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MICROBIOLOGICAL QUALITY CONTROL AT SUPER BOCK GROUP – RISK ASSESSMENT AND IMPLEMENTATION OF NEW MOLECULAR METHODOLOGIES

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Training Placement Report presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Applied Microbiology

By

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Resumo

Na indústria cervejeira, é fundamental assegurar os procedimentos de controlo de qualidade microbiológico para garantir um produto seguro e de alta qualidade. De facto, é sabido que podem ocorrer possíveis contaminações microbiológicas ao longo de todo o processo cervejeiro. Bactérias e leveduras selvagens constituem alguns dos possíveis contaminantes e, dada a sua natureza prejudicial, podem traduzir-se em vários níveis de risco. A falta de controlo pode levar a perdas económicas significativas, assim como diminuir a confiança do consumidor. Esta dissertação providencia uma análise crítica sobre as metodologias em uso na empresa Super Bock Group (SBG), nomeadamente a viabilidade do uso de meios de enriquecimento e a implementação de qPCR no trabalho de rotina no laboratório de controlo de qualidade. Durante 9 meses, 57 amostras, obtidas a partir de vários pontos de amostragem, apresentaram um resultado positivo, ou seja, deteção de UFCs em placa. Subsequentemente, os microrganismos foram isolados e identificados (testes fenotípicos), dos quais 15 foram sequenciados. Os resultados referentes à sequenciação evidenciam fragilidades na amostragem, dado que 21% dos isolados são considerados como falsos positivos, ou seja, microrganismos incapazes de se desenvolver na cerveja (nunca detetados em cervejarias), e nenhum isolado constitui um risco microbiológico (não patogénico, incapaz de deteriorar o produto). Todos os isolados de leveduras selvagens e bactérias ácido-láticas foram analisadas por qPCR, tendo-se obtido resultados negativos em todos. Para testar o sistema qPCR e a aplicabilidade dos meios de enriquecimento, duas estirpes de bactéria e uma de levedura capazes de deteriorar a cerveja foram obtidas de uma fonte externa. De acordo com a frequência de falsos positivos e a natureza restritiva dos meios de enriquecimento, é aconselhado à SBG a adoção de metodologias moleculares mais específicas (ex.: sequenciação, outro sistema qPCR mais robusto, MALDI-TOF MS) para a identificação dos microrganismos e determinar o risco que estes apresentam para o processo cervejeiro.

Palavras-chave: controlo de qualidade, segurança alimentar, cerveja, qPCR

Abstract

Ensuring a proper routine microbiological quality control testing in breweries is crucial for maintaining high product quality and safety. It is known that possible microbiological contaminations can occur throughout all of the brewing procedure. Bacteria and wild yeasts are some of the possible contaminants, due to their spoilage nature, that can be translated into several consequences, with a varying degree of risk. If unchecked, contaminations can lead to significant financial loss and decrease consumer confidence. This dissertation provides a critical analysis of current microbiological methodologies at Super Bock Group (SBG) company and the feasibility of enrichment media and implementation of qPCR into the routine work at the microbiology quality control laboratory. Over the period of 9 months, 57 samples, acquired throughout multiple sampling points, were considered as a positive result, synonym to the detection of CFUs in plate. Subsequently, all microorganisms were isolated and identified (phenotypic tests), of which 15 were sequenced. According with the sequencing results, it was identified some fragilities during the sampling procedure, since 21% of these were considered as false positives, microorganisms unable to develop in beer (never previously detected in breweries), and no isolate constitutes a microbiological risk (non-pathogenic, not a beer spoiler) to the process. qPCR was performed on all isolates of wild yeasts and lactic acid bacteria, which always lead to a negative result. Three external strains of spoilage microorganisms (two bacteria and one yeast) were obtained for qPCR and enrichment media testing. According with the frequency of false positives and the restrictive nature of enrichment media, the adoption more specific molecular methodologies (ex.: sequencing, other PCR systems, MALDI-TOF MS) are advised to the SBG for the proper identification of the microorganisms and distinction of the associated risk.

Key-words: quality control, food microbiology, qPCR, beer

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Finally, I am grateful to every colleague and friends that helped me during the last nine months and throughout my academic life.

Tomorrow will be a day full of uncertainties. Those are untaken opportunities.

You miss 100 percent of the shots you don't take.

Thank you.

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Abbreviations

AAB – Acetic Acid Bacteria

ABP - Actidione, Bromocresol green, Phenyl-ethanol

CFU – Colony Forming Unit

CIP - Clean In Place

DMS - Dimethyl sulfides

ED - Entner–Doudoroff pathway

EMP - Embden-Meyerhof-Parnas pathway

FISH - Fluorescence In Situ Hybridization

GOI – Gene Of Interest

HACCP - Hazard Analysis and Critical Control Points

IPC – Internal Positive Control

LAB – Lactic Acid Bacteria

MALDI-TOF MS - Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry

MPA – Meat Peptone Agar

MRS - De Man, Rogosa and Sharpe agar

MYGP - Malt extract Yeast extract Glucose Peptone agar

MYP - Mannitol egg Yolk Polymyxin agar

PCA – Plate Count Agar

qPCR – Quantitative Polymerase Chain Reaction

SBG – Super Bock Group

UBA – Universal beer Agar

VRBG -Violet Red Bile Glucose agar

WLN - Wallerstein Laboratory Nutrient agar

YPD - Yeast Extract–Peptone–Dextrose agar

1. Introduction

In this first chapter it will be presented the framework of this dissertation, a brief description of the internship in Super Bock Group, as well as the objectives and methodologies.

1.1. Internship outline

The internship was carried out in Super Bock Group's headquarter, located in Leça do Balio (Portugal), more specifically in the central laboratory, throughout 9 months (from September 19th, 2022, until May 31st, 2023)

1.1.1. Super Bock Group

Super Bock Group is the largest Portuguese beverages company with a multi-brand, multi-market strategy, whose core business is based on its operations in the beer and bottled water businesses. Furthermore, SBG is also present in the segments of soft drinks, wines, malt production, marketing and the tourism business, owning two leading attractions in the Trás-os-Montes region: the "Parque de Vidago" and "Parque de Pedras Recreational and Thermal Water Parks" (*Super Bock Group*, 2023).

The company was founded in 1890 as "Companhia União Fabril Portuense das Fábricas de Cerveja e Bebidas Refrigerantes". After many restructurings and name changes, being one of the most relevant ones "Unicer", in November 2017 the company was established as Super Bock Group, a reflection of its most characteristic and well-known beverage, the super bock beer. Regarding the company capital, the majority is Portuguese-owned, 56% being held by Grupo VIACER and 44% by the Carlsberg Group (*Super Bock Group*, 2023). SBG holds a multitude of beverage brands, all divided between beers, wines, water, soft drinks and ciders (Table 1).

Table 1. List containing all SBG brands (as 2023).

Beverages	Brands
Beers	Super Bock, Cristal, Cheers and Super Bock Seleção 1928
Wines	Quinta do Minho, Campo da Vinha, Porta Nova, Vinha das Garças, Vinha de Mazouco, Planura, Monte Sacro, Vini and Vini Sangria
Waters	Vitalis, Água das Pedras, Melgaço and Vidago
Soft drinks	Frisumo, Frutis, Snappy, Guaraná Brasil and Frutea
Ciders	Somersby

The vision statement of the company is “Paixão Local, Ambição Global” (translates into “Local passion, global ambition”), which is reflected upon the solid brand trust from the general public and the ambition to reach new global markets. For this reason, SGB is present across 53 countries worldwide and, according with the 2021 data (*Relatório de gestão e sustentabilidade 2021*, 2022). SBG and its 1232 collaborators, have made 413M€ in sales, which translates roughly into 400M litters of beverages sold.

1.1.2. Internship experience at SBG and objectives

Understanding the brewing process and all of its microbiological aspects (ex.: sampling, propagations, quality control, etc.), as well as the practical approach, were fundamental for this study. This dissertation will mostly focus on the analysis of multiple samples across brewing, from a qualitative standpoint (identification the microorganism), using classic and molecular methodologies and assessing the risk of possible contamination according with the identified microorganism, while surveying the possibility of occurring false positives (detected microorganisms that, under no circumstances, develop in beer). Such findings would allow a better understanding of the current microflora at SBG and improve current quality control procedures.

2. Brewing microbiology review

The ever so popular fermented beverage known as beer has always been intertwined with the human civilization, as far as Man has started his agriculture dependence. Across generations, the brewers have been perfecting their craft and quality of the products and scholars managed to demystify this procedure and even adapting new tool to the process, such as genetic engineering of yeasts. In this chapter, it will be explored the entire brewing process, with a special attention to the possible contaminants within a brewery, the focus of this dissertation. Furthermore, the duality between classic microbiology methods and recent molecular tools for the detection of these contaminants will be taken in consideration.

