An evaluation of the Bacillus content in beer

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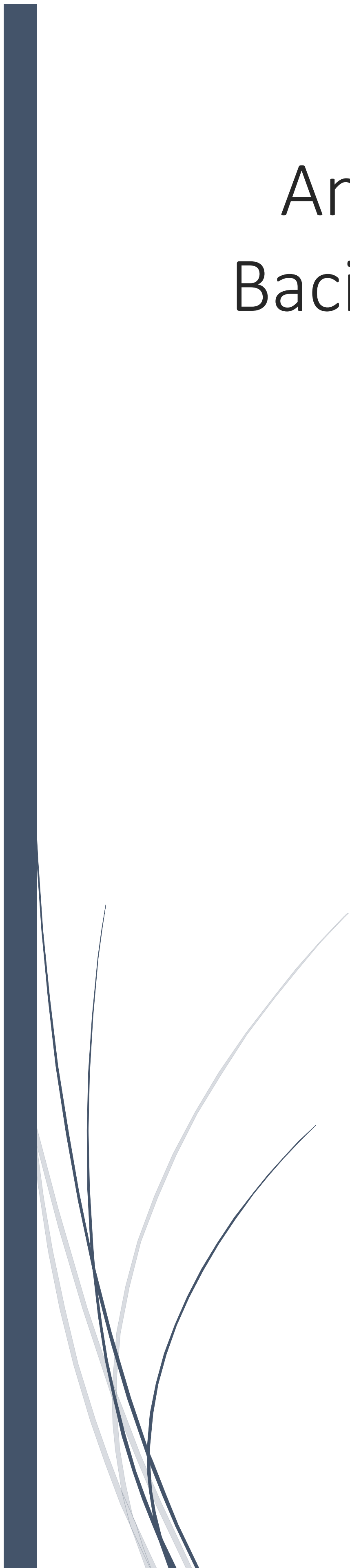

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// Abstract

The aim of this project was to investigate how *Bacillus* can survive in the finished beer. This was evaluated by producing batches of beer with starter cultures that were deemed to be interesting in terms of their *Bacillus* content. This beer, together with beer from microbreweries and store-bought beer were plated onto Bacillus Chromoselect agar, a selective agar for *Bacillus*. Samples were then taken to isolate specific colonies and send them for 16S rRNA sequencing.

The colony count was higher on the produced beer than in the other types. There were minor differences in CFU between the starter cultures. Since the pure cultures presented with radical differences, the similarity in finished beer would indicate that the malt contributes more to *Bacillus* content than the starter cultures. *Bacillus cereus*, or *B. thuringensis*, appeared to be present to a great extent in the produced beer. *B. subtilis and B. smithii* were dominant in the beer from microbreweries based on the 16S rRNA sequencing results. The produced beer had an average of $10^{8.89}$ ±0.2 CFU/330mL, the microbreweries had an average of $10^{4.6}$ ±1.23 CFU/330mL and the store-bought beer had an average of $10^{3.8}$ ±0.22 CFU/330mL. Beer from larger companies was lower than those from microbreweries, this is most likely due to filtration.

The amount of CFU present in these beers were close to what is used in probiotic supplements with *Bacillus*. Since the sequencing showed that some of the survivors were *B. subtilis* it is reasoned that it could have an effect on the microflora in the intestines. This is likely since *B. subtilis* is currently used as a probiotic supplement. There were a lot of bacteria that could not be identified below genus level. There were only two opportunistic pathogens but they were only found once.

Popular Science Summary

Bacillus bacteria are shown to be present in finished beer but what does this mean?

When you take a sip of a beer from your local microbrewery you would imagine it to be safe from a hygiene point of view with no risk of getting sick from drinking the beer. In this project the main goal was to discover if a specific family of bacteria known as *Bacillus* can be alive in beer. Some members can cause sickness but others are used as probiotics. Which ones are alive in the beer?

By checking what is growing on a specific type of nutrient solution the bacteria that are still alive in a bottle of beer are found. Judged by the way they grow and what colour they have the specific type of *Bacillus* can be identified but they were also sent to have their DNA analysed since this is a more specific method. Beer that have been produced in this project had a high number of living bacteria, of which some could probably be harmful. Meanwhile, beer from microbreweries and large commercial breweries had much less bacteria and they had other types which are probably not harmful. The beer from larger companies had lower amounts of *Bacillus* than the ones from microbreweries which could be due to that these companies do filtrations. The microbreweries do not want to do this since the yeast adds a unique flavour profile.

The yeast that were used to produce beer looked different on the nutrient plates than the finished beer. The raw wort, the liquid just before adding yeast, had a lot of growing *Bacillus* on them. These two results together point to the raw materials having more to do with how much *Bacillus* is in the beer than the original idea that the starter cultures were the source of these bacteria. Since these bacteria build a hard shell around themselves when they feel threatened or starved they will survive a lot easier and could thus be present despite the boiling that is a part of the beer production. They would also be much more resistant to the low pH and the alcohol level which usually is enough to destroy microorganisms in the beer.

The number of microorganisms found were close to the amount used in probiotics. Many of the identified samples were a type of *Bacillus* that is used as a probiotic supplement. Since the probiotic supplements have had their effects evaluated in a pill-form and not when taken as a beer it is not known how this would affect the healthy bacteria living inside of you. Judging by the probiotic ability it is not impossible that it could have an effect but there needs to be more knowledge on this subject.

1.0 Introduction

A search on PubMed for "bacillus in beer" returns only two results, out of a total of 69, which to some extent investigates *B. cereus* in finished beer and finds them to be present. *B. cereus* is of great concern during food production due to its' pathogenic nature. The first study [1], investigated how common food pathogens survived into the finished beer. The results showed that *B. cereus* spores that were inoculated at 3-4 log CFU/mL were still present after 28 days despite being stored at 5°C. A search for "bacillus in food" returns a total of 9651 results. The second study [2] found no living bacteria in finished beer other than B. cereus. In addition, there are several more members of the Bacillus genus which may very well have an adverse effect too. The question is whether food producers are more at risk for *Bacillus* contamination than beer producers or if the risks with beer containing *Bacillus* are underappreciated and if there is a need to lift the focus beyond *Bacillus cereus*. Is there a need for more research into this area in order to protect the safety of the consumers?

Beer is a fermented liquid product made from malted cereals. The production of beer largely consists of a few big companies that then proceed to sell the great majority of beer [3]. However, there is an up and rising trend among consumers in the form of microbreweries [4]. The customers desire locally produced with more ingenuity and attention to details. Sweden also has the issue of government-controlled monopoly where beer producers want to sell from the production facility but are not allowed to [5]. The larger companies develop their own yeast strains, something smaller breweries with limited funds or knowledge cannot. Due to this, they buy their strains already prepared for fermentation.

The safety of the food consumers ingest is highly prioritized by companies. There are a multitude of control systems, both forced by government (GXP, HACCP) and voluntarily available in order to create brand recognition (ISO series, TQM). The benefit of beer is that it is generally thought to be a safe product due to a plethora of microbiologically inhospitable factors. For instance, some of the processing steps required for a finished product involve heating [6]. In theory however, all of the raw materials are susceptible to microbial contamination.

During the fermentation the yeast produces high quantities of ethanol, the main goal of an alcoholic beverage. Ethanol is widely accepted as an anti-microbial agent [7]. In addition, there are several other mechanisms by which beer has bactericidal capabilities such as carbon dioxide, lack of oxygen and antimicrobial compounds from the hops. Together they all contribute to providing hurdles for growth of microorganisms. There are also some production specific steps that have the added effect of limiting microbial contamination such as boiling of wort. An illustrative perspective can be seen in *Figure 1*. Theoretically there are some noteworthy organisms capable of growing in beer. They include, but are not limited to, *Zymomonas mobilis*, which can tolerate ethanol amounts upwards of 12% [6], and the microaerophilic *Acetobacter pasteurianus* which can be a nuisance during cask-conditioning of beer. There is also some general concern for any type of organism which is tolerant to pasteurization and those that are spore-forming [6].

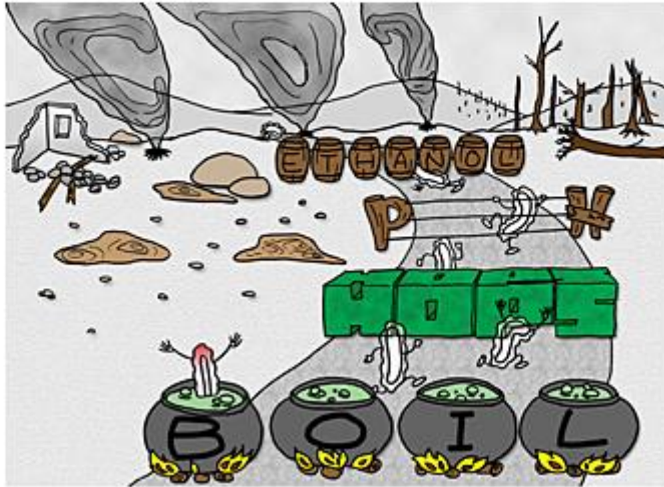

Figure 1:Illustrative description of the different hurdles that are thrown at microorganisms trying to grow in beer. [8]

1.1 Aim

This project is built upon previous research done at the department of food hygiene. During an advanced course research project, the similarity of different starter cultures from different manufacturers were investigated [9]. All of the cultures were intended to produce beer of the type “American Pale Ale” but manufactured by different suppliers. This was analysed through culturing, RAPD and then sequencing by 16S and 18S RNA. The final result showed that there were statistically significant differences in microbial composition.
Another previous project is a master’s thesis done at the department [10]. The research goal was to investigate whether or not beer starter cultures could be a potential source of contamination by foodborne pathogens. The results showed that there was a presence of several pathogenic microorganisms. Of interest is the *Bacillus* family. Especially *B. cereus* which is a spore-forming pathogen and could thus be tolerant to the processing and later on survive in the conditions in beer until ingested.

The main research goal of this master’s thesis is to investigate if anything can survive and grow from the beer. Through use of the same starter cultures that the previous master’s thesis [10] showed to harbour pathogens this will be evaluated. The cultures are analysed at the start of the project to determine the bacterial load of *Bacillus*. These cultures will then be used to brew beer. The finished beer will be evaluated for viable microorganisms which will then be sequenced for identity confirmation. There will also be beer from microbreweries as well as large-scale producers investigated.

1.2 Theoretical Background

1.2.1 The raw materials of beer

Beer essentially consist of three raw materials, malt, hops and water. There are some other ingredients for special types of beer but they are usually small-scale productions. As an example, the brewery might add chili flakes or other odd spices to produce something unique.

Barley is a rapidly maturing cereal plant that is primarily grown in order to satisfy the brewing industry. There are other types of cereals used as long as they can provide the necessary simple sugars for fermentation but barley is the most used type [11]. This plant has been cultivated for a very long time to get very specific qualities for brewing. Its composition is dominated by starch, with the grain product consisting of as much as 65% [11]. This starch is located in starch granules and is made up of amylopectin and amylose at a ratio of 3:1. Once the barley has been harvested it needs to undergo a malting process to produce malt. This process takes place in a large open area where the barley grains can rest spread out on the floor. They are exposed to water in order to increase the moisture content upwards of 40% before being drained of water and then re-moisturized. This takes place a number of times until the desired result is reached. The grain is monitored until signs of germination are observed, this is an indication of the grains becoming ready to grow into a plant from a seed. The germinating grains are moved to a kilning-room where they are exposed to warm air which aims to dry out the grains as fast as possible.

During this malting process, β-amylase is released from storage and α-amylase is synthetized. Additionally, the β-glucan endosperm wall is broken down by β-glucanases. These events add to making the starchy components into more easily nutritiously available simple sugars. The final product here is called malt and is the major raw material for beer. Additionally, the malt can be roasted in order to produce darker beer or smoked with burning peat for whiskey production.

Hops are the plants that probably are the most associated with beer due to the distinct flavour it gives. There are a great variety in the types of hops available which helps the brewer achieve uniqueness in their product. The harvested plants are dried to preserve them over a longer time [12]. The characteristic bitterness attributed to a hoppy flavour comes from the resin-part of the plant, more precisely the soft resin fraction which is further divided into the α-acid fraction and the β-acid fraction. The soft fraction consists of the resins that are hydrophobic. The α-acids precipitate when added to a methanolic lead acetate solution whereas the β-acids remain in the hydrophilic part. Major a-acids are humulone, cohumulone, adhumulone and noteworthy b-acids are lupulone, colupulone and adlupulone [12]. The only difference is the acyl side-chains. There are also the hop oils, and the components therein which have been proven to provide greatly to aroma of beer and are sometimes even solely responsible for the beer style. They consist of over 60 different compounds such as esters, ketones, aldehydes etc. The esters are probably the most important since they have a good chance of surviving the wort boiling and contribute to the aroma of the beer. Additionally, there are organosulfur compounds which have a large flavour activity.

1.2.2 The production process of beer

When the malt is received it is first milled in order to expose the constituents to enzymatic attack during the mashing step. The mashing is a mixing step of ground malt with water which has the ultimate goal of creating a liquid extract that supports yeast growth. Usually the

temperature of the water is upwards of 65°C [13]. When choosing the water for the mash there are a number of parameters to consider which could have an effect on the fermentation. These include pH, hardness, salinity etc. which are needed at optimum levels for the enzymatic activity. The aim is to have roughly 90-95% of the starch converted into fermentable sugars [13]. What now occupies the mashing vessel is referred to as the wort which is boiled in the next step.

The wort boiling is more or less a preservative step, both in terms of microbiology and chemical stability. This is also where the hops are added to be boiled together with the wort, although some brewers practice dry-hopping where hops are added later on. After the boil, the wort can be separated away from any debris left over by the hop and malt. The entire process can be divided into nine different functions [13]:

1. Hinder microbial growth in the wort. A temperature above 100°C will destroy most vegetative cells.
2. Termination of malt-derived enzymes. Some enzymes are not inactivated by the high temperatures in the mashing but they will coagulate in the wort boiling.
3. Concentrate wort by water evaporation.
4. Complete chemical reactions from malting which lowers pH. Calcium phosphate precipitation and bicarbonate ion dissociation takes place which lowers pH to between 5.4-5.2.
5. Coagulate proteins and tannins. Proteins which would otherwise have impacted on the beer characteristics are precipitated due to coagulation and pH changes.
6. Decomposition and loss of unwanted volatile components. Volatile compounds escape with the steam, both desired and undesired ones. This may be counteracted by adding hops towards the end of the boil.
7. Bittering of the wort from isomerizing hop resins. When the α- and β-acids from hops are boiled they will isomerize into iso-derivatives that are highly responsible for the bitter taste of beer.
8. Enhancing the wort colour.
9. Extract essential oils and polyphenols from hops.

