line cleaning, only two elements of the 'Sinner circle' are considered, time and chemical action. In order for a process to be more effective, it must consider all four elements of the 'Sinner circle', time, **temperature**, chemical action, and **mechanical action**. Biofilms are 3D structures, and at the centre highly conserved and quiescent cells are protected from the stresses of the environment. Upon exposure to the environment or fresh nutrition, these cells are resuscitated. This work identified without effective cleaning, cleaning chemicals may only remove the outer layers of a biofilms and expose conserved microorganisms which grow once beer is reintroduced into the line. It was found in this work that planktonic growth increased a week after cleaning and increased duration of cleaning exposed conserved cells which were not killed. This highlights the importance and complexities of effective cleaning, and the compromises of not using all the elements of the 'Sinner circle'. The impact of mechanical action in cleaning was demonstrated in this work, where there was a significant reduction in microorganism resuscitation. Further, the use of increased temperatures during cleaning was found to reduce regrowth, although to a lesser extent than mechanical action. Ultimately, there exists clear evidence by introducing these elements into line cleaning which will improve cleaning effectiveness, and beer quality.

6.4 Sugar profile, pH, %ABV, and organic acids are important in the 'spoilability' of beer

6.4.1 Residual sugars influence the microbiome

Residual sugars in beer range from simple sugars (glucose, fructose) to non-fermentable dextrin, which may contain over 20 glucose molecules. Lager is a more attenuated beer and does not contain high concentrations of residual sugars. On the other hand, ale style beers consist of much higher concentrations of sugars coupled with more dextrin. Attenuated styles of beer with less obvious nutrients require microorganisms with a more versatile carbohydrate metabolism. Lager spoilage has been shown in this work to promote an increased presence of *Lactobacillus* sp. which are able to use multiple carbon sources, ranging from hexoses and organic acids for energy and stress resistance (Tsau *et al.*, 1992b, Miyashita *et al.*, 2015). Whereas the complex sugars in ale, promote the primary growth of microorganisms capable of hydrolysing dextrin. Dextrin is reduced by amyloglucosidase (AMG), releasing glucose and maltose into the media. Ale styles did not show significantly higher glucose concentrations versus lager, and for prolonged spoilage it will be important to hydrolyse dextrin. *Saccharomyces* sp. dominated the microflora of keg ales, and it was subsequently shown that some identified in Chapters II and III were likely to *S. diastaticus*. Similarly, *Brettanomyces* sp. have the capacity to hydrolyse complex sugars, which aligns with the conclusions in Chapter II (Zheng *et al.*, 1994, Menoncin and Bonatto, 2019, Willaert, 2007b). Increased concentration of dextrin in ale is predicted in this work to be a key factor in microbiome selection amidst rapidly depleting glucose concentrations. Whilst conversely, the attenuated nature of lager drives the increased prominence of LAB species.

6.4.1.1 Maltose is poorly used and limits the rate of spoilage

Throughout the work maltose was a poorly used carbon source and may represent a key selective pressure on the microbiome. Irrespective of inoculum source, maltose is not a preferred carbon source for spoilage microorganisms. Maltose addition did not show any significant impact when added to lager beers (Table V-5), nor were forced beers from the trade capable of effectively using maltose (Figure V-3). Maltose transport and metabolism is not common amongst wild yeast, however brewing yeasts can use maltose via the maltose permease transport system. Further, *Lactobacillus* sp. are capable of maltose metabolism, via the maltose phosphorylase enzyme, a process by which glucose is expelled into the extracellular medium. Both the maltose permease (brewing yeasts) and the maltose phosphorylase (bacteria) occur intracellularly. Thus, the process of maltose metabolism is innately slower, compared to glucose, fructose, and sucrose. These sugars are either readily metabolised, or in the case of sucrose, cleaved by by invertase in the extracellular medium (from yeasts) to glucose and fructose. Metabolism of maltose, either via the maltose permease or maltose phosphorylase, is repressed in the presence of glucose. Lager style beers inherently contained less dextrin, but in this work, it was found there to be an abundance of maltose in a range of lagers analysed. Low dextrin levels, depleting glucose, and the presence of maltose may drive the increased presence of *Lactobacillus* sp. in lager style beers. One mechanism of maltose transport is via a maltose/H^+ symporter in *Lactobacillus* sp. and may act as an acid stress resistance mechanism in beer. Moreover, the metabolism of maltose is mediated by maltose phosphorylase that is an ATP-independent process, thus enabling the expulsion of glucose into the extracellular matrix. Glucose, as previously discussed, will repress maltose assimilation in brewing yeasts and LAB. It has been proposed the excretion

of glucose via this mechanism may act to support the proliferation of yeasts incapable of maltose breakdown. However, in the case of beer, it could further act as a method to suppress the maltose transport pathways of competitors *in situ*.