2.1. Brief history of beer

The evolution of the brewing process is closely related with the progress of human civilization. Since its origin, beer is considered as a valuable food item that is continuously being enhanced and tailored to meet human needs. During most of human history, beer has had a low ethanol concentration (Hornsey, 2003), which could lead to intoxication when consumed in high quantities, although this was not its main purpose. Given its inherent characteristics and when access to potable water was limited, beer was frequently the only safe drink.

The genesis of brewing is not clear, although there is some speculation that leads back to the Neolithic man (Hornsey, 2003), as mankind moved away from a nomadic hunter-gatherer lifestyle to an agriculture-based existence. During this time, the first civilizations have flourished in the Fertile Crescent, Mesopotamia and Egypt and with that comes the oldest evidence related with brewing. Chemical analysis of ancient pottery jars has proven that the first traces of a fermented beverage was being produced in Jiahu (China) as early as the seventh millennium BC (McGovern et al., 2004).

During the mentioned period, all the prerequisites were met for the making of beer: the accessibility of suitable grains, a stable source of energy (ex. fireplace) and apt brewing vessels. Still, it is believed that beer was invented by pure chance, a byproduct of the conditions at the time. Since the storage of grains (ex. barley) was in uncovered containers, these were exposed to the elements, such as rain coupled with the temperature, which led to the sprouting of the grains. Natural enzymes present in barley began to degrade starch into fermentable sugars, which coupled with water from the rain and yeasts present in the air, made the fermentation possible.

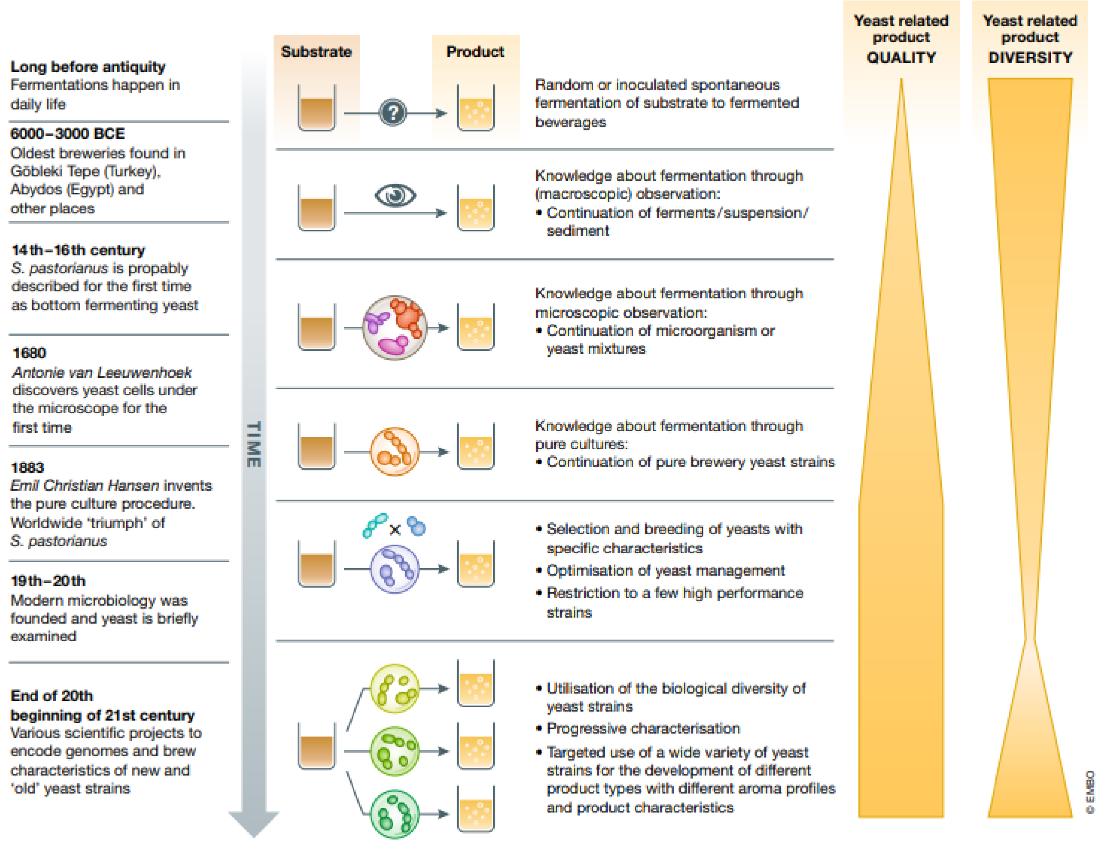

Figure 1. Brewing evolution over time. The most important discoveries and developments during a history of 10,000 years of brewing beer (Harrison & Albanese, 2017; Raihofer et al., 2022).

Across the different ages, the brewing process has been slowly evolving (Figure 1). Up until the end of the Middle aged, the procedure has started to transition from an empirical concept to a theoretical one, with the first record of a description of bottom fermenting yeast (most probably *Saccharomyces pastorianus*). Afterward, brewing technology evolved with the Industrial Revolution, which made possible a higher production volume. With the birth of the modern microbiology, brewing underwent a revolution. The discoveries of Emil Christian Hansen (1842-1909) greatly contributed to the quality and consistency of beer production, which involved proper yeast propagation and management of "pure" cultured yeast and the discovery of a crucial yeast stain for Lager beer, the *S. Carlsbergensis*, now known as a variant of *S. pastorianus*. Discoveries made by Luis Pasteur (1822-1895) allowed the introduction of pasteurization in the process (Smith, 2012).

Nowadays, modern brewing has achieved a balance between advanced biotechnology and traditional knowledge. The brewing industry could be divided between macrobrewers and craft breweries (which are defined as being small, independent and traditional), which vastly diverse when it comes down to quantity produced, diversity and taste. Macrobrewers mostly focus on brewing the highest quantity possible while assuring its quality and constant results/flavor; on the other hand, craft breweries are more experimental. Brewer can play around with alternative yeast strains, raw materials and other food additives in order to achieve a new complexity of flavors. This craft beer movement has gained some traction in recent years; where once large brewers were the dominant force in the beer market, smaller breweries have started to make some headlines. According with the Statista's "Beer Report 2023" (*Market Insights - Beer - Worldwide*, 2023), the overall global beer market, in 2023, amounts to US$610.00b and, in 2021, the total amount of beer produced globally was 1.86 billion hectoliters. Additionally, in the U.S. market, according with the US Brewers Association (*National Beer Sales & Production Data*, 2022), the number of craft breweries has increased from 1509 to 9210 (period 2000-2021). Nevertheless, across generations, beer has offer consumers an unprecedented variety of tastes and flavors, at an affordable price, with an ever-increasing interest in new ways to innovate the current staples in the industry.

2.2. Beer - styles and classification

The definition of "beer" is somewhat ambiguous. The classic designation (first introduced in the Bavarian Purity Law of Germany) is as a fermented beverage made of malted cereals, water, hops, and yeast. A more technical definition would be as an alcoholic beverage produced by the joint or sequential action on starch of amylolytic enzymes and glycolytic enzymes. The last one delimits the crucial biochemistry behind it, saccharification of starch and alcoholic fermentation of sugars, and does not mention any ingredient beyond starch, since they do not differentiate the alcoholic beverages.

The beer industry is vast and diverse and for that reason it is difficult to estimate how many commercial breweries and different kinds of beer exist globally. Usually, beers are classified into different *styles*, which are a combination of different parameters that allow to achieve different and unique beers (*2023 Brewers Association Beer Style Guidelines*, 2023). All existing types are the result of a combination of the ingredients, processing, packaging, marketing and culture. Dunkel, IPA, Stout and Bock are just a few examples of different styles. Overall, beers can be divided in two categories, ale or lager

(Table 2); although there is a number of differences between both production methods, the key difference is the chosen yeast strain.

Table 2. Comparison between ale and lager yeast strains (Harrison & Albanese, 2017; Hill, 2015).

Characteristics	Ale	Lager
Yeast strain	*Saccharomyces cerevisiae*	*Saccharomyces pastorianus*
Fermentation temperature	18 – 22ºC	8 – 15ºC
Optimal growth temperature	>30ºC	<30ºC
Flocculation	Clumps or flocs entrap CO2; lower density floc; floc rises to the surface of the liquid in the fermenter	Higher density floc; floc settles to the bottom of the fermenter
Metabolism	Cannot metabolize melibiose and raffinose	Ability to metabolize melibiose and raffinose

Beyond the described categories, one could consider the existence of a third one: lambic sour beer. This style is typically known as being a traditional Belgium beer and having a unique flavor. These organoleptic characteristics are the result of a spontaneous fermentation. Given that no selected yeast is added and the cooling of the wort is carried out in an open vessel, a plethora of microorganisms are able to be mixed in. Wild yeast, LAB and AAB are able to give a sour character to this beer (Spitaels et al., 2015).