The wort is cooled down after being boiled, it is now referred to as hopped wort which is ready for use as nutrition in the fermentation.

For the fermentation to perform adequately the wort needs to hold a certain set of nutrients. However, addition of supplements is seldom needed [14]. The yeast requires a carbon source which come from the disaccharides that are hydrolysed such as maltose or dextrin but also monosaccharides such as glucose. Nitrogen demand is met by the amino acids and peptides present in the wort. There are plenty of vitamins and minerals in the wort. Some essential ones include biotin, thiamine, nicotinic acid and riboflavin. Among the inorganic ions needed there is sulphur, phosphate and some smaller trace elements that are needed as well as some that act inhibitory. Oxygen is also needed to a smaller extent, more so at the beginning of the process where synthesis of sterols and unsaturated fatty acids is needed for membrane production.

The yeast is pitched into the fermentation vessel. The amount of yeast in proportion to amount of wort is important. Under or over-pitching causes improper yeast growth. The temperature

in comparison to the storage temperature of the yeast is important, to prevent shock. This is typical exhibition of the lag phase that any microorganism is put under when adjusting to a new growth media and the first few hours will be uneventful [15]. Once the yeast has adapted it enters an exponential phase of growth. The last phase of interest is the stationary phase, or the conditioning phase, where the maturation takes place. The yeast reabsorbs un-favourable compounds such as diacetyl and acetaldehyde while also flocculating and falling out. This can be referred to as the yeast cleaning up after themselves and is something that a brewer cannot simply rush past as it would ruin the product.

Fermentation usually takes place in a batch-fermenter which means that all of the wort is mixed with the yeast at once which produces a certain volume of beer. The vessel is temperature controlled depending on which type of yeast will be used as they prefer different environments. The progress of the fermentation is measured by what's known as specific gravity [16]. The gravity in beer refers to the total amount of dissolved solids in water, and in the case of beer it is sugars that are dissolved. The original gravity (OG) is an important measurement since it regulates how far the yeast can ferment the wort. It is a related to the density in the wort in comparison to water which has the value 1. This reading is taken in the brewing vessel right before the yeast is pitched. The final alcohol percentage is thus a function of the OG. As an example, the imperial stout recipe usually can have an OG of around 1.080 whereas an ordinary bitter has 1.032. The resulting alcohol content is 8-12% and 3.2-3.8% respectively and of course the imperial stout will need more malt in the original recipe. The brewers keep track of the specific gravity (SG) for the entire process to keep track of the remaining nutrients and as a way to ensure consistency between batches. The OG and SG are measured with a hydrometer where it is compared to normal water at 1.000. The more the yeast grows and consumes sugars the closer the SG will move to 1. This could be measured by other types of measurements, such as a refractometer and the Brix scale.

1.2.3 Microorganisms of interest

Usually the microorganism one would want in the beer would be brewer's yeast in the form of *Saccharomyces cerevisiae* for ale or *Saccharomyces pastorianus* for lager type beer [17]. There are some types of beer that uses bacteria together with yeast in the brewing process. In the example of sour beer, it incorporates *Lactobacillus* [18]. Aside from this special type of beer, bacteria mostly hold a place of spoilage or pathogenic nature in the brewing process. As can be summarized in *Figure 2*, there are a number of microorganisms that can hold a potential role in the quality and safety of beer as well as enabling processes needed to produce the finished product.

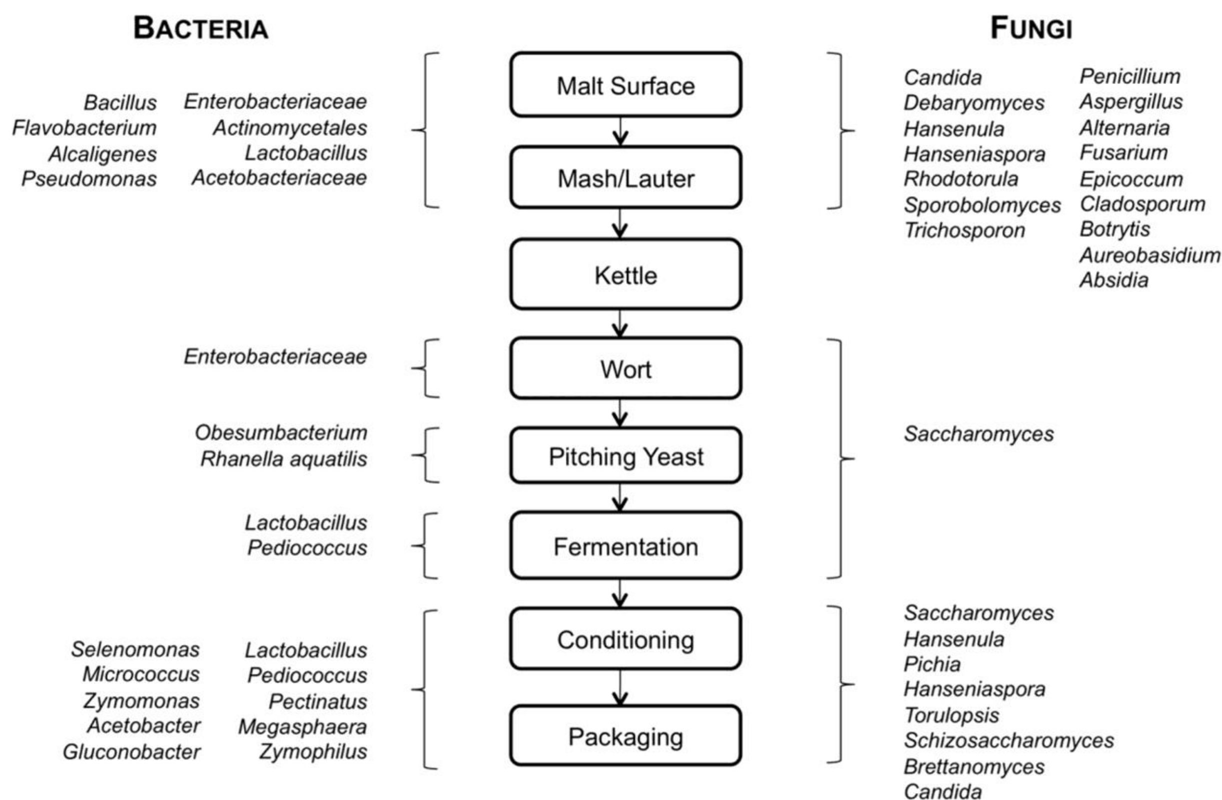

Figure 2: The numerous microorganisms reported to have been found at different stages of the brewing process. [19]

1.2.3.1 Brewer's yeast

Yeast are eukaryotic organisms, equipped with a nucleus and organelles, just like us humans but in comparison to us they are single-celled. The genus *Saccharomyces* is usually referred to as the brewer's (or baker's) yeast. It would be easy to think that the components of a beer, such as the hops or barley, is what gives it the distinct characteristics. A more accurate concept is that the specific strains of yeast used will produce the desired beer, whereas the raw materials are more or less secondary [20]. All yeast in the genus *Saccharomyces* will produce ethanol as a product of fermentative metabolism [21]. However, usually there is a preference for two distinct strains when it comes to brewing beer. There is a distinction between these two types of yeast. One is the top fermenting (ale) *Saccharomyces cerevisiae* and the other is the bottom fermenting (lager) *Saccharomyces pastorianus*. First and foremost, the lager-type is the most prevalent with roughly 90% of worldwide production belonging to this [22]. It is usually brewed at temperatures around 8-15°C and tends to flocculate to a great extent in the bottom of the brewing vessel. Flocculation is the behaviour of yeast to aggregate together [22]. In contrast to the lager-type, the ale-type prefers higher temperatures of 16-24°C and has a lesser degree of flocculation. The higher temperature used in ale-type will cause a greater degree of ester production which in turn affects the taste and smell. The higher temperature enables a faster fermentation and the top-fermenting behaviour enables easier re-collection of the yeast. Currently, the distinction of top and bottom-fermenting is less useful as some ales function as bottom-fermenters. The best division is in temperature of the fermentation process as well as the ability of *S. pastorianus* to ferment the di-saccharide melibiose [23].

In essence, the reaction that is taking place converts simple sugars from the wort into energy, ethanol and CO_2. The CO_2 here giving rise to a natural carbonation [23]. It works according to the following formula: $Glucose + 2\ ADP + 2\ Phosphate \rightarrow 2\ Ethanol + 2\ CO_2 + 2\ ATP$. Simple sugars are metabolized into pyruvate by the first step of glycolysis but without oxygen the fermentative pathway is the next step. This happens instead of further processing in the tricarboxylic acid cycle which would have generated more energy and less carbon by-products. Fermentation, or anaerobic work, is the same pathway, or rather a different version of it, that creates lactic acid when muscles get sore during intense exercise in humans. By keeping the beer in a sealed container after yeast is added, the growing yeast is forced into fermentative metabolism due to lack of new oxygen. However, eventually the level of ethanol will reach a toxic threshold around 10-15% (strain dependant), which is why most beer or wine never go above this range [24].

In the metabolism there a number of compounds produced that affect the flavour of the beer [21]. It is fairly well agreed upon that the different types of yeast do not produce distinct compounds but produce them in different amounts. The fermentation conditions can also have an impact on the amounts produced. Of importance are the vicinal diketones, diacetyl and pentane-dione [25]. They are produced as a result from metabolic intermediates leaking into the wort and being degraded non-enzymatically. The downside to this happening is that these diketones have a buttery or honey-like character which can be undesired. Yeast will to some extent use these ketones in their metabolism to produce higher alcohols, effectively removing the unwanted taste [21]. Another important aspect of flavour is the ester and phenol production. These types of compounds are both attributed to distinct flavours. Esters are usually linked to fruity aromas and taste whereas phenols usually show up as clove-like, medicinal or smoky [26]. These compounds are produced by the metabolism of growing yeast but can also be brought in from the raw materials such as water, malt and wort or even by improper cleaning with chlorine and bromine. In *Figure 3*, the different metabolic pathways as well as their respective excreted products are shown.

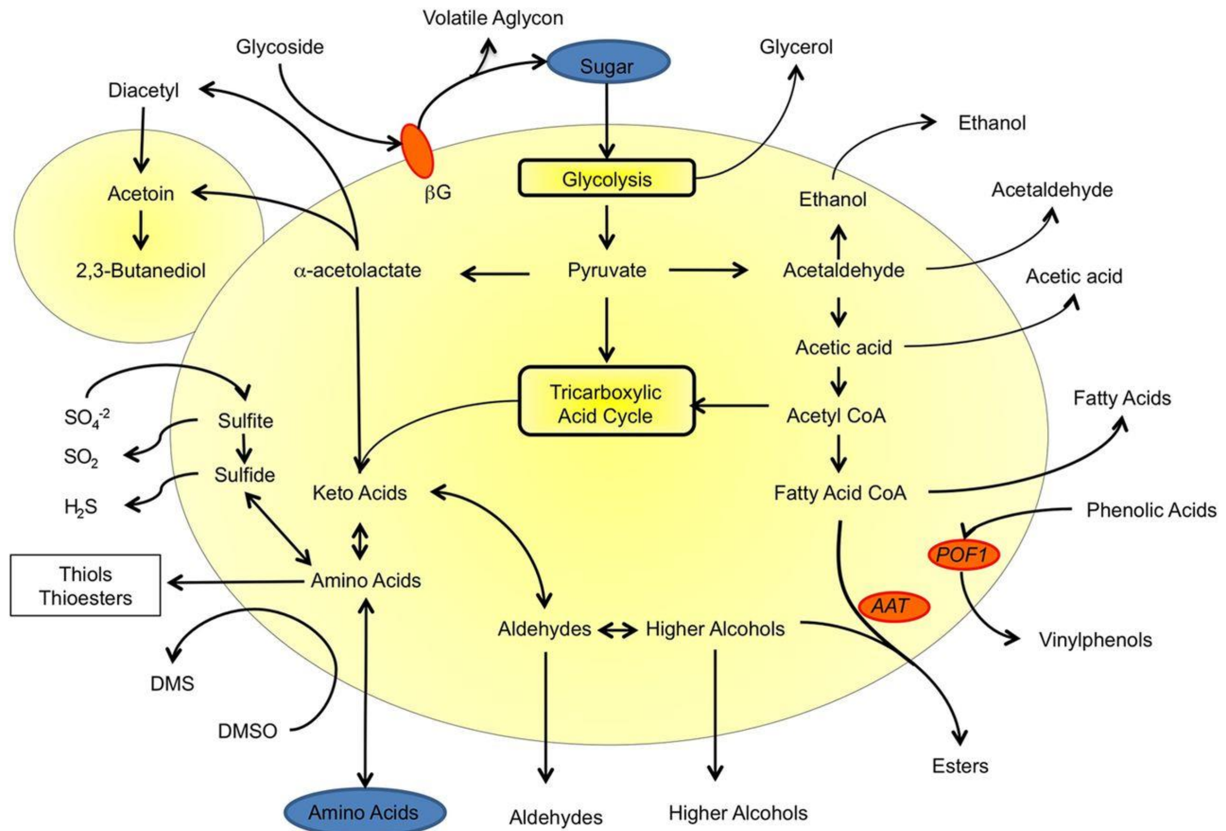

Figure 3: The different ways in which yeast metabolism produces compounds that can affect the environment in and taste of beer [21].

1.2.3.2 Other relevant microorganisms

Foodborne outbreaks are an ever-present risk with almost any type of food item because they contain nutrients that we ourselves need and thus nutrients that pathogens can use to grow. If a product is sold that causes an unbreak it can even cause a company to go bankrupt [27]. It may even cause problems for other companies due to customers becoming more afraid of a certain product category. In the developing world the cost of outbreaks may be even greater than pure economics. Usually a foodborne infection causes diarrhoea which then leads to malnutrition due to improper absorption of nutrients, malnutrition then leads to increased risk of infection and even more diarrhoea. This is what's known as the malnutrition and diarrhoea cycle [27]. All in all, foodborne outbreaks due to bacteria and other pathogens are costly for society, the individual and the companies.

The *Bacillus* family are characterized by aerobic, sporulating rod-shaped, gram-negative bacteria. With the exception of *B. subtilis* they are mostly non-motile. They are usually saprophytic, which means that they mainly consume nutrients from "dead" sources. Some are known to infect humans or other animals, with *B. anthracis* and *B. cereus* being noteworthy while some others only affect insects. They have a wide range of useable applications and metabolic by-products; antibiotics and enzymes are widely purified from them. Additionally, the obligate thermophile *B. stearothermophilus* is used to test heat sterilization procedures [28].