6.4.2 Spoilability is multifactorial and may be predictable

Spoilability has been a common theme of this thesis, with much time spent pondering which factors are driving subtle differences between beers of the same style. Premium lager PL9 was found to be a robust beer, in contrast to PL8 which spoiled easily irrespective of the source of the inoculum. The differences in pH between the two beers is a candidate for this difference, but beers of similarly high pH to PL8 (pH 4.35), were relatively robust (Table V-6). Thus, spoilability of a beer is multifaceted and cannot be predicted from one metric. By using some basic parameters of beer composition - pH, % ABV and residual fermentable concentration - it was possible to rank the spoilage of a selection of lagers.

It was of note that there was a strong relationship between the metabolism of pyruvate and the quality score of the beer, with an enhanced use of pyruvate relating to poorer quality beer. However, it was outside the scope of this work to determine the fate of pyruvate once transported inside the cell. It was clear however, due to other compounds present from the LCMS analysis, the key metabolic pathways across all four styles of beer during forcing was the 'superpathway of glycolysis, pyruvate dehydrogenase, TCA, and glyoxylate bypass'. Further, pyruvates role in acid stress resistance in LAB sp., may be of importance to a beers spoilability. As PL9 beer had a lower pH, its high pyruvate concentrations were possibly converted to acetoin to maintain intracellular homeostasis. Hence, with further supplementation, there was a

significant increase in spoilage as this could be directed to energy production simultaneously.

Ultimately, this work was badly impacted by the pandemic, due to the closures of public houses and university laboratories. This is regrettable. With more time, it would have been possible to investigate whether by measuring key parameters of other beer styles, whether the spoilability of beer is predictable? Using response-surface methodology, several beers would have been screened for key parameters and related to total spoilage using the forcing method. This would have revealed if there are any previously undefined relationships between pH and pyruvate, or residual fermentables and % ABV, etc.

6.5 Future considerations

The work presented here reports some new findings in the microbiology of draught beer together with development and application of methods that could be useful tools for future research. However, there remains many challenges that could with further work prove impactful within the field of beer spoilage

6.5.1 Improve clarity of yeast species data using MinION platform

As discussed within Chapter V, there was a lack diversity for the species data, suggesting either the methodology, database, or both were not sufficient for the generated data. Unfortunately, due to the cost, it was not plausible to investigate this further beyond the necessary experimentation. Future work should look to mitigate this problem. A simple approach would be to take validated species and put them through the platform and analyse the output.

6.5.2 The microbiome of dispense-sourced biofilms and the viability of the microbiome

Due to the limitations of time and money, it was not possible to investigate the microbiome of biofilms from dispense systems. It is clear draught beer biofilms are harbouring a plethora of microorganisms including pathogens and previously unidentified microorganisms. It would be invaluable to reveal the microbiome of dispense-sourced biofilms using culture-independent methods.

The value of next generation sequencing has been proven in this work, however, there is one (if not more) flaw compared to the approach of conventional microbiology. Traditional methods exploit the viability of cells, irrespective of the change of environment with the microorganisms grown on an agar plate. Further, it is recognised that DNA can be present extracellularly and remain intact in non-viable cells. Therefore, the conclusions on novel beer spoilers stop short of confirming their viability and proliferation. There are a number of investments that would prove impactful on future research in this field. The use of viability PCR dyes such as PMAxx^{TM} can specifically bind to the DNA of dead cells (dye is membrane-impermeant) and extracellular DNA and will react when exposed to blue-light. Once exposed, the dye covalently bonds to the DNA inhibiting amplification of the DNA, thus only the DNA from viable cells will be amplified.

Using the MinION NGS platform and different beer styles, future work would endeavour to isolate biofilms, sequence the microbiome, and compare to the planktonic microflora from the same line. Whilst further comparing this to the viable microbiome.