2.3. Brewing overview

Beer is the result of the fermentation of simple sugars from grains. The conversion of the raw material to beer is not as straight forward as one may think, as brewing can be a somewhat complex industrial procedure. As said previously, the main ingredients are water, fermentable carbohydrates, hops and yeasts. Moreover, there could be the addition of adjuncts - any starch or sugar-containing substrates that contribute fermentable carbohydrates. Thus, these fundamental blocks define the character of a beer.

2.3.1. Ingredients

In a brewery, water is used in different steps for different purposes and, overall, for every 3 L of water used, only 1 L of beer is made. Furthermore, beer contains a water percentage of around 90 – 95%, and its quality can influence the organoleptic qualities of the final product (Harrison & Albanese, 2017). Water usage can be divided in four categories (Stewart et al., 2017):

- *Brewing water*; Directly used as a beer ingredient, as it is used to produce wort and to standardize the alcohol content to a desired level (such as in high-gravity brewing). To achieve the desired beer, water must have a certain chemical composition, hence this liquid sometimes undergoes a treatment.

- *Process water*; Preferably potable and softened (Degremont, 1979), used for washing and sterilizing of vessels and pipework and for container cleaning and rinsing.
- *Service water*; Similar to process water, however requires additionally special treatment appropriated to its specific application (ex. pasteurizer).
- *General purpose water*; Involves the rest of water usage, that does not require further treatment, across the industrial site and offices.

Malt consists of grains, usually barley, that have been germinated and then dried through a process known as malting. Barley has a significant amount of protein to enable yeast development and aid in the formation of beer foam, as well as a high starch content that may be converted into fermentable carbohydrates. Beyond these, malt also provides enzymes, proteases to generate free amino nitrogen, lipids, polyphenols/tannins, vitamins and confer color and flavor (Farber & Barth, 2019). Different types of malt can be used to achieve specific characteristics in the beverage being produced.

Hops (*Humulus lupulus*) are the dried flowers from the female hop plant and impart bitterness, aroma, flavor and longer shelf life to beer (Cattoor et al., 2013). These distinct characteristics come from a substance found within the hop cones (the flower-like structures of the hop plant), called lupulin. This soft resin contains alpha and beta acids which are associated with flavor and bitterness, as well as antimicrobial properties. Under high temperature conditions, alpha acids are isomerized to iso-alpha acids, the main component for beer bitterness (Sun et al., 2022).

Finally, the main catalyst for brewing, the yeast is responsible for the fermentation, being a similar process for both types of beer. These eucaryotic organisms are able to utilize wort sugars and free amino nitrogen to produce alcohol, carbon dioxide, new yeast cells and aromatic compounds (Stewart et al., 2017). Under aerobic conditions, yeast metabolizes glucose into two pyruvate molecules (EMP pathway) and then via the Krebs cycle and oxidative phosphorylation to carbon dioxide and water with a large energy gain. As a lack of oxygen prevails, the Krebs cycles comes to a halt, despite the EMP pathway still working. As pyruvate, an acidic compound, is accumulated, yeast removes this by metabolizing it into acetaldehyde and, subsequently, ethanol and CO_2.

Once all the sugars are depleted, the yeasts flocculates, which further allows an easier removal of it.

2.3.2. Brewing process

Understanding the brewing process is crucial to acknowledge the possible microbiological risks. Each individual step is presented in the Figure 2. The first step of the process is the conversion of barley into malt. Here, the main objective is the production of a sufficient number of enzymes that degrade starch, protein and other components of the grain. The mashing process involves mixing the malt with water, inside of a mash turn. By controlling the temperature, it is possible to enhance the activity of the enzymes which will allow the hydrolyzation of the non-soluble, complex carbohydrates into soluble fermentable compounds. Eventually, the solids settle out and the enzymes (natural or added) are inactivated, which leaves the wort. Here, the objective is to collect the maximum amount of sugars from the raw material. The left solids (spent grains) can be sold as a sub-product of the process (as animal feed) and the liquid is transferred into the brew kettle.

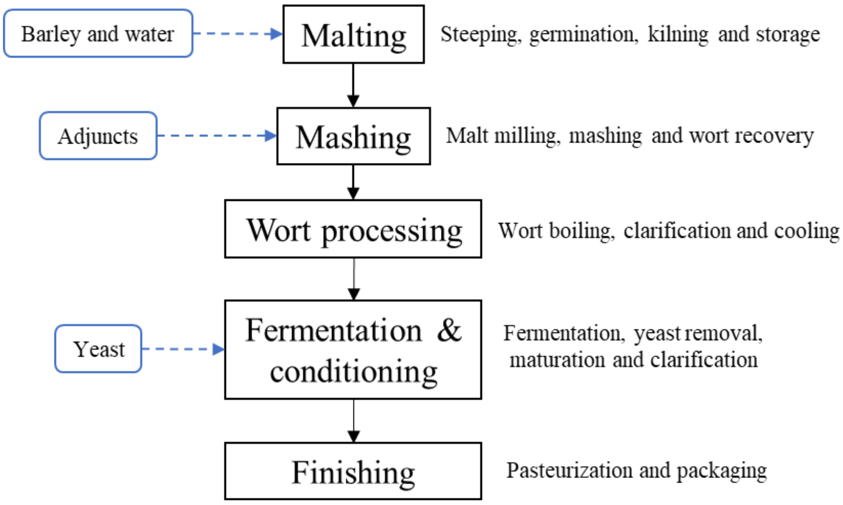

Figure 2. Brewing process and each associated sub processes ("17 - Microbiology," 2004; Harrison & Albanese, 2017; Stewart et al., 2017).

On this tank, hops are added and the mixture undergoes a heat treatment. This step satisfies a multitude of crucial objectives: (1) nearly killing any of the remaining microorganisms, (2) inactivating any remaining enzyme, (3) extracting bittering compounds (early addition), oils and aroma compounds (late additions) from hops, (4) coagulate the excess proteins, (5) enhancing color development, (6) removal of unwanted volatiles, such as dimethyl sulfide, and (7) concentration of sugar by water evaporation

(Stewart et al., 2017). Afterwards, through a heat exchanger, the wort is cooled to a certain temperature apt to the type of yeast used next. Air or oxygen is added to the wort, before the fermentation occurs, which is essential for healthy yeast growth and optimal fermentation. At this stage, wort is ready for being fermented, since this complex medium contains fermentable sugars (glucose, fructose, sucrose, maltose and maltotriose), unfermentable dextrins, free amino nitrogen, ions, vitamins, and so on.

Once the malt is transferred into the cylindroconical fermenter vessel, yeast can be pitched in order for the fermentation to occur. Despite being a procedure with a plethora of parameters to be aware and control, what it is crucial to know is that yeasts will consume the carbohydrates and free amino nitrogen to produce carbon dioxide, alcohol, new yeast cells and flavor compounds. Throughout this step, a variety of chemical properties are tracked (ex.: solids and ethanol content, degree of fermentation, pH, etc.) as well as the amount of yeast cells and their viability (Farber & Barth, 2019).

A proper yeast handling in a brewery is crucial, from a quality standpoint, and can be highly cost efficient. Before the fermentation, in order to ensure an adequate and healthy yeast population, a pure culture of yeast is used to start a propagation. This procedure can be divided into three main phases: cell culture, laboratory and commercial propagation (Figure 3); all must abide to sterile conditions to prevent any external contaminations. Pure cells cultures may be ordered from a cell culture bank or university and these can be either cryopreserved (-80ºC), which ensures the long-term viability, or stored in slants (4ºC), up to four months, either in malt agar or YPD medium - most common (Farber & Barth, 2019).