The *Bacillus* family is probably most known for *B. anthracis.* It has a well-deserved bad reputation due to causing the very severe disease referred to as anthrax (Swedish: Mjältbrand). This disease state is caused by toxin production from dormant spores that have

been turned into vegetative cells and can result in skin, lung and intestinal infections which lead to septicaemia, oedema and it is not uncommon with fatal results. The spore-form of this bacteria can even be sent through a letter and spread the disease to everybody who comes into contact with it, as happened in the US in early 2000s [29]. Thankfully, outbreaks of *B. anthracis* are quite rare due to regular control methods of livestock which are the most common carriers [30, 31]. Additionally, the development of a human and animal vaccine lowers the risk. In terms of food safety *B. anthracis* is of course a problem but the occurrence is as mentioned very low.

The remainder of the genus can be involved in a multitude of clinical complications. It will most likely start out as a low-grade infection of some organ or tissue but can, if left untreated, proceed to systemic infections known as bacteraemia/septicaemia which has severe consequences and could lead to septic shock. Most vulnerable are of course those with compromised immunity or already existing conditions which raise the susceptibility such as alcoholism or diabetes. It could also be a result of an unaided infection in an otherwise healthy individual [28]. A noteworthy organism is *B. cereus.* It is a toxin producing spore-former with resistance to normal heat treatments that are usually applied to food production. An infective dose is between 10^5-10^8 CFU [28]. Once ingested it can follow one of two possible routes. Diarrhoea and abdominal pain can set in around 8-16 hours after ingestion of spores with fluid loss as a result. The other way it can manifest is through vomiting and general nausea. The outcome of ingestion depends on the state the cells are in. If they are in spore form they will become vegetative in the intestines and cause an immune response there which causes diarrhoea whereas already formed toxins in the food will induce vomiting once they reach the stomach. The other members of the genus do not produce a toxin, in contrast to *B. cereus*, but will still produce very similar clinical effects however, the toxicity of these are much less understood or investigated.

While some members of *Bacillus* are known for harmful abilities there are those that are being applied in probiotic solutions, either by themselves or in a mixture with other probiotics such as *Lactobacillus* and *Bifidobacterium* [32]. Most notably associated with probiotics are *B. subtilis* and *B. coagulans*. They are usually administered in amounts between 10^7-10^{10} CFU/capsule. In clinical studies these types of probiotic bacteria have among other things reduced IBS severity and caused a reduction in antibiotic-associated diarrhoea.

The main reason for their infectious nature is the ability to sporulate, one that they share with *Clostridia sp.,* and that they can survive for decades, if not centuries, in this state while also being highly resistant to normal disinfection methods. Sporulation is the process by which bacteria encapsulate themselves in order to survive an unfavourable environment. This is a protection mechanism that changes them from the normal vegetative state, where the metabolism is fully activated and growth by cellular fission takes place, to the spore-state where metabolism is dormant and the DNA and cell is protected from radiological, chemical and biological threats [33]. This means the bacteria can survive where normal bacteria would not and it is therefore of great use in an evolutionary perspective. The spore formation is initiated when the cell experiences a starvation period from any of the essential nutrients; carbon, nitrogen or phosphorous. Instead of the normal binary fission the mother cell creates what is known as a pre-spore, as can be seen in *Figure 4*. The mother cell encapsulates the pre-spore within itself where it becomes a protoplast. The mother cell then coats the pre-spore with layers of cell wall. The normal cell wall becomes thicker in its peptidoglycan layer and

outside of this there also comes the addition of a multi-layered protein shell. This process is terminated by the lysis of the mother cell and a newly formed spore is released although it still continues to increase the amount of cross-linking in the protein shell sometime after lysis.

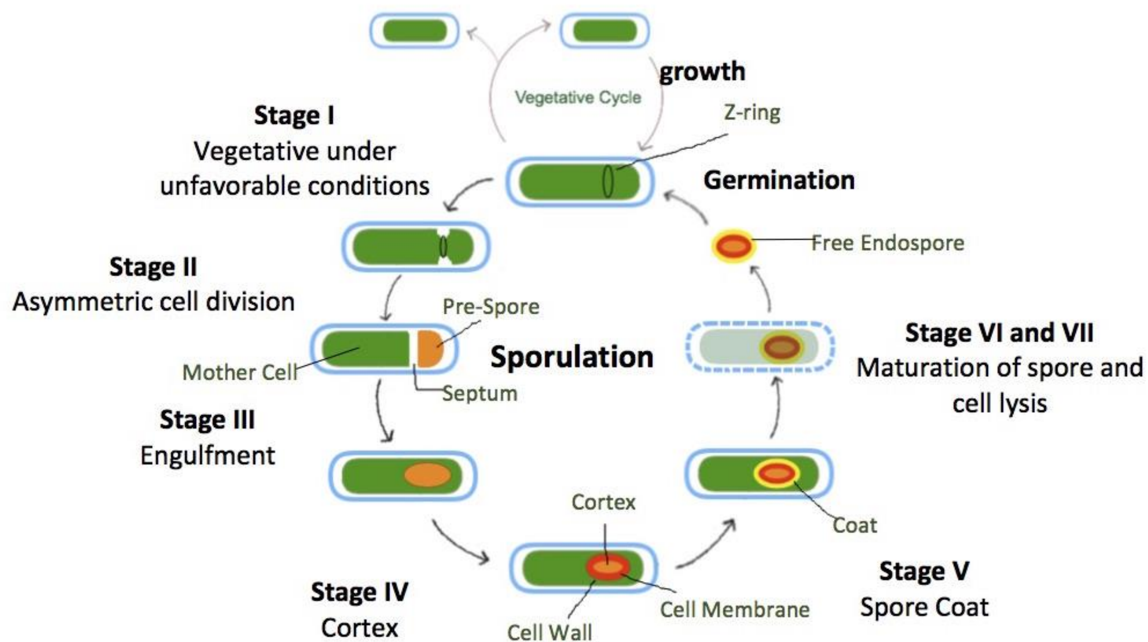

Figure 4: The different stages of sporulation in bacteria when reacting to unfavorable conditions [34].

1.2.3.3 Inhibition of microbial growth in beer

Beer has several factors that all contribute to making it inhospitable to microbial growth, many produced as a result from the fermentation metabolism, not least due to the yeast consuming nearly all nutrients during the fermentation process. There are also several heating steps and also a total boil in the processing which usually means microorganisms are destroyed.

Ethanol is perhaps one of the best known anti-microbial agents. It's used to maintain sterility in research labs everywhere. The anti-microbial effects were described as early as the mid-1900s [35] where higher ethanol contents were shown to act increasingly inhibitory. The main reason why ethanol has such a good effect in beer is due to the already existing low pH. Ethanol weakens the cell membranes by which pH homeostasis becomes increasingly hard to maintain [8]. This has been observed at normal beer alcohol percentages of 5%.

Next to ethanol content, pH is probably the second to largest contributor to inhibition. As with a lot of types of food items in general the pH can usually be a big predictor of shelf-life. A low pH, in bear usually between 3.4-4.8, will cause intracellular acidification in microorganisms [8]. Since so many of the enzymatic pathways are reliant on a constant pH this will have large effects on growth. Depending on the species and strain there are methods to alleviate themselves of pH problems mainly by pumping out cations which reduces the membrane potential. However, this is highly dependent on ATP to carry out the transport which means that the cells can only keep up the pH reduction methods for so long before they will die from lack of energy. The organism will waste all energy on equalizing pH that it can't

manage basic metabolism needs. The pH also works synergistically with hop acids and enables them to exert their effect much better [36].

The inhibition by the iso-α acids from hops works in several ways [37]. However, the effect seems to be mainly, or entirely, directed at gram-positive bacteria such as *Lactobacillus*. Against *Bacillus*, another gram-positive bacterium, it has been observed to induce leakage of cell membranes, thus inhibiting active transport of nutrients [8]. Another inhibitory effect is through action as a proton ionophore. This dissipates the ion gradients across the cytoplasmic membrane and causes unfavourable changes in intracellular pH. Even more so if the extracellular pH is already low. Additionally, it disrupts the ability of the cell to take in leucine which is essential for normal cell function and can eventually have cytotoxic effects. There are some specific strains of *Lactobacillus* that have a special gene which renders the hop-effect useless [38].

Due to the fermentation process itself and if the bottling is done correctly there should be no, or very little, O_2 available for bacteria to grow which shifts the spoilage and pathogens to those that can survive anaerobically. In general, a lower O2 level reduces the chance of pathogenic growth [39]. The general equilibrium between CO_2 and its corresponding acid, H_2CO_3, lowers the pH when more CO_2 is produced in the fermentation (or added through extra carbonation) [8]. Furthermore, it has been shown that lower levels of dissolved CO_2 lead to shortened shelf-life [40, 41].

In addition to the various intrinsic factors of beer there are some extrinsic from the processing methods. The largest contributor to microbial death in processing is the wort boil where the nutrient rich solution is boiled for an extended period of time. In theory this will kill spores as well as vegetative cells [8]. The heating during mashing is not as severe and will not be as devastating for the organisms. Since these steps take place before the fermentation it might be worth applying a quick pasteurization or filtration process after the fermentation is performed. There could be contamination from the starter cultures which are not dealt with by the wort boil.

1.2.4 Methods

When investigating if there are viable microorganisms in a food item or on a surface the usual go-to method is to take a sample and apply it onto an agar plate. The main benefit is the gel-like structure even at low concentrations of agar in water. Thus, the gel has properties of water while still being more rigid and easier to work with [42]. There are essentially two types of gels. The general-purpose agar which allows growth of a wide range of organisms whereas the selective media are more discriminatory and allow only specific bacteria to grow. The general-purpose may lack nutrients that hinder growth of specific types of bacteria but the selective have added compounds that actively inhibit growth of undesired organisms. Additionally, there is the elective media which allows specific organisms to grow faster than others while not necessarily inhibiting them.

Since this project is based on the previous work done with starter cultures, the preferable media are those used in these studies. For isolation and enumeration of *Bacillus*, the Bacillus Chromoselect agar is used. This media allows different *Bacillus* species to be distinguished from one another by colour, shape and size based on what biochemical reactions they can facilitate. The fact sheet provided by SIGMA-Aldrich for Bacillus Bacillus Chromoselect agar shows what the different kinds of structures/colours means in terms of what type of bacteria

can produce them. *Bacillus subtilis* can produce green to light green colonies. *B. cereus* and *B. thuringensis* produces light blue to blue colonies with *B. thuringensis* having irregular margins while *B. cereus* has smooth ones. *B. megaterium* is responsible for production of yellow mucoid colonies while *Enterococcus faecalis* and *Staphylococcus aureus* also produce yellow colonies but without the mucoid appearance. Finally, *B. coagulans* produce small pinkish colonies [43]. Tryptic soy agar (TSA) is a general growth agar that allows a total viable count of all bacteria [44]. Violet red bile dextrose (VRBD) agar is commonly used for gram-negative bacteria and generally those in the *Enterobacteriaceae* family [45]. Finally, for enumeration of the brewers yeast the malt agar is used due to its lowered pH which reduces growth of bacteria while allowing fungi to flourish [46].

In order to determine what type of *Bacillus* is growing on the media/ in the food item genomic sequencing can be done. One way of doing it is through what is known as 16S rRNA sequencing which is based on differences present on ribosomal DNA among bacteria. rRNA is a part of the ribosomes which, together with ribosomal proteins, assist with translation of RNA to proteins. One major contributing factor to phylogeny arrangements is metabolic abilities and processes. Since protein synthesis is a highly central part in the metabolism, due to the production of the necessary enzymes, the ribosomal constituents will also differ together with these metabolic pathways. Thus, the DNA which is transcribed into this rRNA will also differ and sequencing can be done by analysing this specific part of the bacterial DNA [47]. These differences are evolutionary linked, which means that the phylogenetic tree can be reconstructed by 16S sequencing since the relationship between different prokaryotes can be investigated. Another great advantage to this method is that there are multiple whole genome databases established as well as more specific ones with the 16S genes [48]. This means that a novel isolate can be matched with a known sample and thus the identity of the therein existing bacteria be revealed. The gene is also involving both conserved and variable regions which enables creation of highly specific or universal primers for purification.

There is a caveat to bear in mind when applying this technique. Some bacteria can be hard to distinguish this way, such as *B. globisporus* and *B. psychophilus* which have >99,5% similarity, or type strains of *Edwardsiella* which have 99,35-99,81% similarity but are easily distinguished biochemically [49]. In the *Bacillus* family, worth noting is also the difficulty of identifying *B. cereus* by 16S sequencing.

The Polymerase Chain Reaction (PCR) is one of the most useable techniques in molecular biology. The range of use varies greatly, from the forensic analyst trying to amplify a DNA sample taken from a crime scene in order to have more DNA for further testing to the microbiologist wanting to investigate whether or not the cells have become transfected with the correct gene sequence [50]. The basis for PCR is the normal replication mechanism that is constantly being used in all living cells. With knowledge of a few nucleotides on both borders of the region of interest primers can be constructed which direct DNA to be copied between them. The process relies on a heat-stable polymerase, the taq-polymerase which has been isolated from thermophilic organisms. The procedure is quite straight forward. A mixture of the polymerase, oligonucleotide primers and nucleotides, as well as the DNA to be investigated, are put in a thermal cycler. A heating and cooling protocol is followed which has a number of effects. First and foremost, without a topoisomerase available, the strands must be separated which is induced by breaking the hydrogen bonds with a heating to 94°C during the denaturation step. This enables the primers to anneal to the DNA once temperature is

lowered to 50-60°C. The now annealed primers allow the polymerase to attach and start the extension process once temperature is up to 74°C [51]. These three steps, denaturation, annealing and extension, are repeated until a sufficient amount of amplification is generated. In *Figure 4* the general procedure is shown with the amplification of a small DNA fragment. Designing of primers is extremely important. Too short might induce hybridization on other parts than the desired sequence whereas too long might cause problems with the cycling scheme.