6.5.3 Investigate the impact of the ‘Sinner circle’ on cleaning at the on-trade

It was intended to tackle the question of introducing the elements of the ‘Sinner circle’ into draught beer dispense and its efficacy for removing biofilms. By using the method developed in Chapter IV, this method was be used to assess elements of the Sinner circle (time, temperature, mechanical action, and chemical action). These elements were investigated using the biofilm assay for dispense-sourced biofilms, however, optimising these four elements as a collective was not conducted. Further, should this work prove promising, applying this work to the public houses and assessing its impact on draught beer quality should be assessed.

6.5.4 The relationship between residual sugars and dominant microbiome

The work reported here proposed several theories relating the residual sugars and the microbiome of beer styles and proposing the sugars direct the microflora in different beer styles. However, how the microbiome changes in response to carbon source depletion have not been investigated. Such an approach would track changes in the microbiome during forcing, whilst tracking the concentration of fermentable sugars and dextrin.

6.5.5 Quantifying tricarboxylic acids in beers and tracking consumption versus spoilage

In this work it was only possible to track organic acid concentrations in beers using LC-MS analysis. Future work could investigate key energy producing organic acids in beer, tracking the concentrations during spoilage. In this work pyruvate assimilation has been shown to relate to the degree of spoilage during forcing. This would be worth developing and exploring further.

6.5.6 A 'spoilability' predictor – is it possible?

There remain undefined relationships between the antimicrobial elements of beers and spoilage. Using design of experiment tools such as response surface methodology, it would be fascinating to explore the relationships between these factors and the spoilability of a beer. Future work should screen a broad-spectrum of beers, quantifying fermentables and dextrin, pH, %ABV, iso-alpha-acids, organic acids concentrations, amino acids and trace nutrients (e.g. vitamins). Challenging these beers with a range of microflora from the on-trade, quantifying the defined parameters and measuring response, could reveal previous unknown relationships to spoilage. This could be further used to develop a tool to predict (and then minimise) the spoilage of beers, but importantly, assess the beers suitability to draught dispense.

6.6 Concluding remarks

Draught beer quality has been a largely ignored area in brewing and beer research. Following on from the work by Dr James Mallett, the work reported here has utilised next-generation sequencing tools, investigated beer spoilability, and draught beer biofilms. In this thesis the complexity of draught beer microbiology and the contribution of beer styles was evaluated using both culture dependent and culture independent approaches. Further, a method was developed to quantify the influence of draught dispense parameters on biofilm formation and microbiome. Finally, this work has sought to understand the environmental niches of a beer style or brand driving its spoilability. For unexpected reasons, the final section falls short on identifying the relationship between beer composition and spoilage. It is suggested that future work should develop these finding and investigate their relationships *in-situ* and the impact on spoilage, using draught beer microflora.

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Appendix

Appendix A: Chapter IV

Figure IV-3 Statistics

Appendix C: Table 1: ANOVA statistics for Figure IV-3 of Chapter IV. Assay demonstrated no variance between the means across 16 repeats of n=3 for each style.

Style	*df*	*F*	*P*	Sig. dif? ($p < 0.05$)
Lager	15	1.919	0.2681	No
Stout	15	1.281	0.3728	No
Keg Ale	15	5.171	0.0925	No
Cask Ale	15	5.391	0.1028	No

Figure IV-4 Statistics

Appendix C: Table 2: Two-way ANVOA comparing with and without nutrient replenishment from Figure IV-4. Between all styles there is a significant variance between the means.

Style	Type III Sum of Squares	*df*	Mean of Squares	Sig. dif? ($p < 0.001$)
Lager	0.0408	6	0.006800	Yes
Stout	0.02828	6	0.004713	Yes
Keg Ale	0.1185	6	0.01975	Yes
Cask Ale	0.02667	6	0.004445	Yes

0

Figure IV-7 Statistics

Appendix C: Table 3: Multiple t-tests of aerobes v anaerobes for each beer brand. Only cask ales demonstrate significant difference

Beer	Sig. dif? (*p* < 0.05)	*P* value	Mean of Aerobes (2)	Mean of Anaerobes (1)	Difference	SE of difference	*df*
PL3	No	0.371029	0.09677	0.07973	0.01703	0.01692	4
SL6	No	0.595277	0.1066	0.09727	0.0093	0.01614	4
SL10	No	0.246570	0.1833	0.1387	0.04463	0.03291	4
SC1	Yes	0.007421	0.172	0.07563	0.0964	0.01923	4
SC4	Yes	0.002144	0.2305	0.1053	0.1252	0.01779	4
ST1	No	0.452933	0.08507	0.07777	0.0073	0.008790	4
KA6	No	0.100509	0.08357	0.06913	0.01443	0.006785	4
PKA1	No	0.475206	0.07423	0.06780	0.006433	0.008173	4
C2	No	0.262799	0.1118	0.09937	0.0124	0.009523	4