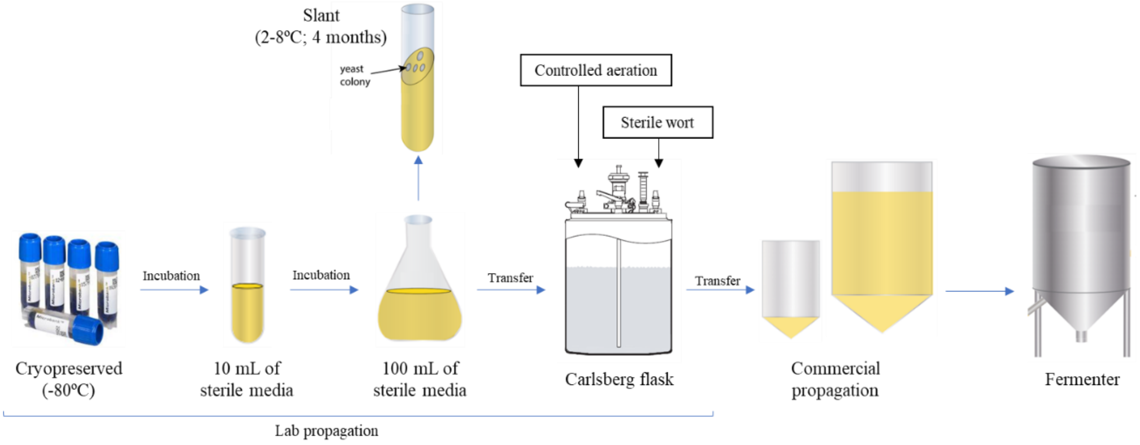

Figure 3. Typical propagation of yeast in a brewery.

At the end of fermentation, often in the same vessel, before yeast removal, *lagering* (or maturation) occurs. During this this phase, beer undergoes several biochemical and flavor changes that contribute to its overall quality, such as the reduction of acetaldehyde and diacetyl. Afterwards, bright beer is produced due to a clarification step, where particles are removes via centrifugation or filtration. Once the yeast is removed, it can be transferred into a storing vessel and can be reused for another fermentation. Cell viability, quantity, temperature and testing possible contamination must be kept in check before repitching.

The final stage, finishing, involves several (some optional) processing steps in preparation for distribution to the consumer. Such possible steps include: chill-proofing (addition of proteases to prevent haze development) (Harrison & Albanese, 2017), carbonation or alternatively bottle conditioning (adjust the final carbon dioxide level) and pasteurization, either tunnel or flash (improves the shelf life of the final product). Regardless, beer is properly filled in the desired contained (keg, bottle or can).

Parallel to this whole procedure, a separated system, built into the design of the plant, is capable to maintain the plant cleanliness and a hygiene control. Clean in place (CIP) is the process of cleaning the equipment and involves different automated steps. Among the brewery industry there is no single identified best procedure (Laing et al., 2021); hence each individual brewery tends to adapt the program to the facility, always striving to optimize its effectiveness. A cleaning cycles usually involves, in order: prerinse (water), alkaline (caustic) detergent wash, intermediate rinse, acid wash and final rinse (Farber & Barth, 2019). Nevertheless, the objective is always the same, the sanitization of the equipment, removal of undesired materials that could contribute to off--flavors or interfere with the proper operation and reduce the microbiological risk (surface biofilms).

In order to assure the quality of the final product and homogeneity between batches, certain control measures are employed. Routine quality control procedures can trigger correctives measures to the process when abnormal situations occur. Within the brewery, four key areas comprise the quality control: biological, chemical-analytical, sensory analysis and field quality (related with packaging). The biological area includes all related with yeast management, fermentation monitoring and microbiological testing.

2.3.3. Microbiological control in a brewery

Beer is widely considered as a microbiological stable beverage (Sakamoto & Konings, 2003). An environment with reduced levels of oxygen (less than 0.1 ppm), high CO_2 content (0.5% w/v), low pH (3.8-4.7) and ethanol (0.5-10% w/w) prevent the growth of many microorganisms (Hill, 2015). The presence of hop bitter compounds also provides an extra layer of defense against bacteria, given their antibacterial properties (Bartmańska et al., 2018). Additionally, this beverage is also considered to be a poor medium due to the lack of present nutrients, result of the fermentative activity of brewing yeast. Given these antimicrobial hurdles and the processing methods, only a limited number of species can potentially have a negative impact on a range of quality characteristics in beer. Moreover, it is widely assumed that foodborne pathogenic microorganisms cannot survive in beer (Vriesekoop et al., 2012), although there have been instances where some species could survive for some time (Menz et al., 2011) and grow in alcohol-free beer (L'Anthoën & Ingledew, 1996).

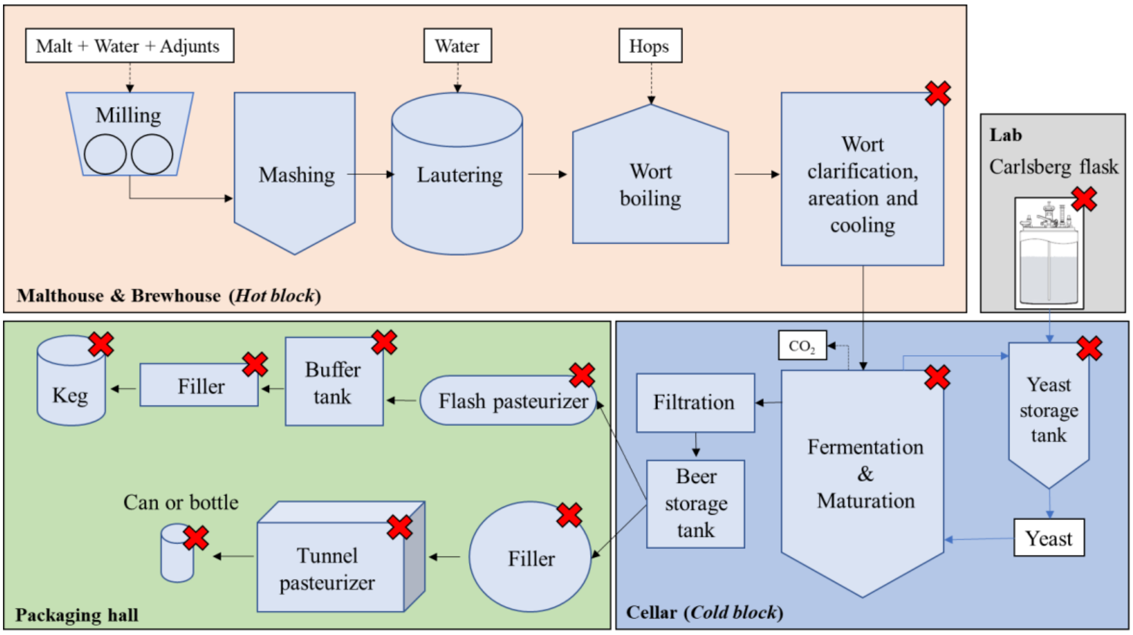

Figure 4. Schematics of the brewing procedure - steps with a red mark are considered vulnerable to possible microbiological contamination ("17 - Microbiology," 2004; Vaughan et al., 2005).

Beer stability is essential for maintaining flavor, quality, and customer satisfaction. The detection of any possible contaminants throughout brewing is necessary; even if there are preventive measures, unpredictable spoilage can still occur and these assessments can save time and money. Other deteriorating characteristics include: changes in alcohol content and attenuation, off-flavors, gushing, excessive acidity and turbidity (Farber & Barth, 2019). It is assumed that after boiling, beer is free of live microbes. Thus, a brewery needs to define what are the most (microbiological) vulnerable points (Figure 4), what aspects to test (according with the type of sample) and their frequency.

The stage of the processing (and its characteristics) influences the type of microorganisms that can occur and their due spoilage ability; if aseptic conditions are assured during sampling, one can predict what possible contaminants are expected. Positive detection leads to corrective measures; one such case could happen in the yeast storage tank. Prior to pitching, a sample of the brewer's yeast is tested for the presence of LAB and wild yeast; a positive for LAB implies that the yeast must undergo an acid wash procedure (Munford et al., 2020) and if positive for wild yeasts, the entire vat content must be discarded since there are no recorded methods capable of eliminating these contaminants. The following chapters go more in depth about possible contaminants.

2.4. Microbiological risk - contaminants

2.4.1. Gram-positive bacteria

2.4.1.1. Characteristics of lactic acid bacteria (LAB)

LAB such as *Lactobacillus* and *Pediococcus*, can display some resistance to hop bitter compounds, being the predominant beer spoilers (P. Ashtavinayak & H. A. Elizabeth, 2016). Furthermore, these bacteria confer certain undesired characteristics to the final product, such as an increase in turbidity, some acidity and off-flavors.

The *Lactobacillus* genera is quite diverse, comprising 261 recorded species (at March 2020) (Zheng et al., 2020) with little commonality. Being non spore forming, oxidase and catalase negative, facultatively anaerobic, having a rod shape and the ability to produce lactic acid, either as an exclusive or at least a major product, are the only transversal taxonomical characteristics. The lactobacilli, in general, have a poor growth in the presence of air. These grow best in rich complex media since they are auxotrophic for a variety of nutrients. Their optimum temperature is 30-40ºC, although it is also possible to have some bacterial growth over a range of 5-53ºC. They also are aciduric with an optimum growth pH of 5.5–5.8; in general, they can grow at a pH <5 (Batt, 2014).