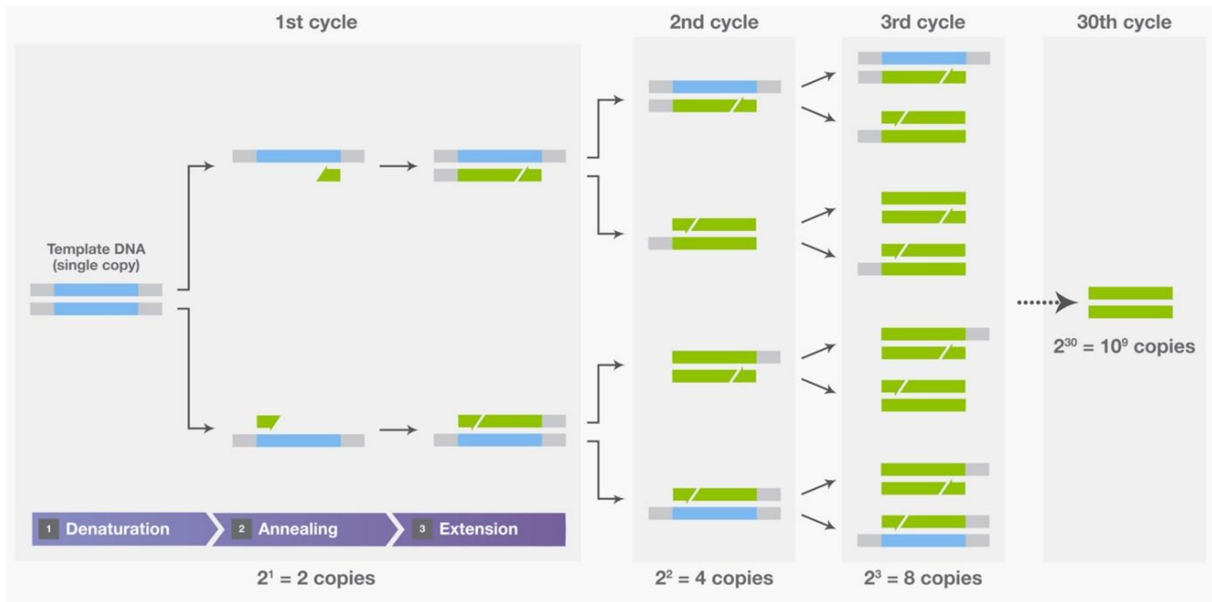

Figure 5: General result from a PCR run with the different stages of action. [51]

3.0 Material and Methods

3.1 Analysis of starter cultures

The starter cultures used for this project were the 7 from the previous work that had exhibited the greatest amount of contamination by *Bacillus* [10]. These starter cultures are all commercially available and used by both microbreweries as well as home-brewers. The cultures are summarized in *Table 1*. There are five freeze-dried yeast cultures and two liquid cultures with a suggested wort amount of 20L. One of the liquid cultures, Wyeast 3056, has a nutrient pack in it for a smaller lag-phase in fermentation. Three bags of each culture were purchased from shop.humle.se and stored in the fridge at 8°C.

Table 1: Overview of the different starter cultures used in the project.

Product name	Manufacturer	Culture type	Package size
Safale S-04	Fermentis	Freeze-dried	11.5g
Saflager W-34/70	Fermentis	Freeze-dried	11.5g
Empire Ale M15	Mangrove Jack	Freeze-dried	10g
Belgian wit M21	Mangrove Jack	Freeze-dried	10g
Californian lager M54	Mangrove Jack	Freeze-dried	10g
Pilsner Lager WLP800	White Labs	Freeze-dried	40mL
Bavarian Wheat Blend 3056	Wyeast	Freeze-dried	125mL

The starter cultures were plated onto Bacillus Chromoselect agar (referred to as Bacillus Chromoselect) (Sigma-Aldrich, St. Louis, USA) and VRBD agar (Merck Millipore, Darmstadt, Germany).. The procedure for enumeration of starter cultures onto agar plates was taken from the previous project [10]. 0.33g of the dry cultures was aseptically taken from each bag, in each triplicate set, into a sterile dilution tube with 9mL of peptone water whereas 0.33mL of the liquid cultures was taken after a thorough mixing of the bag. Peptone water is a mixture of 1g bacterial peptone with 8.5g NaCl per litre of water that is then autoclaved in the dilution tubes. The reason for taking 0.33g or 0.33mL from each bag of the triplicates is to create a more representative image of the microbrewers production. Since a microbrewer will most assuredly brew a batch greater than 20L, they will require more than one bag (which the cooperating brewers confirmed to be true). Due to this, an average of three bags was used to get a result more closely linked to the real situation of one type of starter culture. The tube with starter culture and peptone water was then vortexed thoroughly before 1mL of liquid was transferred into another tube with 9mL peptone water. This was done until tubes with 1:1000000 (10^6) dilution was produced.

For the plating of the dilution series, 0.1mL of the culture was transferred onto an agar plate. Based on the total colony count in the previous project [10], the dilutions chosen for Bacillus Chromoselect agar was 10^{-2} and 10^{-6} whereas for the VRBD agar 10^{-1} and 10^{-2} were chosen. A first run with 10^{-1} of gave too high colony count on Bacillus Chromoselect which justified a higher dilution of the starter cultures. The Bacillus Chromoselect plates were put in an incubator at 30°C for 48h whereas the VRBD were at 37°C for 24h. After this incubation period, three of the cultures were chosen to produce beer from. They were chosen to get a broad amount of different colony shapes, colours etc. into the brewing process. The cultures that were chosen and the reasons were: Safale S-04 due to the huge amount of yellow/white

dot colonies present on 10^{-2} dilution; Pilsner Lager WLP800 due to the presence of yellow/white dots as well as the large mucus colonies; Bavarian Wheat Blend 3056 due to the green appearance of the media.

Additionally, the plates with growth were photographed and stored. These photos were then used for future references.

3.2 The brewing process

The finished beer should be as representative as possible of what could be done in the microbrewery as well as in the process of a home-brewer. Every part of the brewing process up until fermentation was performed in the 20-Litre Braumeister automated system [52]. The manual parts of adding malt and setting up the equipment followed strictly the hand-book developed by the manufacturer. The brewing equipment automates the different temperature steps in the mashing and wort boil to reduce human error in release of the necessary nutrients. The program used was the one described in the guide-book that accompanied the equipment.

1. 70 min, 63℃
2. 5 min, 73℃
3. 5 min, 78℃
4. Wort boil, 70 min, 102℃

The raw materials used were purchased from Humlegården.se. The malt was 4.5kg of Pale Ale Malt [53] and 0.25kg of Cara 120 [54]. Finally, the hop used was 15g Polaris with 16.9% alpha-acid [55]. The amount was calculated based on a desire to stay in the middle of the standard IBU ranges (Beer bitterness) which typically are between 10-50 [56]. This beer would situate itself around 33 using a specific calculator for IBU [57].

After the boil the wort was separated into 20 fermentation bottles with 900mL in each. These bottles [58] had been left under a UV-light, the same one used for maintaining sterility in a PCR room, for 4 hours to sterilize them. 18 of these bottles had yeast cultures added to them with 6 bottles designated for each yeast culture type of S-04, Pilsner Lager WLP800 and Bavarian Wheat Blend 3056. The amount added of each yeast culture was calculated to be 1.875mL for Bavarian Wheat Blend 3056, 0.61mL for Pilsner Lager WLP800 and 0.1725g for S-04. This could be calculated based on one bag being intended for 20L of wort and scaling this down to 0.9L. These amounts were taken from each bag in a triplicate set and then added to the fermentation bottle with the wort. So, to clarify with an example: Bag 1, Bag 2, Bag 3 of Pilsner Lager WLP800 had 0.61mL taken from each, mixed thoroughly and then added to the Bottle 1 of Pilsner Lager WLP800 fermentation. The same procedure was repeated for Bottle 2-6 and then applied similarly with other, above mentioned, amounts for Bavarian Wheat Blend 3056 and S-04.

The two bottles left over out of the 20 was left untouched and kept with the other bottles as a control sample of pure wort, additionally, the OG was measured by brix value to be 13.5° as a reference for checking how well the fermentation has proceeded later. The normal values for finished beers are between 1.000-1.020 FG or 0-5.1° brix [59, 60].

For the Bavarian Wheat Blend 3056 there was an extra step required due to the smack pack nutrient bag. This smack pack was broken and the bag was allowed to rest for an hour to

allow the yeast to adapt and then mixed thoroughly before being added to the fermentation bottle as per above mentioned method.

The bottles, with added yeast, were then stored in a climate-controlled room at 21°C until there was no more bubbles forming in the fermentation tubes placed in the bottles. Cessation of bubbling was taken as an indication that the fermentation was over.

3.3 Analysing the viability of bacteria in beer

After 6 days the bubbling had stopped in all of the fermentation bottles and thus they were capped and moved into a fridge storage at 8°C. 10mL samples were taken from each bottle into sterile stubs for storage and further analysis. 0.1mL of these samples were transferred, undiluted, onto agar plates and spread by way of glass beads. The agars used were; Bacillus Chromoselect (Sigma-Aldrich, St Louis, USA), TSA (Sigma-Aldrich, St Louis, USA), VRBD, Malt (Sigma-Aldrich, St Louis, USA), Rogosa (Sigma-Aldrich, St Louis, USA), The temperature as well as time spent in the incubator can be seen in *Table 2*. All of the agar types were prepared according to the label on the container which means some were autoclaved and some were only boiled to mix.

Additionally, the brix value and pH were measured.

Table 2: Types of agar used for analysis of brewed beer.

Agar	**Incubation temperature**	**Duration**	**Purpose of agar**
Bacillus Chromoselect	30°C	48h	*Bacillus*
TSA	30°C	72h	Total viable count
Rogosa	37°C	72h	*Lactobacillus sp.*
Malt	Room temp	7 days	Fungi
VRBD	37°C	24h	*Enterobacteriaceae*

After the incubation period the plates were checked and any colonies counted. All plates were photographed for easier referencing later. The plates with Bacillus Chromoselect were decided to be redone, as the undiluted samples were too rich in *Bacillus* to distinguish anything. Decision was made to redo the brewed beer samples on Bacillus Chromoselect with 10^{-3} and 10^{-4} dilutions.

Based on the plates with Bacillus Chromoselect agar, 10 colonies were chosen at random from each type of yeast culture to isolate for possible sequencing later on. The origin plate as well as colour/shape of the colony was noted down for future reference. These colonies were put on fresh Bacillus Chromoselect agar and incubated per *Table 2* directions. After this incubation time they were checked for purity to make sure only one type of bacteria was growing on the plate. The plate was then scraped clean with a loop and deposited into Hogness freezing media, recipe displayed in *Table 3*, which had been prepared and autoclaved. These samples were then kept in -80°C freezer until needed.

Table 3: The contents of Hogness freezing media.

Chemical	Amount
K2HPO4	0.17g
KH2PO4	0.04g
Tri-sodium-citrate-dihydrate	0.3g
MgSO4*7H2O	0.05g
Glycerol 99.5%	24.3mL
Water	175mL

Beer from microbreweries was donated to the project and it was made sure that the beer was "fit for consumption" (beer that was otherwise to be sold) in order to investigate the *Bacillus* load in beer that costumers would consume. This meant that the beer had been deemed, by the microbreweries, to have the correct taste and odour. The beers were not pasteurized or filtered. Two types of beer from 5 microbreweries were analysed and three bottles of each were used for additional statistical stability. In addition to the microbrewery beer there were 4 more types of beer that were bought from Systembolaget. These were: two beer types that were produced by large well-known companies; one alcohol free beer that was produced by one of these companies; one sour-beer which had *Lactobacillus* and lowered pH to 3.3. These were also analysed in triplicate bottles. The dilutions used for microbrewery beer were 0, 10^{-1} and 10^{-3} while the other beers had 10^{-2} instead of 10^{-3}.

The analysis of real beer produced by companies followed the same one as for the brewed beer except that it was only plated on Bacillus Chromoselect and VRBD. To ensure the integrity of any beer and companies involved the samples and results were labelled as B#: #. Here B# represents the brewery and the second # represents the beer type. Additionally, during all plating there were duplicates of each sample made and there was a duplicate control sample with nothing added to make sure the agar itself was not contaminated. Following the results, 10 colonies from each type of beer were isolated for sequencing.

3.4 Identification of colonies

The isolates were re-plated on new Bacillus Chromoselect agar to ensure purity. The growth was transferred to 1.5mL tubes with 1mL of autoclaved MilliQ water. Small glass beads that had been sterilized under UV light were added. These tubes were then shaken for 45 minutes to disturb the cell membranes and release DNA. After having been shaken, the tubes were centrifuged at 14G for 1 minute.

A PCR reaction was done to amplify the 16S regions of the DNA. The master mix used had the following reagents for 1 sample of DNA: 18.375µL nuclease free water, 2.5µL TopTaq buffer, 0.5µL dNTP mix, 0.5µL ENV1 primer (Seq: AGAGTTTGATZZTGGCTCAG), 0.5µL ENV2 primer (Seq: CGGZTACCTTGTTACGACTT) and 0.125µL Taq polymerase. To this master mix 2.5µL of sample was added. The primers were produced by Eurofins and target the 16S region for amplification in all types of bacteria. The dNTP mix and Toptaq were produced by Qiagen. All mixtures were diluted according to manufacturers instructions. The PCR was run for 25 cycles according to the TopTaq25 program which is shown in *Table*

4. Here, the steps 2-4 are repeated 25 times. The PCR result was confirmed by gel electrophoresis to see if the DNA in the PCR product was of the correct size. After confirmation, the PCR product was loaded onto a 96 well plate and sent off for Sanger-sequencing at Eurofins.

The sequencing results were examined to see the quality of them. Some sequences needed to be cut before BLAST analysis but the great majority used pre-cut suggestions from the sequencer where optimal certainty of the DNA sequence where presented. The sequences were edited with the Bioedit software. The sequences were submitted to http://metasystems.riken.jp/grd/ where the resulting identification along with id % recorded. Isolates were positively identified on species level if only one species was present above 99% whereas between 95-99% identity only indicated the correct genus. Isolates with identity scores below 95% were discarded. Additionally, any resulting sequences which were below 500 bp long were discarded as to avoid uncertain interpretation of the results and those which did not present a clear read were also discarded.

Table 4: Temperature schedule in the TOPTAQ25 program which was used to enumerate the 16S rRNA gene when preparing for sequencing.