Figure IV-11 Statistics

Appendix C: Table 4: T-tests were carried out to compare the impact of increased temperature on line cleaning effectiveness. Only 7^{o}C and 55^{o}C were compared, all styles showed a significant difference between the means and thus cleaning was improved at 55^{o}C

Style	Sig. dif? (*p* < 0.05)	*P* value	Mean of 7^{o}C	Mean of 55^{o}C	Difference	SE of difference	*df*	Adjusted *P* Value
Lager	Yes	0.001753	0.0693	0.0185	0.0508	0.01037	7	0.004265
Stout	Yes	0.001424	0.03242	0.01062	0.0218	0.00481	9	0.004265
Keg Ale	Yes	<0.000001	0.0478	0.009535	0.03827	0.003167	9	0.000003
Cask Ale	Yes	0.043133	0.05605	0.01231	0.04374	0.01859	9	0.043133

Figure IV-12 Statistics

Appendix C: Table 5: T-tests were carried out to compare the impact of mechanical action on the effectiveness of line cleaning. For each style there was a significant improvement in the effectiveness of line cleaning after the introduction of mechanical actions versus a static control.

Style	Sig. dif? ($p < 0.05$)	*P* value	Mean of Control	Mean of Mechanical Action	Difference	SE of difference	*df*	Adjusted *P* Value
Lager	Yes	0.001372	0.05708	0.01878	0.0383	0.007994	10	0.001847
Stout	Yes	0.000056	0.03447	0.004	0.03047	0.004571	10	0.000113
Keg Ale	Yes	0.000011	0.04572	0.01076	0.03496	0.004015	10	0.000045
Cask Ale	Yes	0.005846	0.02727	0.0124	0.01487	0.004142	10	0.005904

Appendix B: Chapter V

Table VII-1: Spoilage screen beers were supplemented with five sugars at concentrations equal to the number of potential glucose molecules. OD660 was measured after 96 hrs (n=3). [a]Largest impact on OD of the sugar across all beers. [b]Lowest impact on OD of the sugar across all beers. [c]Largest impact on the individual beer across the five sugars used. [d]Lowest performance on the individual beer across the five sugars.

	Sugar (concentration)				
Beer	**Glucose (1mM)**	**Fructose (1mM)**	**Maltose (0.5mM)**	**Sucrose (0.5mM)**	**Maltotriose (0.33mM)**
SL1	0.453 ± 0.02	0.466 ± 0.008	0.441 ± 0.018[d]	0.454 ± 0.021	0.507 ± 0.023[c]
PL8	0.512 ± 0.014[a]	0.519 ± 0.016[a]	0.526 ± 0.007[c]	0.506 ± 0.017[ad]	0.524 ± 0.017[a]
SL3	0.407 ± 0.022[d]	0.447 ± 0.018	0.44 ± 0.005	0.495 ± 0.02[c]	0.486 ± 0.016
PL9	0.405 ± 0.012	0.394 ± 0.007	0.578 ± 0.087[c]	0.322 ± 0.03	0.312 ± 0.008[d]
SL5	0.407 ± 0.015[c]	0.388 ± 0.132	0.405 ± 0.024	0.38 ± 0.063	0.369 ± 0.034[d]
SL6	0.388 ± 0.009	0.4 ± 0.037	0.371 ± 0.018[d]	0.497 ± 0.013[c]	0.496 ± 0.034
PL1	0.465 ± 0.044	0.47 ± 0.077	0.69 ± 0.026[ac]	0.445 ± 0.011	0.424 ± 0.016[d]
PL2	0.277 ± 0.034[bd]	0.309 ± 0.019[bc]	0.278 ± 0.03[b]	0.309 ± 0.009[b]	0.288 ± 0.011[b]
PL3	0.489 ± 0.025[c]	0.469 ± 0.004	0.476 ± 0.017	0.473 ± 0.019	0.447 ± 0.025[d]
PL6	0.31 ± 0.098[d]	0.387 ± 0.034	0.435 ± 0.007[c]	0.422 ± 0.011	0.406 ± 0.056

Table VII-2: Spoilage screen beers were supplemented with five sugars at concentrations equal to the number of potential glucose molecules. OD660 was measured after 96 hrs (n=3). [a]Largest impact on OD of the sugar across all beers. [b]Lowest impact on OD of the sugar across all beers. [c]Largest impact on the individual beer across the five sugars used. [d]Lowest performance on the individual beer across the five sugars.