Beers with high pH values and a low ethanol concentration can be spoiled by some hop sensitive lactobacilli species, such as *Lacticaseibacillus casei*, *Loigolactobacillus coryniformis* and *Lactiplantibacillus plantarum*. Despite the low number of incidents related with these species (Hill, 2015), they should be considered as risk since they cause diacetyl off flavor in beer. Species like *P. inopinatus* and *P. claussenii* have also been recorded as possible beer contaminants (Dobson et al., 2002; Iijima et al., 2007). Pediococci are facultatively anaerobic to microaerophilic, oxidase and catalase negative and, unlike lactobacilli, *Pediococcus* can be seen in culture perfectly round cocci, in pairs and tetrads.

The knowledge of extensive diversity of the genus *Lactobacillus*, which includes the genus *Pediococcus* as an integral part, was only possible through the use of genotypic methods, such as 16S rRNA-based phylogeny. Recent genomic analysis, plus some physiological and phylogenetic considerations, have replaced the older differentiation of LAB with a simpler one, between homofermentative and heterofermentative (Zheng et al., 2015). Homofermentative LAB are capable of metabolizing hexoses via the EMP pathway to pyruvate as the key metabolic intermediate, and heterofermentative LAB metabolizes hexoses via the phosphoketolase pathway to pyruvate and acetyl phosphate as key intermediates (Figure 5). Heterofermentative strains produce a mixture of end products, including lactate, glycerol, ethanol and acetate.

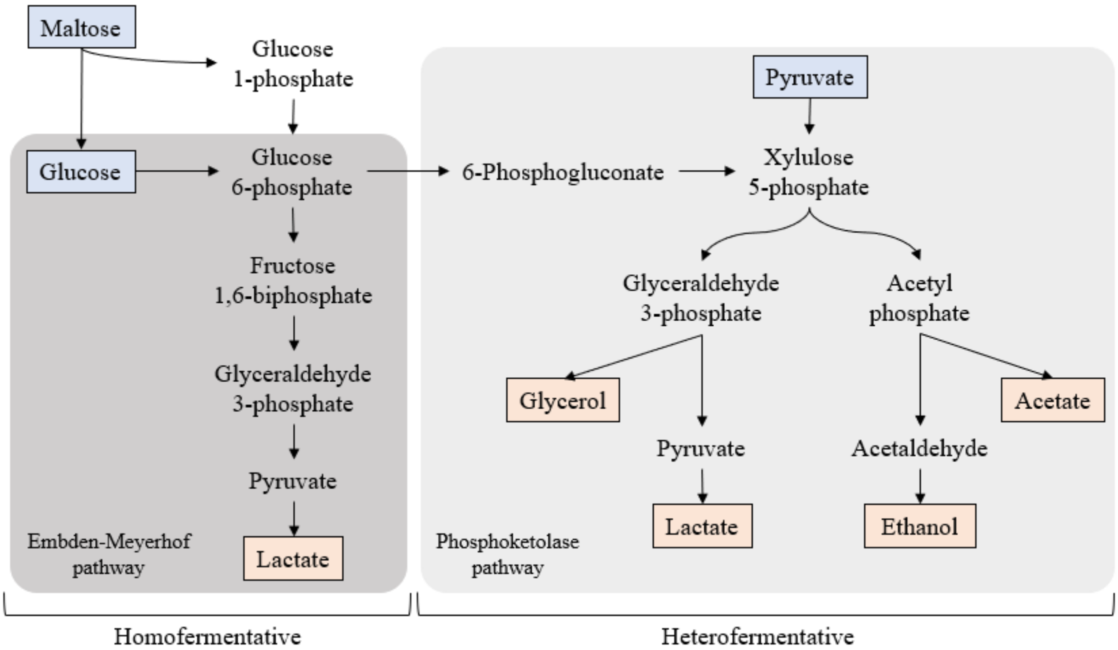

Figure 5. Homofermentative and heterofermentative sugar metabolism in lactic acid bacteria (Gänzle, 2015).

It is noteworthy that a recent study (Zheng et al., 2020) has proposed the reclassification of *Lactobacillus* into 25 different genera, based on the evaluation of different parameters within whole genome sequences. Despite this, the generic term ‘lactobacilli’ is used to designate all organisms that were classified as *Lactobacillaceae* until 2020. It is known that most microbiological spoilage incidents are caused by different LAB. Among these, *Levilactobacillus brevis*, *Fructilactobacillus lindneri* and *Peddiococcus damnosus* are considered as the most widespread beer spoilers. Moreover, *L. brevis* has been reported as being the most frequently detected LAB species in spoiled beer products, as well as in fermentation and maturation processes (Back, 2005). However, depending on the strain and source of isolation, the beer-spoilage of ability *L. brevis* differs considerably; some strains are highly resistant to disinfectants and can tolerate multiple pasteurization processes (Rachon et al., 2018). Hence, the intraspecies differentiation of beer-spoilage ability in *L. brevis* is important in the brewing industry. Beyond the awareness for these high thermal treatment tolerant strains, one must consider those that are resistant to hop compounds.

The addition of hops during the brewing procedure enhances the anti-microbial properties of beer against gram-positive bacteria. Alpha and beta acids, when in contact with gram-positive bacteria, cells are ultimately killed since the enzymatic activity and nutrient transport are compromised. These molecules function as proton ionophores, dissipating the transmembrane proton gradient, decreasing cytoplasmic pH, and squelching proton motive force (Behr & Vogel, 2009). Additionally, further oxidative stress is caused to the bacterial cell, because iso-alpha-acids participate in transmembrane redox reactions in association with manganese.

Regardless, some LAB have managed to adapt to the harsh beer environment and develop a resistance to hop bitter compound. This tolerance has been correlated with the presence of genes *horA* (ATP-dependent transporter protein), which purges hop compounds from the cell, and *horC* (Suzuki et al., 2006). Opposite to gram-positive bacteria, alpha and beta acids do not have a noticeable impact on gram-negative bacteria; on yeasts there is little to no inhibition. In the case of *S. cerevisiae* there is an active relegation of these compounds within the vacuole, expulsion across the cell membrane and modification of the cell wall structure in response to hop stress.

Given the unpredictability of possible contaminations, due to a multitude of factors, if LAB are found in a brewing environment, these should always be subjected to a confirmation for the presence of the relevant genes, to establish the potential risk of spoilage. Additionally, different hop compounds have varying effects on LAB (Michel et al., 2020); it is possible to inhibit a known microorganism with a specific hop compound.

The identification of microorganisms can be carried out through phenotypic or genotypic methods. Among LAB, the analysis of a few traits helps to distinguish these. The characteristics are presented in the following table.

Table 3. Characteristics for discrimination of LAB (Batt, 2014).

Characteristics	Lactobacilli	*Lactococcus*	*Leuconostoc*	*Pediococcus*
Tetrad formation	-	-	-	+
Co2 from glucose	+/-	-	+	-
Growth at 10 °C	+/-	+	+	+/-
Growth at 45 °C	+/-	-	-	+/-
Growth at 6.5% NaCl	+/-	-	+/-	+/-
Growth at 18% NaCl	-	-	-	-
Growth at pH 4.4	+/-	+/-	+/-	+
Growth at pH 9.6	-	-	-	-
Lactic acid isomer	D, L, DL	L	D	L

The differentiation of some species within *Lactobacilli* is possible through the identification of some traits (Table 4). While phenotypic characters are generally important for species description, the known characteristics for most lactobacilli are too limited to be able to serve as the unique identifier. Furthermore, it is advised the use of standardized and commercially available system, such as API 20 STREP and API 50 CH (bioMérieux), for a more accurate and reliable result. Additionally, the implementation of additional genotypic techniques allows a more precise identification.

Table 4. Characteristics for discrimination of different beer spoilage lactobacilli (Batt, 2014; Zheng et al., 2020).