Degrees [°C]	Time at specific temperature [min]
94	3
94	1
50	0.75
72	2
72	10
4	After completion of all cycles

4.0 Results

4.1 Viable count of starter cultures

The result showed that the dry starter cultures were very similar to each other in appearance on the Bacillus Chromoselect agar. They all exhibited streaks of white colonies on the lowest dilution which turned out to be yellow/dots when diluted further. Blue dots were present on some of them but not to a large extent. The liquid cultures were different from each other when diluted more but at 10^{-1} they looked fairly similar. At 10^{-2} dilution the Pilsner Lager WLP800 showed similarities to the dry yeast cultures with lots of dot colonies but at 10^{-6} there was a single large mucus structure present which did not occur on the dry ones. The Bavarian Wheat Blend 3056 was the only one which presented with a greenish tint to the agar

which occurred at all dilution levels used. The cultures that were decided to be used in producing beer were; S-04, Pilsner Lager WLP800 and Bavarian Wheat Blend 3056. A summary of the observed characteristics of colonies found in starter cultures is found in *Appendix 1*.
The VRBD plates were all empty.

4.2 Analysis of beer

4.2.1 In-house-produced beer

The produced beers had brix values of 7-8° and pH of 4.4-4.5, see *Appendix 2* for specific numbers.

The growth on plates with the produced beer showed a very diverse set of colony colours on Bacillus Chromoselect. The total colony count from Bacillus Chromoselect agar can be seen in *Figure 6*. The produced beers had an average value of $10^{8,85}$ CFU/330 mL (σ=0.2). On average there were 28% (σ=4%) colonies on the plates of blue colour dots, 67% (σ=3.1%) of white/yellow dot structure and 5% (σ=1.5%) of some type of green dot. There were also some white mucoid structures but they were not present on all that many plates and were negligible at an average of 0.07% (σ=0.05). The blue colonies varied greatly in the colour scheme from a deep dark blue to a very light blue, almost white appearance. An example image can be seen in *Appendix 3* which shows bottle 1 from Bavarian Wheat Blend 3056 and in *Appendix 4-7* the colony count of the different types can be seen.

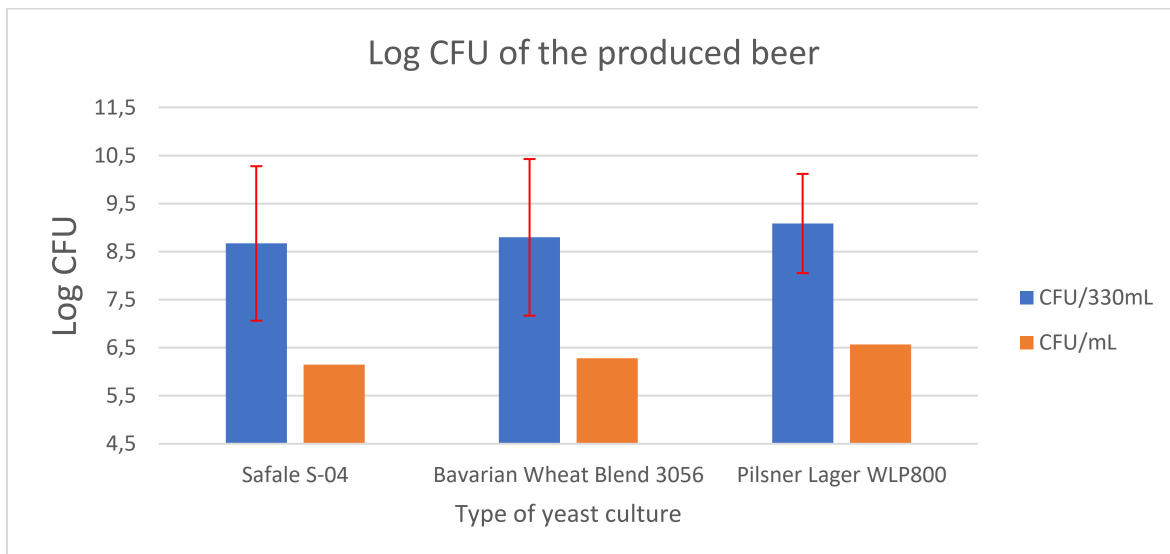

Figure 6: Logarithmic scale of Colony Forming Units in the produced beer made in-house on Bacillus Chromoselect agar. The main unit is /330mL due to beer being consumed per bottle with the usual size of 330mL. Also /mL shown since this is normal unit for CFU.

Overall, there was a great similarity in the results from the produced beer. The composition of the plates was similar in terms of the colony structure and colour. The changing variable between the plates was for the most part the number of colonies.

On Rogosa agar there was no growth for both S-04 and Pilsner Lager WLP800 but Bavarian Wheat Blend 3056 had an average of 2400 CFU/mL (σ=1600) in the 6 bottles. On the Malt agar there were only yellow colonies indicating pure yeast growth for all bottles. VRBD was

negative on all bottles with yeast culture. The control, the wort without added yeast, had growth on Bacillus Chromoselect, VRBD and Malt. VRBD showed a CFU/mL of 400. Bacillus Chromoselect had a purple/blue appearance with a lot of growth, see *Appendix 8*. The Malt agar had colonies of mould on it.

Bottle number 4 of the type Pilsner Lager WLP800 shattered before the second sampling with 10^{-3} and 10^{-4} was performed which meant that it was excluded from these results. Therefore, this yeast culture type only had 5 fermentation bottles while the other two types had 6.

4.2.2 Microbrewery beer

The beer from microbreweries produced a different result than the one obtained from in-house brewing of beer. The average CFU/330mL was $10^{4.6}$ (σ=1.25).There were yellow mucoid structures on almost all of the plates, as can be seen in *Appendix 9*. An example of the mucoid structure is shown in *Appendix 10* with B2:1:1, undiluted. While the blue dot colonies were dominating the in-house produced beer, in the microbrewery beer the blue colonies were very low in numbers as seen in *Appendix 11*. The number of yellow/white dots, *Appendix 12,* were low except for a select few plates from one brewery which had great numbers.

A lot of the beer types were similarly scattered with mucoid colonies but there were some which were very low or even entirely free of these structures. B1:2, B3:1 and B4:1 had very low levels of mucoid structures compared to the rest of the samples while B4:2 and B5:1 had none. In the case of yellow/white dot colonies, B1:1 was the only one without any while B3:2 and both beer types from B5 had a large amount of these colonies. In contrast to the samples from in-house produced beer there was a visual difference in composition and amounts between the beers on Bacillus Chromoselect agar. The total CFU in one bottle was calculated and the amounts are shown in *Figure 7* where they range from $10^{2.5}$-$10^{6.5}$. The reason for reporting per 330 mL bottle instead of per mL, as with the in-house beer, is that the microbrewery beer is intended to be drunk by the bottle and that the amounts found here were smaller than the in-house beer. The standard deviation was also calculated and shown to be relatively small compared to the amount of CFU present. The pH range was small and varied between 4.3-4.6.

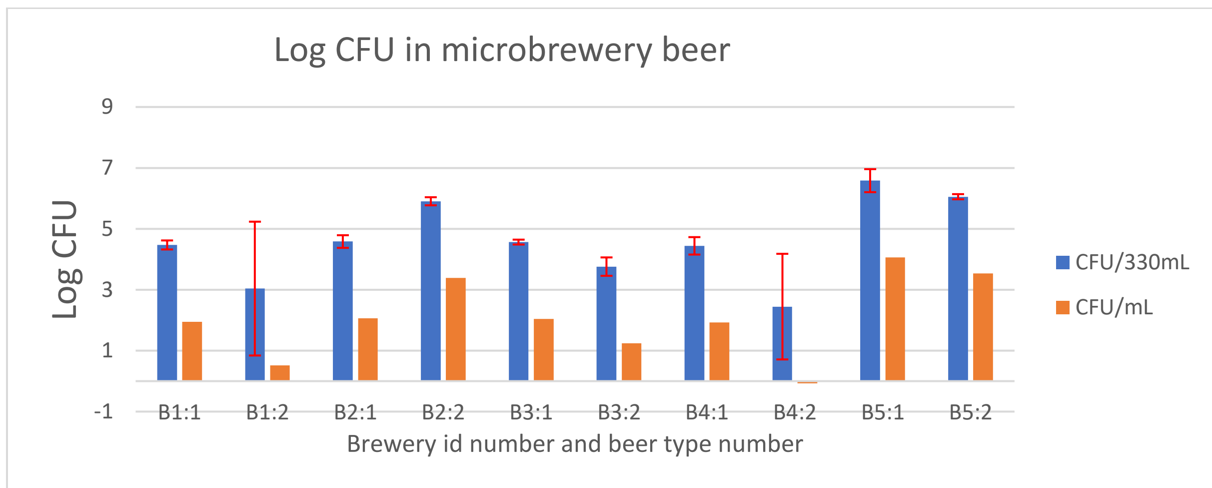

Figure 7: Total CFU of microbrewery beer calculated from Bacillus Chromoselect plates.

4.2.3 Commercial beer and sour beer

Compared to the other beers tested the sour beer and store-bought were low in CFU/330mL with an average of $10^{3.81}$ CFU (σ=0.22). B8 is the largest, but not by much, at $10^{3.87}$. There appears to be no difference between alcohol containing and alcohol-free version of beer from the same company, B6 and B7, which both are situated around $10^{3.8}$ CFU/330mL. The sour beer has the lowest at $10^{3.78}$ CFU/330mL. The total CFU are displayed in *Figure 8*. The control plates, 5 of them, had an average of 2 colonies on each.

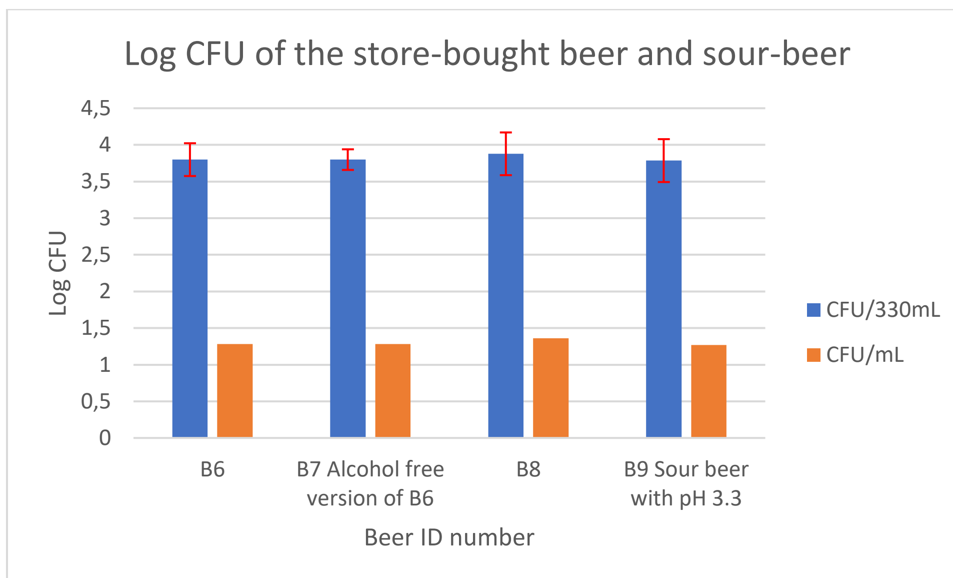

Figure 8: CFU of store bought mass produced beer and sour beer

4.2.4 Statistical analysis

There are some statistically significant differences between the types of beer that were tested. These were evaluated in Sigmaplot by comparing many groups with ANOVA on ranks analysis or rank sum test for two groups, both with a cut off value at P=0.05.

For the produced beer there was a statistical difference between Pilsner Lager WLP800 and Safale S-04 (P=0.004), which means that WLP800 is significantly larger in CFU, but no other difference could be found in this group. See *Appendix 13* for analysis results.

For the store-bought beer and microbrewery beer the ales are compared together and lagers together. Between the ales, B1:1, B5:1, B1:2, B2:2, B3:2, B4:2, B5:2, there were some differences (P=0.007). B5:1 was significantly greater than the rest and B5:2 was together with B2:2 in second place since there was no significant difference between them. See *Appendix 14* for analysis. Between the lager beers, B3:1, B4:1, B6, B7, B8, there were no statistical differences. See *Appendix 15*. Additionally, between the ale-type and lager-type beers there were no statistical differences. See *Appendix 16*.

Between B6 and B7, where B7 is the alcohol-free variant of B6 there was no significant difference. See *Appendix 17.*

There were no significant differences between the store-bought beers (B6-B9). See *Appendix 18*.

Between the store-bought beers from large companies, that is to say B6-B8, and the beer from microbreweries there was a statistically significant difference in favour of the store-bought beer having a lower CFU/330mL (P=0.018). See *Appendix 19*.

4.2.5 16s rRNA sequencing results

Out of the 140 samples sent for sequencing, 10 for each beer from microbreweries and store-bought beer, 77 presented with bad sequences. 38 of these had impure results where a clear sequence could not be established and 39 of the sequences proved to be shorter than 500bp.

The full list of matched identities can be seen in *Appendix 20*.
Generally, there were overwhelmingly *Bacillus* species in the identification. For the microbrewery beer it was dominated by *B. subtilis* whereas in the store-bought beer. Other *Bacillus* members were one unclear result between *B. amyloliquefaciens/velezensis* as well as *B. licheniformis* and *B. pumilis*. There were a lot of isolates that could not be distinguished further than the *Bacillus* genus due to more than one species above 99%. However, many of them still had Bacillus subtilis as the highest identity score. Some findings that were not *Bacillus* include other members of the *Bacillales* order, *Ornithinibacillus* and *Lysinibacillus*. There were also the unrelated *Micrococcus luteus, Enhydrobacter aerosaccus*, *Exiguobacterium sibiricum* as well as *Pseudomonas* and *Staphylococcus epidermis*.

There was no clear correlation to the morphology from Bacillus chromoselect agar and the sequencing identification. That is to say that the colonies that were identified to be *Bacillus subtilis* by sequencing did not appear as green colonies when on the agar. None of the sequence results showed any *Bacillus megaterium* which the mucoid colonies should have been according to the data-sheet for the agar.

Based on the sequencing results of positively identified *Bacillus subtilis*, a rough estimation of the amounts of this species can be made in the microbreweries. This was calculated since this species was identified several times. This is seen in *Figure 9*. Staphylococcus epidermis was present at 5.05 log CFU in B5:2 and Micrococcus luteus was present at 2.76 log CFU in B3:2. Note that this is a very rough estimation since not all 10 isolate samples that were sent

for sequencing yielded results and in some cases only very few bacteria were identified at species level out of the 10 samples.