	Sugar (concentration)				
Beer	**Glucose (100µM)**	**Fructose (100µM)**	**Maltose (50µM)**	**Sucrose (50µM)**	**Maltotriose (33µM)**
SL1	0.514 ± 0.012[ac]	0.487 ± 0.013	0.5 ± 0.014	0.471 ± 0.005	0.432 ± 0.031[d]
PL8	0.478 ± 0.013[d]	0.506 ± 0.011[a]	0.526 ± 0.006[ac]	0.496 ± 0.011	0.494 ± 0.03[a]
SL3	0.429 ± 0.009	0.434 ± 0.019	0.457 ± 0.009	0.486 ± 0.019[c]	0.195 ± 0.01[bd]
PL9	0.426 ± 0.014[c]	0.37 ± 0.018	0.424 ± 0.027	0.305 ± 0.01[bd]	0.335 ± 0.036
SL5	0.449 ± 0.019[c]	0.379 ± 0.089	0.392 ± 0.048	0.354 ± 0.04[d]	0.432 ± 0.044
SL6	0.388 ± 0.02	0.424 ± 0.032	0.344 ± 0.02[d]	0.51 ± 0.01[c]	0.46 ± 0.059
PL1	0.467 ± 0.007[c]	0.449 ± 0.009	0.427 ± 0.015	0.455 ± 0.001	0.415 ± 0.005[d]
PL2	0.345 ± 0.033[b]	0.309 ± 0.014[b]	0.303 ± 0.021[bd]	0.352 ± 0.044[c]	0.311 ± 0.016
PL3	0.475 ± 0.02	0.505 ± 0.006	0.476 ± 0.019	0.511 ± 0.03[ac]	0.429 ± 0.009[d]
PL6	0.398 ± 0.019	0.345 ± 0.027[d]	0.407 ± 0.023	0.418 ± 0.065[c]	0.41 ± 0.016

Table VII-3: Spoilage screen beers were supplemented with five sugars at concentrations equal to the number of potential glucose molecules. OD660 was measured after 96 hrs (n=3). [a]Largest impact on OD of the sugar across all beers. [b]Lowest impact on OD of the sugar across all beers. [c]Largest impact on the individual beer across the five sugars used. [d]Lowest performance on the individual beer across the five sugars.

	Sugar (concentration)				
Beer	**Glucose (10µM)**	**Fructose (10µM)**	**Maltose (5µM)**	**Sucrose (5µM)**	**Maltotriose (3.3µM)**
SL1	0.454 ± 0.024	0.456 ± 0.027	0.453 ± 0.007[d]	0.498 ± 0.014	0.448 ± 0.006[d]
PL8	0.48 ± 0.022	0.408 ± 0.029[d]	0.527 ± 0.016[a]	0.51 ± 0.019	0.481 ± 0.014
SL3	0.41 ± 0.033	0.436 ± 0.026	0.388 ± 0.044[d]	0.493 ± 0.023	0.448 ± 0.037
PL9	0.416 ± 0.006	0.412 ± 0.015	0.394 ± 0.055	0.301 ± 0.009[b]	0.286 ± 0.007[bd]
SL5	0.423 ± 0.032	0.363 ± 0.048	0.398 ± 0.021	0.392 ± 0.034	0.331 ± 0.06[d]
SL6	0.36 ± 0.03	0.354 ± 0.1	0.352 ± 0.015[d]	0.419 ± 0.162	0.502 ± 0.01[a]
PL1	0.46 ± 0.015	0.489 ± 0.029[a]	0.395 ± 0.003[d]	0.503 ± 0.039	0.437 ± 0.013
PL2	0.296 ± 0.011[b]	0.339 ± 0.081	0.295 ± 0.004[bd]	0.318 ± 0.015	0.301 ± 0.021
PL3	0.499 ± 0.03[a]	0.472 ± 0.028	0.436 ± 0.039[d]	0.511 ± 0.022[a]	0.441 ± 0.01
PL6	0.361 ± 0.018	0.309 ± 0.047[bd]	0.44 ± 0.032	0.431 ± 0.049	0.4 ± 0.063