Genera	Species	Fermentation type	Relation to oxygen	Growth temperature specifications	Lactic acid isomer(s)
Fructilactobacillus	*F. lindneri*	Heterofermentative	Microaerophilic	15 - 37°C	DL
Latilactobacillus	*L. curvatus*	Homofermentative	Facultatively anaerobic	15 - 45°C	DL
Lacticaseibacillus	*L. casei*	Homofermentative	nd	10 - 45°C	DL
Lactiplantibacillus	*L. plantarum*	Homofermentative	Facultatively anaerobic	15 - 45°C	DL
Lentilactobacillus	*L. buchneri* *L. parabuchneri*	Heterofermentative	Facultatively anaerobic	Most at 15°C (some at 45°C)	DL
Levilactobacillus	*L. brevis* *L. paucivorans*	Heterofermentative	Facultatively anaerobic	Most at 15°C (but not at 45°C)	DL
Loigolactobacillus	*L. coryniformis*	Homofermentative	nd	15 - 45°C	DL
Schleiferilactobacillus	*L. harbinensis*	Homofermentative	nd	15 - 42°C	L
Secundilactobacillus	*S. malefermentans*	Heterofermentative	Facultatively anaerobic	15 - 45°C	DL

A plethora of media have been described as successful for the identification of lactobacilli. Media such as UBA, all-purpose Tween 80, modified Homohiochii media or Raka-Ray agar are all a possibility. However, MRS agar has been proven to be the most optimal medium for this purpose (Kubizniaková & Matoulková, 2016). Depending on the specific composition of the medium, the addition of an inhibitor, such as antibiotic actidione, is advised since it allows the inhibition of yeasts, molds and some gram--negative bacteria.

2.4.1.2. Spore forming bacteria

Spore forming bacteria are another type of bacteria that can be isolated from work-in-process products and finished beer products. Their detection is related with the fact that these are difficult to completely eradicate from brewing environments, due to their strong resistance to heat treatment and disinfectants. Genera such as *Bacillus* and *Paenibacillus* have been reported in such facilities (Brozova et al., 2018a).

Bacteria of the genus *Bacillus* are generally considered to be hazardous contaminants of various foods and food ingredients. Given their vast availability in nature and thermoresistant spores, they can slip into the industrial process in the middle of the raw materials. These bacteria are aerobic or facultative anaerobic, spore forming and mostly catalase positive. Some species, in later stages of growth, are gram-variable. Cells are rod-shaped with rounded or straight ends and can occur singly, in pairs, or in shorter chains. Spores are formed inside the cell (endospore) and may be located anywhere within the cell.

In *Bacillus*, spore formation takes place when the cell reaches the stationary growth phase. Sporulation may be induced by nutritional deprivation, or cell density and is affected by numerous factors, such as temperature, pH, aeration, and availability of various nutrients. During the sporulation process, a vegetative cell (the progenitor) gives rise to two specialized cells, that differ in cell type, both from each other and from the parent cell (Jenson, 2014).

Some *Bacillus* bacteria can survive mashing and wort boiling (Back, 2005), however if the wort is boiled for more than 45 minutes, all vegetative cells and their spores are destroyed. Since during soaking and germination of barley there is an increase of microbial microflora, some microorganisms will be present on green malt (Bokulich & Bamforth, 2013). Some *Bacillus* are able to survive when exposed to high temperatures and appear on properly kilned malts. Malts exposed to lower temperatures (in order to kill microorganisms) are more vulnerable to contaminations. Furthermore, these might also enter the brewing operations with malt surrogates (unmalted cereals, starch extracts, syrups and concentrates, malt extracts, etc.) or with improperly treated and stored wort extract (Brozova et al., 2018a). Given their production of metabolites, such as butyric acid, and their high degree of proteolytic activity, these microorganisms may cause irreversible damage to these substrates.

Another aspect to considered when dealing with a *Bacillus* contamination, during the brewing process (especially during mashing) is their ability to reduce nitrate to nitrite. Nitrous acid can be formed in the acidic aqueous medium, from the nitrite ions, soluble in water, and further react to form nitrogen oxides.

As said previously, despite some strains being able to survive to a high temperature treatment, beer (as a finished product) does not give them the proper condition for their development due to low pH and presence of hop compounds, hence these microorganisms are generally not considered hazardous brewing contaminants. However, some spore forming bacteria strains containing the *horA* gene (resistant to hop compounds) have been discovered (Haakensen & Ziola, 2008). Such is the case for *Bacillus cereus*, *Bacillus licheniformis*, *Paenibacillus humicus*, and *Staphylococcus epidermidis*, of which are considered as a possible cause for beer spoilage. Furthermore, *B. cereus* is considered as a food pathogen, thus is considered as a major health risk.

Table 5. Some discriminatory characteristics of major beer spoilage spore forming bacteria (Brozova et al., 2018a; Jenson, 2014).

Species	Spores (oval or cylindrical)	Anaerobic growth	Lecitinase activity	Casein hydrolysis	Starch hydrolysis	Voges-Proskauer test
B. cereus	Central, not swelling	+	+	+	+	+
B. licheniformis	Central to terminal, not swelling	+	-	+	+	+
B. subtilis	Central, not swelling	-	-	+	-	-
Brevibacillus brevis	Subterminal swollen	-	-	-	-	-
Paenibacillus macerans	Terminal, swollen	+	-	+	+	-

Some of the major *Bacillus* relevant for brewing are *B. cereus*, *B. licheniformis*, *B. subtilis*, *Brevibacillus brevis* and *Paenibacillus macerans*, among others. Some characteristics for their differentiation are be shown in Table 5. Moreover, a plethora of different spore forming bacteria strains were found in alcohol-free and lager beers, even strains containing the *horA* and *horC* genes (Munford et al., 2017). Despite this, most strains found were in a negligible number of isolates and those with a beer spoilage ability were not capable of growing in beer (given the low pH). Still, these can be regarded as indicators of raw materials quality and of efficiency of hygiene regimes in breweries. The most suitable medium for the isolation of bacilli is MYP agar (Brozova et al., 2018b).

2.4.1.3. Other gram-positive bacteria relevant in brewing

Other common contaminants belong to the genera *Staphylococcus*, *Kocuria* and *Micrococcus* (Nuñez, 2014). Usually, these bacteria are not considered as major spoilage microorganisms, however they can be typically found in brewery environments. Some important characteristics for the discrimination of the genera can be shown in the following table.

Table 6. Characteristics for discrimination of various gram-positive bacteria (Matoulková & Kubizniaková, 2018).

Characteristics	*K. kristinae*	*K. varians*	*Micrococcus*	*Staphylococcus*	*Pediococcus*
Cell shape	Cocci	Cocci	Cocci	Cocci	Cocci
Cell arrangement	Single, pairs, tetrads	Tetrads	Single, pairs, tetrads	Single, pairs, irregular clumps	Single, pairs, tetrads
Cell size (µm)	0.7–1.2	0.9–1.5	0.5–1.0	0.5–1.0	0.5–1.0
Relationship to oxygen	Facultative anaerobic	Aerobic	Aerobic	Facultative anaerobic	Microaerophilic
Metabolism	Respiratory	Respiratory	Respiratory	Respiratory, fermentative	Fermentative
Production of acids	+	+	-	+	+
Production of pigments	+	+	+	+/-	-
Catalase	+	+	+	+	-
Oxidase reaction	+	-	+	-	-

Cocci, such as *Staphylococcus epidermidis* and *Staphylococcus saprophyticus*, are sensitive to hop compounds and are unable to grow in low pH media (<4.5). Still, these can survive for long periods of time and sometimes be detected in routine quality control analysis.

Kocuria kristinae has been reported as beer contaminant, with a low potential of spoiling beer due to its sensitivity to hop compounds, ethanol, and pH. Additionally, this specific species is facultatively anaerobic, unlike the other *Kocuria* species. The intensity of their growth is affected by the oxygen content in the product. In this case, spoilage occurs with relatively high pH and low bitterness beer products (Hill, 2015) . *Kocuria varians* is another possible contaminant that can survive in beer for a long period of time, although it does not damage the product. The risk of damage to beer by *Kocuria* is relatively negligible. The main issues related with this contaminant are their detection is an indication of a poor hygiene procedure and the misplacement possibility for other microorganisms during routine quality control analysis; usually mistaken by *Pediococcus*.

In aerobic condition, the optimal media for the incubation of *Kocuria* and *Micrococcus* are MPA and PCA. UBA is also a valid possibility, which can be used to distinguish from *Pediococcus* if incubated in both conditions (aerobic and anaerobic). These genera grow in the form of flat, colorless, smooth shiny colonies with a smooth rim.

2.4.2. Gram-negative bacteria

Beer is microbiologically stable due to several intrinsic microbiological properties, however, there is still the possibility of contamination of gram-negative bacteria. A number of species were detected in the brewing environment and have been linked to the cause of spoiling the end product (Table 7). Beer spoilage gram-negative can be divided between two categories. The first consists of anaerobic bacteria, such as *Pectinatus*, *Megasphaera*, *Selenomonas* and *Zymophilus*, which are mainly an issue in unpasteurized beer. The other are aerobic and facultative anaerobic bacteria, such as acetic acid bacteria (AAB), *Zymomonas* and certain *Enterobacteriaceae* species.

Table 7. Spoilage effects and metabolic products produced by gram-negative beer spoilage bacteria (P. Ashtavinayak & A. Elizabeth, 2016).