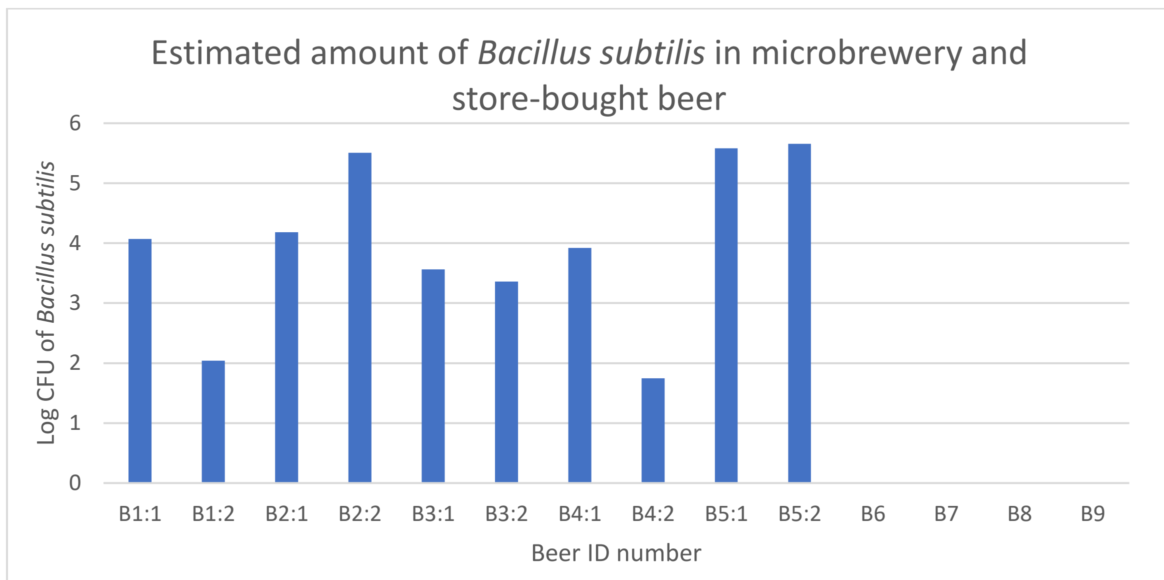

Figure 9: An estimation of the amounts of CFU of *Bacillus subtilis* present in beer from microbreweries and store-bought beer. Based off of 16S rRNA sequencing results.

5.0 Discussion

The reason for bottles breaking apart with the produced beer is most-likely two-fold. The first part is the high brix value of 7-8º while, as mentioned before, a normal brewing process should end up at 0-5.1º depending on the type of beer. This meant that there were still a lot of unutilized sugars present that bacteria and the yeast could use to grow. Another factor is that Pilsner Lager WLP800 is a Lager type beer and should have thus been kept fermenting at around 10 ℃ instead of the 21℃ it was kept at. This could explain why these bottles were the first to break apart from too much pressure. The yeast had grown poorly in the hot environment and had a lot of nutrients left when it reached a more favourable temperature which caused an increase in pressure. With the air-locks now removed and caps on the bottles there was no way to release the pressure and the bottles cracked. Closer attention should have been paid to the brix value rather than relying only on the cessation of bubbling in the air-locks. However, this scenario might occur at a home-brewer who is new to the craft and thus could be somewhat illustrative of the resulting beer gained there. The high brix-value could very well be the reason for the large difference in CFU between the produced beer and the other beer types analysed since those usually are much lower in brix.

There was a great difference in starter culture appearance on Bacillus Chromoselect plates contra the appearance of the finished beer. If the starter cultures had been to blame for the bacterial content it would have been expected to be differences between these different beers and there should be some connection between the type of growth pre vs post fermentation. When taking into account the similarities between starter culture beer together with the Bacillus Chromoselect plates of the control with untouched wort this indicates that the raw materials, i.e. the malt, have a greater contribution to the *Bacillus* load than the starter cultures. The sack with malt was usually only used during a brewing course offered at the institution and could thus have been there for some time. This should not have influenced the

result to a greater extent since *Bacillus* on grains are spores and not in a viable state [61]. One additional thought about this result is that the CFU could be overinflated a bit in the control wort by having a solution with pure nutrients together with a presumed original amount of *Bacillus.* It would have been a good idea to sample the control wort on a Bacillus Chromoselect plate right after the wort boil and compare it to the one that had been left in the climate room.

The total CFU calculation in the microbrewery beer has a big nuisance. The mucoid colonies were very difficult to count correctly due to how they are structured. It was not always clear where one colony had fused together with another or just one that had grown in a weird pattern. This could mean that the number of mucoid colonies are underestimated since it would be a greater chance to count two colonies as one. A better way of doing it could have been to take pictures every 12h or 24h of the total 48h spent in the incubator in order to better see developing colonies before they grow into each other. There was also the problem of blue/yellow/white dot colonies growing underneath the mucoid colonies which made the isolations harder to do and might also have reduced the CFU count of these types.

There was never any growth on VRBD in finished beer, except for the raw wort. The same was true for the mould on malt agar with raw wort which did not occur in the produced beer on malt agar. This would indicate that these organisms are outcompeted by the yeast or severely inhibited by the lowered pH.

The problem with errors in the dilution series does not have a clear explanation. Since it was observed in the microbrewery samples that the dot colonies decreased while the mucoidal colonies stayed constant there is no way of knowing what the error is. If the dilution method had been erroneous it should in theory also have afflicted the dot colonies. Every time there was a step of mixing dilutions they were vortexed for 2-3 seconds which should provide ample time for homogenization. It might be that they aggregate together easily when in spore-form and thus clump together. Since the pipette tip is lowered approximately 0.5cm beneath the surface there could be a greater concentration there.

The store-bought beer had a very low colony count with the numbers being between 0-4 colonies per plate. These low numbers add some uncertainty to the CFU that was calculated. With such low numbers one randomly occurring extra colony on a plate will inflate the results much more than if the numbers had been ten times greater on each plate. This means that the colony count on these beers could be much lower. The control plates that accompanied this run of plates had an average of 1.5 colonies on it. The real CFU is thus most likely lower than what is presented and it could very well be that the store-bought beer is lower than all of the other beers tested.

From the statistical analysis it is obvious that B5 had the greatest problem with bacillus content and they were also the only brewery which presented with large numbers of yellow/white dot colonies. Also interesting is that there was no difference between the alcohol-free and alcohol containing beer of the same brand which would suggest that ethanol has a small effect on survivability in beer. The alcohol content of the alcohol-containing beer was 4.2% while the alcohol-free beer was at the legal maximum of 0.5%. There was a statistical difference between the store-bought beer and the ones from microbreweries. This could be due to the fact that these larger companies often pasteurize or at least filter their beer. The yeast that is left in microbrewery beer often adds to the flavour profile that is desired and

a filtration step could be costly to implement since bacteria are smaller than yeast. The yeast would thus need to be re-added later on somehow. The limiting factor of the statistical analysis is that there were only three replicates of each type of beer for the microbreweries. For better statistical soundness it would have been more adequate with six replicates as was the case with the in-house produced beer.

The sequencing results showed that there was an overwhelming presence of *Bacillus* in the samples from both microbreweries and store-bought beer. However, there was a difference in the species of *Bacillus* with the store-bought beer having mainly unspecified *Bacillus* whereas the microbrewery beer had *B. subtilis*. This was surprising since *B. megaterium* was never detected in the sequencing despite the data sheet for Bacillus Chromoselect agar indicating the large yellow mucoid colonies to be this species. Since sequencing of 16S rRNA is a more precise method compared to general plating, the results of the sequencing are deemed to be more reliable. *B. subtilis* is one of the two species of *Bacillus* that are used in probiotic supplementation and as such, the presence of this bacteria in beer should not be a cause for concern. The amount of *Bacillus subtilis* that was calculated to be present in the beer seems to correspond somewhat with the amount of yellow dot colonies. But since

The other Bacillus species were only identified in single isolations. *B. amyloliquefaciens* has been investigated as growth addition to increase yield of chicken production with successful results if added to their diet as a probiotic [63]. This is probably due to increased nutrient uptake but no studies were found on humans. *B. velezensis* has been investigated as a plant-protective bacterium which for instance inhibits spoilage by *Fusarium* species [64]. It naturally produces an alpha amylase and a restriction enzyme which could inhibit growth of harmful bacteria [65]. This could be why the addition of it to plants cause greater growth [66]. *B. pumilis* is used as a probiotic supplement for humans in conjunction with the previously mentioned *B. coagulans* and *B. subtilis* but some strains of it can cause a disease state similar to *B. anthracis* as well as be hostile to plant life [67]. This analysis is not robust enough to distinguish the strain for certain but it is unlikely to be the disease causing one as that would have harmed the plant and been noticed in the production of malt.

There is no substantial information available concerning *Ornithinibacillus*, *Exigobacterium sibiricum, Pseudomonas* and *Enhydrobacter aerosaccus* in relation to pathogenicity.

Of some concern are the last two types of bacteria found. *Microccus luteus* has been known to cause skin infections, at least in immunocompromised patients such as those with HIV or leukemia [69]. *Staphylococcus epidermis* is known as an opportunistic pathogen [70]. It is present on human skin and can thus be transferred quite easily. For instance, infections of *S. epidermis* through catheters is causing troubles due to antibiotic resistance and biofilm formation.

The microbial species discovered through sequencing of isolates show a clear favour for species which have no pathogenicity. Surprisingly, many isolates from microbrewers were the probiotic *B. subtilis*. However, since the probiotic function is established when administered as a supplement not much can be said of exactly how it would behave when absorbed in the intestines along with the beer. At least the amounts of *B. subtilis* ingested when consuming a few bottles are close to the probiotic levels in supplements and it is not unlikely that it would have an effect on the microflora and thus the consumer. The unspecified *Bacillus* bacteria are unknown since they could not be distinguished to one species above 99% identity. Further

analysis should be done to evaluate the full *Bacillus* presence in beer as well as how the consumption of Bacillus bacteria, whether probiotic or not, in this format affects the gut flora.

6.0 Conclusion

The aim of this project was to investigate how the *Bacillus* flora present in starter cultures could survive into the finished beer and also find out if this is a problem occurring in commercially available beer. After having seen what happened with in-house produced beer the results point to the malt being more responsible for the *Bacillus* load than the starter cultures themselves. The negligible statistical difference, in absolute values, between yeast styles indicates that this is true.

In the microbrewery beer the main species identified was the probiotic *Bacillus subtilis*, but the probiotic function when administered through beer is unknown. There were a lot of bacteria which could not be identified past the genus level. There were also two potential pathogens found that were surviving in beer, *Staphylococcus epidermis* and *Micrococcus luteus*, which indicates that this subject should be investigated further.

In contrast to previous studies, which looked exclusively at *B. cereus*, this project found other *Bacillus* species as well as other types of bacteria growing in finished beer.

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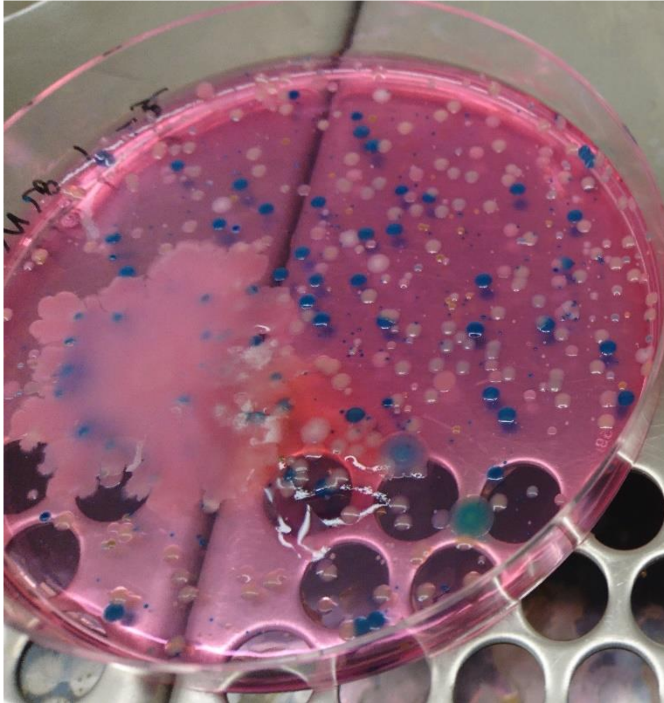
8.0 Appendix

Starter culture	Type of growth on the plates	Colour of agar ($10^{-1}/10^{-2}/10^{-6}$)
Safale S-04 **(dry)**	Plenty of white streaks which, on the 10^{-6} dilution, was shown to be yellow/white dots. Blue dots present on 10^{-1} dilution.	Red/Orange/Yellow
Saflager W-34/70 **(dry)**	Streaks of white and a blue dot. Large mucus colony on 10^{-6} dilution. Blue dots on 10^{-1}.	Red/Orange/Orange-Yellow
Empire Ale M15 **(dry)**	Plenty of white streaks which, on 10^{-6} dilution, was shown to be yellow/white dots. Big blue spots on 10^{-1} dilution.	Red/Orange-red/Orange-Yellow
Belgian wit M21 **(dry)**	Plenty of white streaks which, on 10^{-6} dilution, was shown to be yellow/white dots. Blue dots present on 10^{-1}	Red/Orange-Red/Orange-Yellow
Californian lager M54 **(dry)**	Plenty of white dots but nothing on 10^{-6} dilution.	Red-Yellow/Orange/Orange-Yellow
Pilsner Lager WLP800 **(liquid)**	White dots on 10^{-1}, 10^{-2} and no dots but a very large mucus colony on 10^{-6}.	Red/Orange-Red/Orange
Bavarian Wheat Blend 3056 **(liquid)**	White streaks on 10^{-1}. Blue/light blue and yellow dots on 10^{-2} and 10^{-6}.	Red-Green/Green-Yellow/Orange

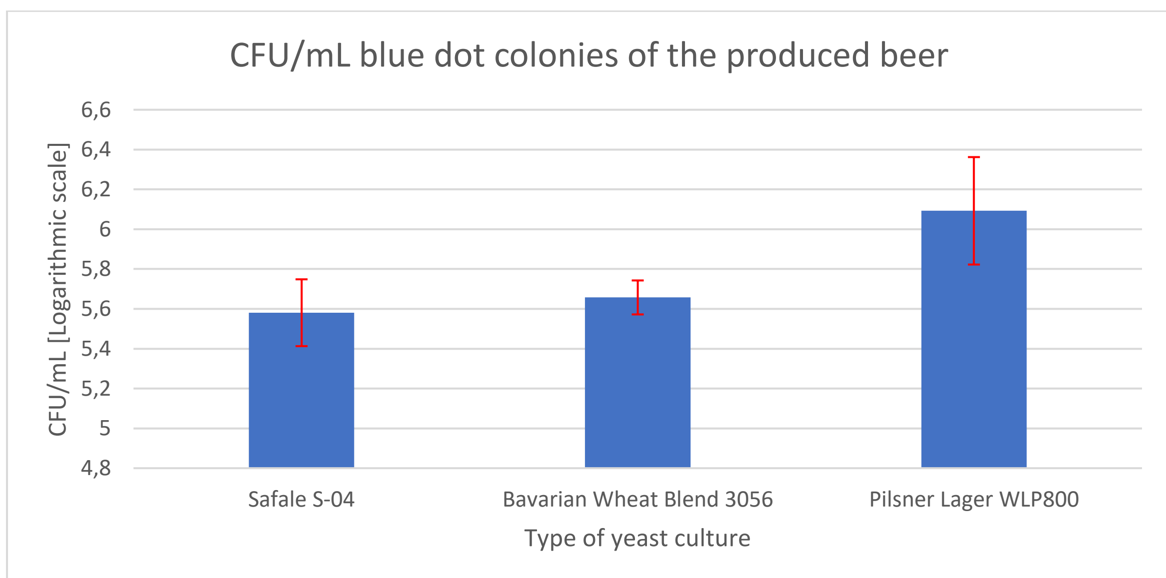
Appendix 1: Appearance of colonies found growing on Bacillus Chromoselect agar from pure yeast cultures based on 10-2 and 10-6 dilutions.