Bacteria	Occurrence in brewery environments	Aroma (*Off-flavors*) and odor	Metabolic products
Acetic acid bacteria			
Acetobacter	Wort, beer dispenses and cask condition ales and barrel aged ales, brewery biofilm.	Sour, vinegar	Acetic acid
Gluconobacter	Wort, beer dispense and cask condition ales	Sour, vinegar	Acetic acid, acetate
Enterobacteriaceae			
Citrobacter	Brewing liquor, fermenting wort	Parsnip, sulphury	DMS, diacetyl, lactic acid, acetaldehyde
Klebsiella	Fermenting wort, biofilm	Unpleasant odour	4-vinylguaicol, DMS, diacetyl.
Obesumbacterium	Pitching yeast and fermenting wort	Parsnip, sulphury	DMS, diacetyl, higher alcohols and N-nitrosamines, acetoin
Rahnella	Pitching yeast, initial stages of fermentation (wort)	Fruity, sulphury,	DMS, diacetyl, methyl acetate, ethyl acetate
Zymomonas	Primed beers (not found in lagers)	Fruity, rotten apple, rotten egg	Acetaldehyde and H_2S
Obligatory anaerobes			
Megasphaera	Low alcohol, unpasteurized beer, beer filling area, biofilm	Unpleasant odor	H2S, butyric acid, isobutyric acid, caproic acid, valeric acid, isovaleric acid.

Pectinatus	Low alcohol unpasteurized beer, beer filling area, biofilm	Rotten egg, unpleasant odor	Acetic acid, propionic acid, lactic acid, succinic acid, H2S, acetoin, methyl mercaptan and other sulfur compounds
Propionispira	Pitching yeast or brewery waste	Unpleasant odor	Acetic acid and propionic acid
Selenomonas	Pitching yeast	Unpleasant odor	Acetic, lactic, and propionic acids.

2.4.2.1. Characteristics of acetic acid bacteria (AAB)

The AAB belong to the family of *Acetobacteraceae* as a branch of acidophilic bacteria. AAB are non-sporulating, gram-negative bacteria which usually have strictly aerobic metabolism with oxygen as a terminal electron acceptor (P. Ashtavinayak & A. Elizabeth, 2016). These mesophilic microorganisms are oxidase negative and catalase positive. Morphology consists of cocci or rod-shaped cells of various lengths occurring individually or in chains. The optimal temperature for their development is between 25ºC to 30ºC, and never above 37ºC. For their development, oxygen is required and an optimal pH, between 5.0 to 6.5 (Wang et al., 2015). In regard to nutrient requirements, AAB are classified as nutritionally undemanding microorganisms and are able to metabolize ethanol and a plethora of carbohydrates (glucose, arabinose, fructose, galactose, etc.) as a carbon source.

Oxidation of ethanol to acetic acid is one of the most important metabolic characteristics of AAB. First, ethanol is oxidized to acetaldehyde, followed by another oxidization to acetic acid; the reactions are carried out by cytoplasmic membrane-bound enzymes (Lynch et al., 2019). AAB can also metabolize carbohydrates already present in beer, through three different metabolic pathways (Guillamon & Mas, 2011). Nonetheless, energy is produced due to the oxidation of NADPH, via respiratory chain, and ATP formation by oxidative phosphorylation ("17 - Microbiology," 2004). Some important different characteristics from these genera can be shown in the Table 8. Due to metabolic differences, *Acetobacter* can better develop in beer, when compared with *Gluconobacter* (Kubizniaková et al., 2021).

Modern beer production has an emphasis on the removal oxygen in post fermentation processes (Vriesekoop et al., 2012). For this reason, plus the inherent characteristic of beer, the development of AAB is very low. The ever-increasing implementation of effective cleaning and sanitization procedures further contributes for lowering the risk of AAB contamination. Still, AAB are considered as a possible member of potentially occurring microbial communities in breweries, of which can appear throughout the whole industrial process. Moreover, AAB can be classified, according to their specific activity, as: possible beer spoilage microorganisms, indicator of other contaminants (microorganisms) or being part of production consortium (Kubizniaková et al., 2021).

Table 8. Defining characteristics of AAB relevant in brewing (Gomes et al., 2018).

Characteristics	*Acetobacter*	*Gluconobacter*
Ox. of ethanol to acetic acid	+	+
Ox. of acetic acid to CO_2 and H_2O	+	-
Ox. of lactic acid to CO_2 and H_2O	+	-
Formation of acid from (D)-Fructose	-	+
Production of water-soluble brown pigment	-	+/-
Major metabolites produced in beer	acetic acid	acetic acid/acetate

The presence of AAB can be traced if the end product is spoiled. Due to their metabolism, the production of bacterial carbohydrates induces an undesired (and unpleasant) change in taste and flavor (Storgårds, 2000). Furthermore, there is a possibility for formation of slime and turbidity, plus the production of acetic acid decreases the alcohol content. These microorganisms can enter in the industrial process through raw materials or returning empty kegs from consumers, which were not properly washed/sterilized. Still, while AAB can be a rare occurrence in beer production, for draught beer that is not the case. Due to the higher exposition to oxygen and step temperatures differences, AAB spoilage incidents are more associated with dispensing system in restaurants, bars, etc (Jevons & Quain, 2021).

Another risk related with the detection of AAB lies within their ability to form biofilms, as they are one of the first microorganisms attaching to various types of surfaces. Their appearance can be seen as a consequence to the insufficient and poor cleaning procedures, especially in certain places where it is hard to access (ex: corners, folds, etc.) and have residues (ex.: process intermediates, finished beer, etc.). that allow a favorable environment for biofilm formation. AAB, coupled with some enterobacteria (Kubizniaková et al., 2021), under aerobic conditions are able to create a biofilm, which causes slime coating of the surface (Storgårds, 2000). Moreover, since AAB have the ability to produce polysaccharides, a suitable environment for the development of other microorganisms, such as LAB, *Pectinatus*, *Megasphaera* and yeasts, is formed (Maifreni et al., 2015). Here, LAB can accumulate lactic acid which, for anaerobic bacteria, can serve as an energy and carbon source.

Recommended media for their detection is Carr medium, which contains bromocresol green as a pH indicator. The formation of acetic acid is then indicated by a color change from the originally blue-green to yellow where the subsequent re-bluing indicates re-increased pH due to the production of CO_2 and water (Kim & Goodfellow, 2015). Other viable possibilities consist of Frateur medium, WLN medium and UBA (if used more regularly), in aerobic conditions at optimal temperature for each medium.

2.4.2.2. Characteristics of ***Enterobacteriaceae***

Within gram-negative bacteria, *Enterobacteriaceae* are another possible risk for the brewing environment. Several species belonging to this family have been isolated from brewery environments with the propensity of wort spoilage rather than beer (P. Ashtavinayak & A. Elizabeth, 2016). Such genera related with wort spoilage are *Citrobacter, Hafnia, Klebsiella and Obesumbacterium.* These are considered as indirect beer spoilage microbes, due to their inability to grow in finished beer, or hygiene indicators. Growth can occur during the initial stages of the brewing process, causing unwanted off-flavors in the final product (Matoulková et al., 2018).

Coliform bacteria are indicators of hygiene and sanitation in breweries; their presence in water is an indication of a poor water treatment. The determining factor separating coliform from *Enterobacteriaceae* is the ability of coliform to ferment lactose while the *Enterobacteriaceae* family ferments glucose (Patel et al., 2014). Enterobacteria are rod-shaped and facultatively anaerobic with both a respiratory and a fermentative type of metabolism. Relevant species that occur in breweries (Table 9) are catalase positive and oxidase negative.

Obesumbacterium proteus the best-known enterobacterial contaminant which has only been found in breweries. This species is classified as anaerobic or facultatively anaerobic, while being catalase positive (weak reaction) and oxidase negative. The morphology is defined as a short rod, but it can be variable in the of yeast in fermenting wort. Within *O. proteus* there are two distinct taxa, known as biogroups 1 and 2. A previous study (Priest & Barker, 2010) has done phylogenic analysis on both biogroups and concluded that biogroup 1 strains were closely related to *Hafnia alvei* (common pathogen) and biogroup 2 strains, since they had distinct phenotypic characteristics, were reclassified into *Shimwellia pseudoproteus sp. nov.* It should be noted that strains from the biogroup 2 are more common in breweries (Priest & Barker, 2010) and have not been reported from any other source.

Table 9. Occurrence and damaging characteristics of enterobacteria order in the brewing process (Patel et al., 2014).