Yeast culture	Average pH	Average Brix value
Safale S-04	4.41	8
Bavarian Wheat Blend 3056	4.29	7
Pilsner Lager WLP800	4.44	8

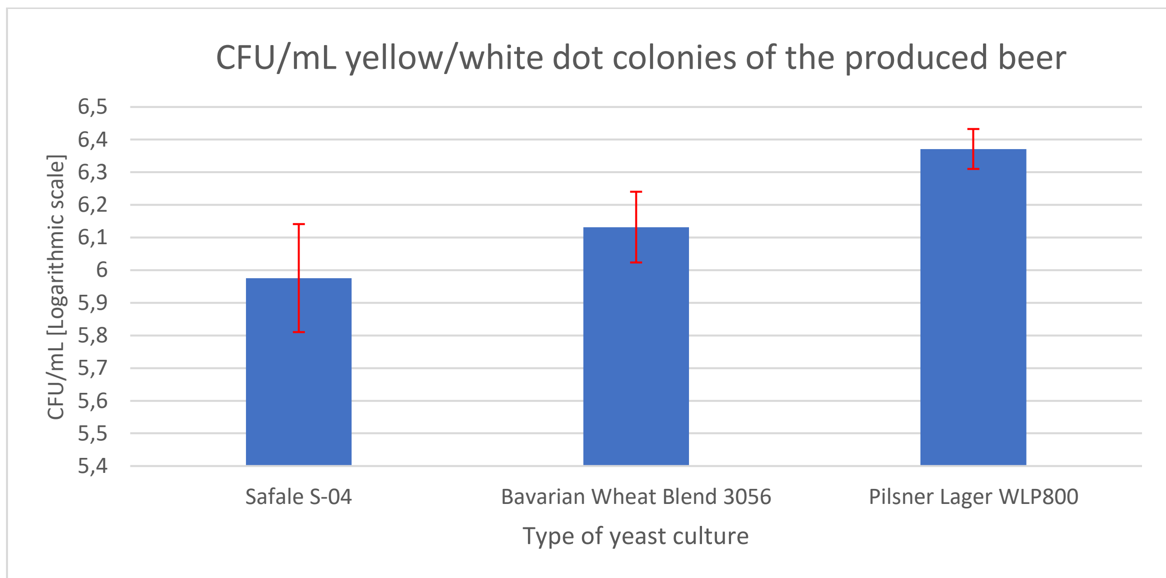
Appendix 2: Values of beer parameters taken from the in-house produced beer.



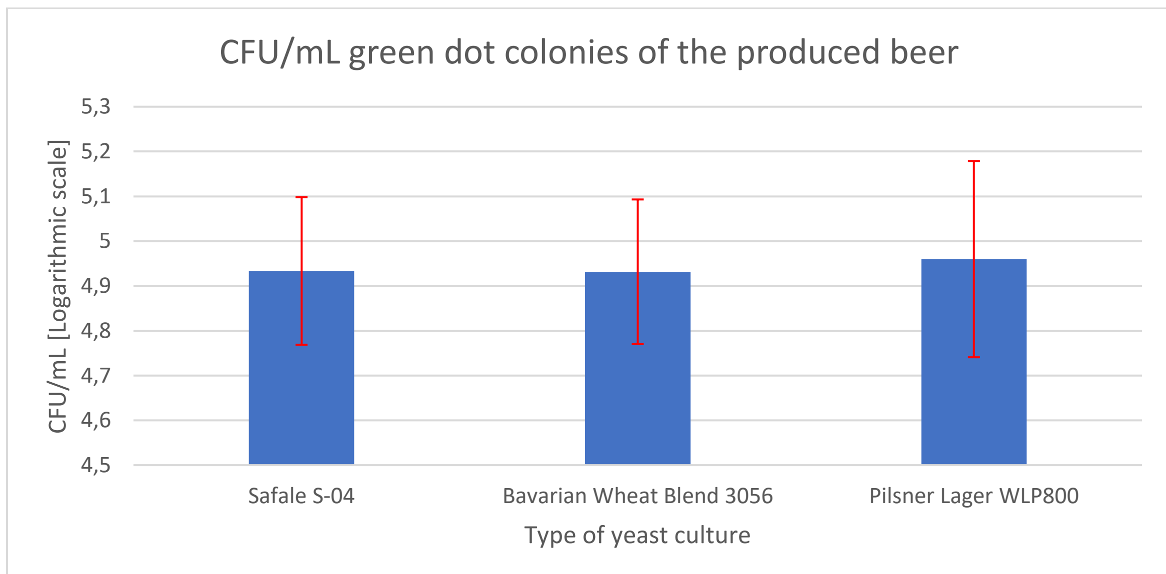
Appendix 3: Plate with bottle 1 from Bavarian Wheat Blend 3056 at dilution 10-3 which shows the diversity in structure and colour present in the samples from the in-house produced beer on Bacillus Chromoselect agar.



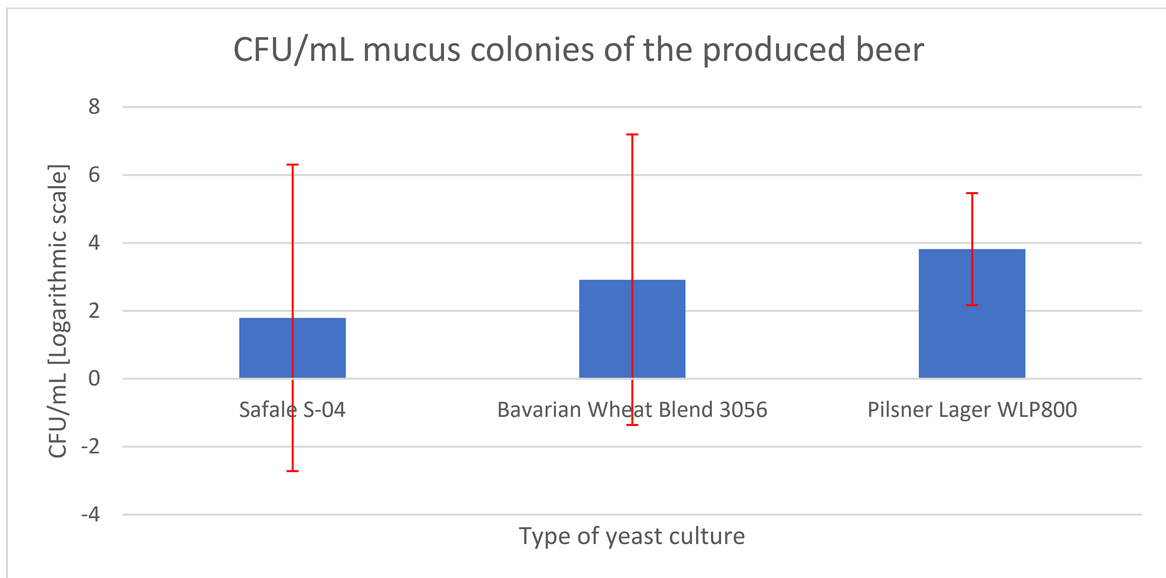
Appendix 4: CFU/mL for the blue dot colonies present on beer brewed in-house.



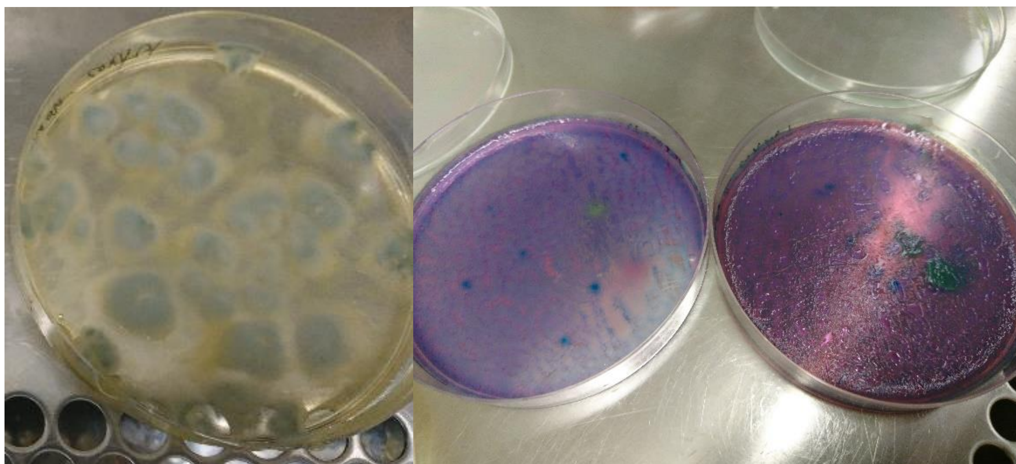
Appendix 5: CFU/mL for the yellow/white dot colonies present on beer brewed in-house.



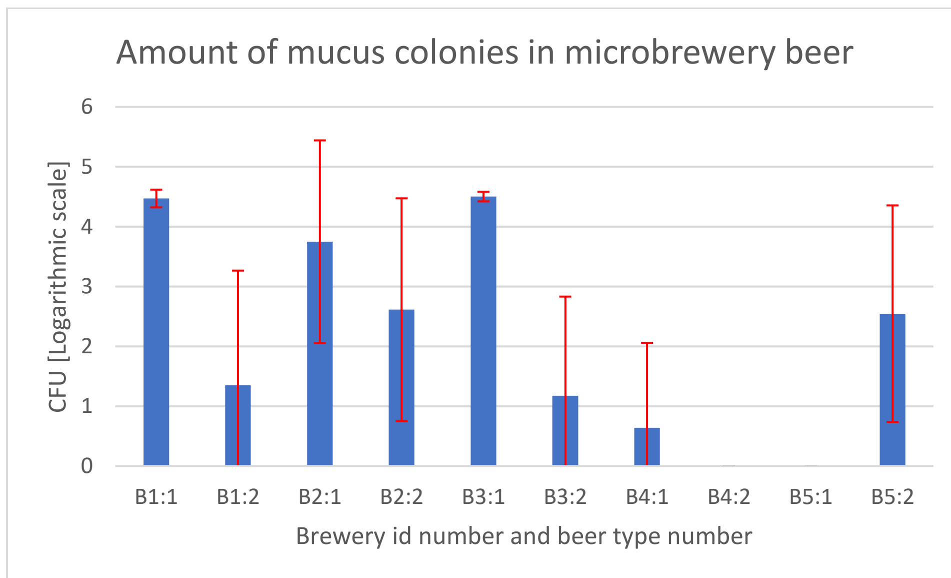
Appendix 6: CFU/mL for the green dot colonies present on beer brewed in-house.



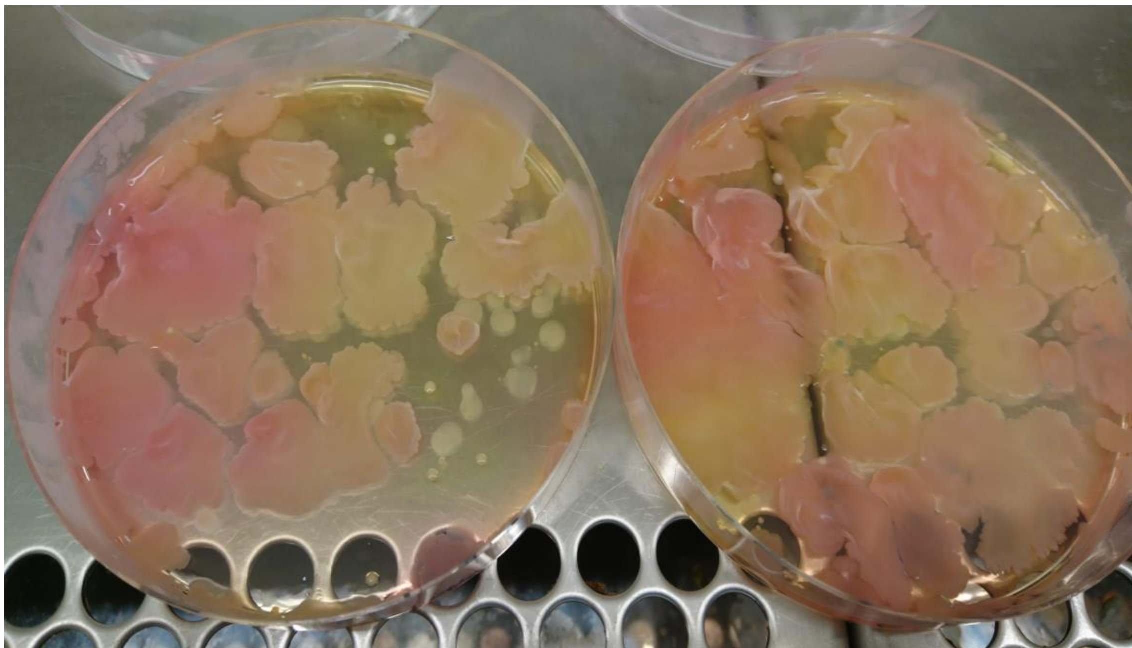
Appendix 7: CFU/mL for the mucoid colonies present on beer brewed in-house.



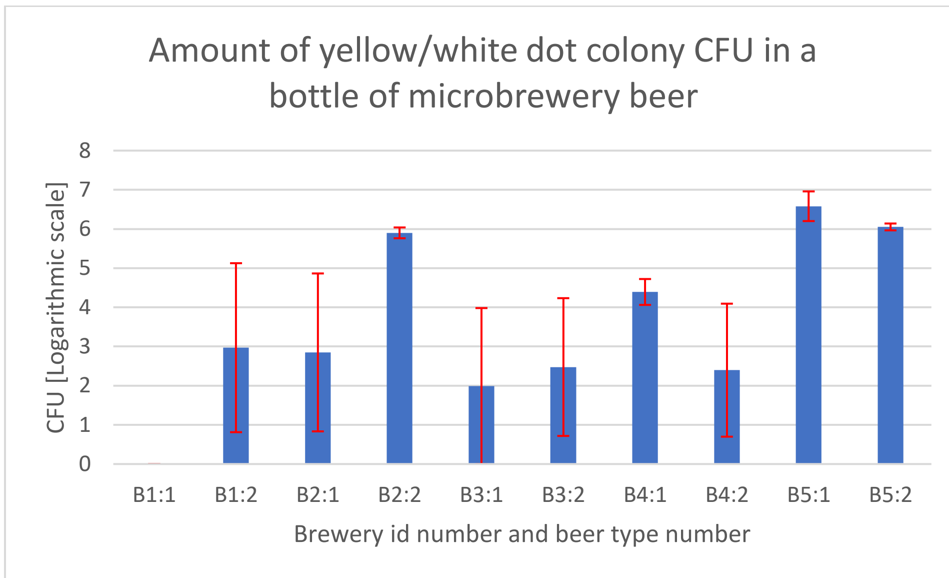
Appendix 8: To the left is the control sample of wort on Malt agar and to the right is wort on Bacillus Chromoselect agar. The Malt agar has mouldy growth and Bacillus Chromoselect has a lot of purple growth.



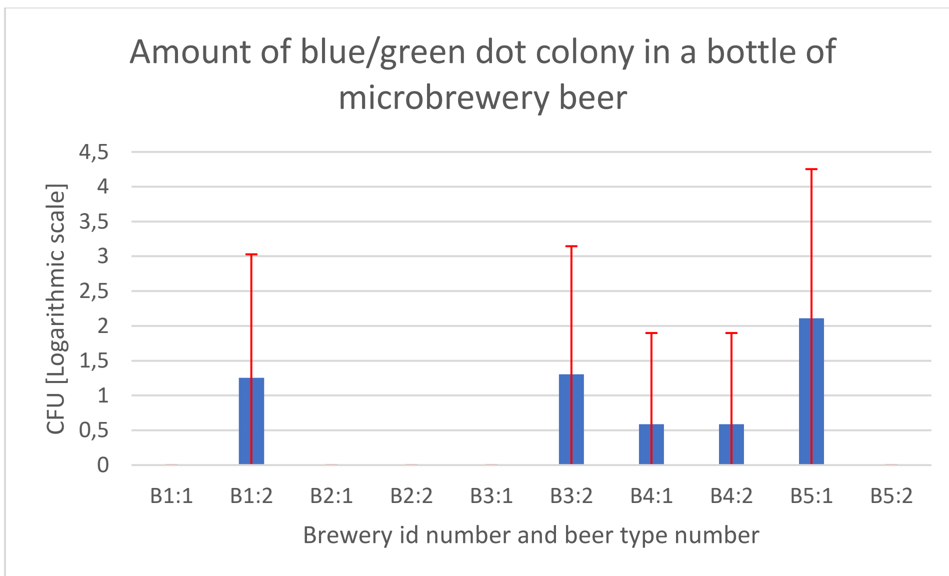
Appendix 9: Amount of mucus colonies present in the beer from microbreweries.



Appendix 10: Mucoid structures present many of the microbrewery plates. Here shown from B2:1:1 undiluted.



Appendix 11: Amount of yellow/white dot colonies present in the beer from microbreweries.



Appendix 12: Amount of blue/green dot colonies present in the beer from microbreweries.

Kruskal-Wallis One Way Analysis of Variance on Ranks den 26 maj 2018 07:57:03

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk): Passed (P = 0,247)

Equal Variance Test (Brown-Forsythe): Passed (P = 0,521)

Group	N	Missing	Median	25%	75%
Col 1	6	0	8,668	8,579	8,826
Col 2	6	0	8,790	8,729	8,867
Col 3	5	0	9,040	9,013	9,229

H = 10,837 with 2 degrees of freedom. (P = 0,004)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0,004)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Dunn's Method) :

Comparison	Diff of Ranks	Q	P	P<0,050
Col 3 vs Col 1	9,833	3,216	0,004	Yes
Col 3 vs Col 2	7,167	2,344	0,057	No
Col 2 vs Col 1	2,667	0,915	1,000	No

Note: The multiple comparisons on ranks do not include an adjustment for ties.

Appendix 13: ANOVA analysis on the produced beer where Col 1 is Safale S-04, Col 2 is Bavarian Wheat Blend 3056 and Col 3 is Pilsner Lager WLP800.

Kruskal-Wallis One Way Analysis of Variance on Ranks den 26 maj 2018 13:29:43

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk): Failed (P < 0,050)

Group	N	Missing	Median	25%	75%
Col 1	3	0	4,496	4,320	4,598
Col 2	3	0	6,389	6,278	7,078
Col 3	3	0	4,092	0,000	5,023
Col 4	3	0	5,969	5,768	5,979
Col 5	3	0	3,669	3,669	3,941
Col 6	3	0	3,519	0,000	3,820
Col 7	3	0	6,057	5,965	6,141

H = 17,707 with 6 degrees of freedom. (P = 0,007)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0,007)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparison	Diff of Ranks	q	P	P<0,050
Col 2 vs Col 6	49,500	4,606	0,019	Yes
Col 2 vs Col 5	44,000	4,758	0,010	Yes
Col 2 vs Col 3	38,500	4,970	0,004	Yes
Col 2 vs Col 1	30,000	4,804	0,004	Yes
Col 2 vs Col 4	16,000	3,373	0,045	Yes
Col 2 vs Col 7	11,000	3,395	0,016	Yes
Col 7 vs Col 6	38,500	4,164	0,038	Yes
Col 7 vs Col 5	33,000	4,260	0,022	Yes
Col 7 vs Col 3	27,500	4,404	0,010	Yes
Col 7 vs Col 1	19,000	4,006	0,013	Yes
Col 7 vs Col 4	5,000	1,543	0,275	No
Col 4 vs Col 6	33,500	4,325	0,019	Yes
Col 4 vs Col 5	28,000	4,484	0,008	Yes
Col 4 vs Col 3	22,500	4,743	0,002	Yes
Col 4 vs Col 1	14,000	4,320	0,002	Yes
Col 1 vs Col 6	19,500	3,122	0,121	No
Col 1 vs Col 5	14,000	2,951	0,093	Do Not Test
Col 1 vs Col 3	8,500	2,623	0,064	Do Not Test
Col 3 vs Col 6	11,000	2,319	0,229	Do Not Test
Col 3 vs Col 5	5,500	1,697	0,230	Do Not Test
Col 5 vs Col 6	5,500	1,697	0,230	Do Not Test

Note: The multiple comparisons on ranks do not include an adjustment for ties.

A result of "Do Not Test" occurs for a comparison when no significant difference is found between the two rank sums that enclose that comparison. For example, if you had four rank sums sorted in order, and found no significant difference between rank sums 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed rank sums is a procedural rule, and a result of Do Not Test should be treated as if there is no significant

Appendix 14: Statistical analysis of the ale style beers. Column 1-7 are as follows: B1:1, B5:1, B1:2, B2:2, B3:2, B4:2, B5:2.

Kruskal-Wallis One Way Analysis of Variance on Ranks den 26 maj 2018 13:51:42

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk): Passed (P = 0,277)

Equal Variance Test (Brown-Forsythe): Passed (P = 0,386)

Group	N	Missing	Median	25%	75%
Col 1	3	0	4,539	4,535	4,621
Col 2	3	0	4,470	4,146	4,709
Col 3	3	0	3,908	3,519	3,970
Col 4	3	0	3,820	3,669	3,908
Col 5	3	0	3,970	3,519	4,146

H = 10,240 with 4 degrees of freedom. (P = 0,037)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0,037)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparison	Diff of Ranks	q	P	P<0,050
Col 1 vs Col 4	26,500	3,421	0,110	No
Col 1 vs Col 3	24,500	3,923	0,028	Do Not Test
Col 1 vs Col 5	20,500	4,322	0,006	Do Not Test
Col 1 vs Col 2	3,500	1,080	0,445	Do Not Test
Col 2 vs Col 4	23,000	3,683	0,046	Do Not Test
Col 2 vs Col 3	21,000	4,427	0,005	Do Not Test
Col 2 vs Col 5	17,000	5,246	<0,001	Do Not Test
Col 5 vs Col 4	6,000	1,265	0,644	Do Not Test
Col 5 vs Col 3	4,000	1,234	0,383	Do Not Test
Col 3 vs Col 4	2,000	0,617	0,663	Do Not Test

Note: The multiple comparisons on ranks do not include an adjustment for ties.

A result of "Do Not Test" occurs for a comparison when no significant difference is found between the two rank sums that enclose that comparison. For example, if you had four rank sums sorted in order, and found no significant difference between rank sums 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed rank sums is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the rank sums, even though one may appear to exist.

Appendix 15: Statistical analysis of the lager-style beers. Column 1-5 are as follows: B3:1, B4:1, B6, B7, B8.

Mann-Whitney Rank Sum Test den 29 maj 2018 19:30:27

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk): Passed (P = 0,915)

Equal Variance Test: Failed (P < 0,050)

Group	N	Missing	Median	25%	75%
Col 1	7	0	4,471	3,038	6,054
Col 2	5	0	3,878	3,799	4,503

Mann-Whitney U Statistic= 16,000

Yates continuity correction option applied to calculations.

T = 31,000 n(small)= 5 n(big)= 7 P(est.)= 0,871 P(exact)= 0,876

The difference in the median values between the two groups is not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,876)

Appendix 16: Statistical analysis of difference between the calculated CFU values for ale-type and lager-type beers. Col 1 are the Ale-type and Col 2 are the lager-type.

Mann-Whitney Rank Sum Test den 29 maj 2018 19:24:12

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk): Passed (P = 0,348)

Equal Variance Test: Passed (P = 0,587)

Group	N	Missing	Median	25%	75%
Col 1	3	0	3,908	3,519	3,970
Col 2	3	0	3,820	3,669	3,908

Mann-Whitney U Statistic= 3,500

Yates continuity correction option applied to calculations.

T = 11,500 n(small)= 3 n(big)= 3 P(est.)= 0,825 P(exact)= 0,700

The difference in the median values between the two groups is not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,700)

Appendix 17: Statistical analysis of the alcohol containing and alcohol-free variant from the same large brewery. Col 3 is B6 and Col 4 is B7 (alcohol-free).

Kruskal-Wallis One Way Analysis of Variance on Ranks den 27 maj 2018 10:19:38

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk): Passed (P = 0,784)

Equal Variance Test (Brown-Forsythe): Passed (P = 0,718)

Group	N	Missing	Median	25%	75%
Col 1	3	0	3,908	3,519	3,970
Col 2	3	0	3,820	3,669	3,908
Col 3	3	0	3,970	3,519	4,146
Col 4	3	0	3,669	3,519	4,169

H = 0,329 with 3 degrees of freedom. (P = 0,955)

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0,955)

Appendix 18: Statistical analysis of store-bought beer. Col 1-4 are as follows: B6, B7, B8, B9.

Mann-Whitney Rank Sum Test den 29 maj 2018 19:36:02

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk): Failed (P < 0,050)

Group	N	Missing	Median	25%	75%
Col 1	50	20	4,539	4,054	5,966
Col 2	9	0	3,908	3,594	3,970

Mann-Whitney U Statistic= 49,000

Yates continuity correction option applied to calculations.

T = 94,000 n(small)= 9 n(big)= 30 (P = 0,004)

The difference in the median values between the two groups is greater than would be expected by chance; there is a statistically significant difference (P = 0,004)

Appendix 19: Statistical analysis of difference between the calculated CFU values for microbrewery beer versus store-bought beer (not including sour-beer). Col 1 are the microbrewery beers and Col 2 are the store-bought beers.

Beer ID	Identified bacteria by BLAST in the isolations	Percentage of identification (%)
B1:1	*Bacillus amyloliquefaciens /velezensis*	100
	Bacillus subtilis	100
	Bacillus subtilis	100
	Bacillus subtilis	100
	Bacillus subtilis	99
B1:2	*Bacillus pumilis*	100
	Bacillus	99
	Bacillus subtilis	99
	Bacillus	98.90
B2:1	*Bacillus*	98.51
	Bacillus	98.51
	Bacillus subtilis	100
	Bacillus subtilis	99.53
	Bacillus	98.05
	Bacillus subtilis	99.03
	Bacillus subtilis	99.84
B2:2	*Bacillus subtilis*	100
	Bacillus subtilis	100
	Bacillus	95.75
	Bacillus	95.24
	Ornithinibacillus	95.99
	Bacillus subtilis	99.65
	Bacillus subtilis	99.65
B3:1	*Bacillus subtilis*	99.76
B3:2	*Micrococcus luteus*	99.22
	Bacillus subtilis	99.73
	Bacillus	98.44
	Bacillus subtilis	100
	Bacillus	95.75
	Bacillus subtilis	100
	Bacillus subtilis	100
B4:1	*Bacillus subtilis*	99.86
	Bacillus subtilis	100
	Bacillus subtilis	100
B4:2	*Bacillus*	95.56
	Bacillus subtilis	99.74
	Bacillus subtilis	100
B5:1	*Bacillus*	95.00
	Bacillus subtilis	100
B5:2	*Micrococcus*	98.38
	Bacillus subtilis	99.88
	Enhydrobacter aerosaccus.	99.16
	Staphylococcus epidermis.	100

	Bacillus subtilis	100
	Bacillus subtilis	100
	Bacillus	98.58
	Bacillus subtilis	100
B6	*Exiguobacterium sibiricum*	100
	Pseudomonas	98.11
	Bacillus	95.44
	Lysinibacillus	96.65
B7	*Bacillus*	95.49
	Bacillus	95.42
	Bacillus	95.64
	Bacillus	95.49
	Bacillus	95.61
B8	*Bacillus*	95.62
	Bacillus	95.42
	Bacillus	95.51
	Bacillus licheniformis	99.74
B9	*Bacillus smithii*	95.49

Appendix 20: The confirmed bacterial species present in isolated samples taken from beer along with the identity score. Identity was given by uploading the DNA sequence to http://metasystems.riken.jp/grd/seqsearch.html.