Microorganisms	Occurrence and general characteristic	Damaging characteristics
Obesumbacterium proteus *Shimwellia pseudoproteus*	•Yeast contamination; •Multiply at the early stages of main fermentation; •Relative tolerant to ethanol (< 6 %); •Survive in yeast, can contaminate following batch.	•Production of DMS, higher alcohols, diacetyl •Reduction of nitrates to nitrites (non-volatile N-nitrosamines formation); •Slowdown of fermentation, higher pH of final beer.
Citrobacter freundii	•Contamination of pitched wort; •Sensitivity to ethanol; •Unable to survive the main fermentation.	•Production of organic acids and DMS; •Increase of initial rate of wort fermentation.
Klebsiella oxytoca *Raoultella terrigena*	•Contamination of pitched wort; •Sensitivity to ethanol; •Unable to survive the main fermentation.	•Production of 4-vinylguayacol, acetoin, 2,3-butandiole and DMS
Enterobacter cloacae *Serratia marcescens*	•Contamination of water; •Multiply at the early stages of main fermentation.	•Production of sulfur and phenolic compounds (and DMS in the case of Enterobacter) •Reduction of nitrates to nitrites (non-volatile N-nitrosamines formation)
Rahnella aquatilis (belongs to the *Yersiniaceae* family)	•Yeast contamination; •Multiply at the early stages of main fermentation, mainly in ales; •Relative sensitivity to ethanol; •Survive in yeast, can contaminate following batch.	•Production of diacetyl, DMS, acetaldehyde and ethyl acetate •Increase of initial rate of wort fermentation; •Higher pH of final beer.

This microorganism is usually found in pitching yeast and fermenting wort. It has not been reported in beer and it cannot survive in a pH below 3.9 (Stewart et al., 2017). Still, if these are present during the fermentation, there is a competition against yeast for nutrient. Therefore, the fermentation rate is slower and there is an accumulation of metabolites, such as acetoin, lactic acid, propanol, among others.

One important characteristic of certain *Enterobacteriaceae*, especially *O. proteus*, is having the ability of reducing nitrate to nitrite in fermenting wort, since during anaerobic respiration, nitrates are used as electron acceptors (Smith, 1994). Moreover, much like with *B. cereus*, there is a possibility to occur the formation of N-nitrosamines, if the produced nitrites react with secondary amines present in the wort.

Regarding the wort analysis, for the detection of enterobacteria it is advised the use of VRBG agar. The medium selectivity is due to the presence of bile salts and crystal violet. Typical, colonies on VRBG must be further confirmed: oxidase reaction (negative) and glucose fermentation (positive) for confirmation as *Enterobacteriaceae*. Another possible option is the use of UBA implemented with actidione (for suppression of yeasts).

2.4.2.3. Characteristics of *Zymomonas*

Bacteria of the genera *Zymomonas*, and more specifically *Z. mobilis*, are considered a risk in beers containing adjunct sugars (Vriesekoop et al., 2012). Overall, these bacteria are gram-negative, catalase positive, aerotolerant ant facultatively aerobic. Regarding their morphological aspect, *Zymomonas* are short plump rods which occur singly, in pairs and sometimes in chains or rosettes. Moreover, these are ethanol tolerant of up to 16% (v/v) and resistant to hop compound (Lee et al., 2010). They are able to develop in beers with pH over 3.4 and temperature of 25 to 30ºC. Their ability to spoil beer resides in their production of acetaldehyde and hydrogen sulfide, giving the beer an aroma of rotten eggs.

Zymomonas are able to metabolize certain carbohydrates, such as glucose, fructose and sucrose, via ED pathway, but not maltose and maltriose, producing ethanol and carbon dioxide (Yang et al., 2009). Additionally, strains from this genera are unable to utilize the glycolytic pathway due to the absence of 6-phosphofructokinase, which converts fructose-6-phosphate into fructose-1,6-bisphosphate. *Z. mobilis* is a unique aerobic microorganism that uses the ED pathway anaerobically instead of the EMP pathway. Furthermore, *Z. mobilis* does not have two enzymes of the Krebs cycle (2--oxoglutarate dehydrogenase and malate dehydrogenase). Still, this species still is able to produce important building blocks including oxaloacetate, malic acid, and fumaric acid through alternative metabolic pathways (Lee et al., 2010).

Spoilage associated with *Zymomonas* is more often associated with ciders, due to their composition. However, these microorganisms have been found in breweries (beer, surface of brewery equipment and on brushes of cask-washing machines). Usually, if these are present in cask or keg beers, beyond the unpleasant odor, the liquid will also present heavy turbidity. In lager beers, given the low temperatures at which these are processed, there strains pose no risk (Yanase, 2014).

Detection of *Zymomonas* is based on its characteristic ability to produce carbon dioxide from glucose and its ethanol tolerance. In a brewery environment, for their detection (routine), it should be used UBA medium, under anaerobic condition, at 28 to 30ºC.

2.4.2.4. Characteristics of strictly anaerobic beer-spoilage bacteria

Gram-negative, strictly anaerobic bacteria detected within the brewing environment include four different genera: *Pectinatus*, *Megasphaera*, *Selenomonas* and *Propionispira* (formerly known as *Zymophilus*) (Ueki et al., 2014). Overall, these bacteria are mesophilic, oxidase negative, non-sporulating with a strictly fermentative type of metabolism (Matoulková & Kubizniaková, 2014). These beer spoilage bacteria cause less frequent incidents when compared to LAB. While *Pectinatus* and *Megasphaera* are considered as direct beer spoilage bacteria, that is not the case for *Selenomonas* and *Propionispira*, since these were only found in pitching yeasts. In order to differentiate these genera some characteristics are presented in the Table 10.

Table 10. Characteristics of multiple anaerobic microorganisms possibly isolated during the brewing processes (Haikara & Helander, 2006).

	Genus			
	Megasphaera	*Pectinatus*	*Propionispira*	*Selenomonas*
	Species			
Characteristics	*M. cerevisiae* *M. paucivorans* *M. sueciensis*	*P. cerevisiiphilus* *P. frisingensis* *P. haikarae*	*P. paucivorans* *P. raffinosivorans*	*S. lacticifex*
Cell morphology	Cocci	Curved rods	Curved rods	Curved rods
Catalase	-	- (except *P. haikarae)*	**-**	-
Metabolites produced (major metabolites in bold)	Acetic, **butyric**, caproic, isobutyric, isovaleric, propionic, and valeric acids, **H_2S**	Acetic, **propionic**, succinic and lactic acids, acetoin, **H_2S**, organic sulfur compounds	Acetic and propionic acids (H_2S by *P. raffinosivorans*)	Acetic, **lactic**, and propionic acids

Spoilage by these bacteria is characterized by the increase in turbidity of beer, although it can only be noted after 4–6 weeks. Furthermore, *Pectinatus* and *Megasphaera* confer to the final product an intense odor reminiscent of rotten eggs, given their ability to metabolize different carbon sources (Matoulková & Kubizniaková, 2014). The presence of these genera is usually associated with their survivability in biofilms across the industrial process; as stated previously, this biofilm creation and optimal microenvironment could be related with AAB.

For routine analysis, beyond the possibility to use qPCR for the detection of these genera, another technique is used, different from the membrane filtration, given its unreliability (Haikara, 1985). The objective of the "shelf-life test" is to observe a possible increase of the turbidity (or sedimentation), after the beer is stored for up to six weeks at room temperature. Still, if a faster analysis is required, liquid media, such as MRS or Raka-Rey broths, are recommended.

2.4.3. Wild yeasts

Yeast species that are not deliberately manipulated, by the brewer, and, subsequently, uncontrollable by the manufacturer during the process, are considered as wild yeast. This definition includes brewing yeasts, used for a different style of beer, which could be related with a possible cross contamination, and nonbrewing yeasts. Despite the variety of wild yeasts that could appear in breweries (Table 11), their only common feature is being predominantly unicellular. The impacts of wild yeasts on the process include poor performance of the brewing yeasts, the development of off-flavors and aromas and a final product with an atypical low terminal gravity and high alcohol content (Vaughan et al., 2005).

Some of these wild yeasts can only develop under certain conditions, such is the case for *Pichia membranefaciens*, a common contaminant which is strictly aerobic and cannot ferment carbohydrates in an anaerobic environment. Other possible aerobic contaminants are *Brettanomyces* and *Dekkera* species, which have the ability to produce acetic acid. Wild yeasts, such as *Saccharomyces*, *Torulaspora* and *Zygosaccharomyces*, have the ability to compete with the culture yeasts for nutrients, which, if not controlled, it could eventually displace the desired yeast. For the detection of both *Saccharomyces* and non-*Saccharomyces* it is advised the use of common yeast media, such as MYGP agar supplemented with copper sulphate. Nevertheless, it should be noted that the concentration of copper sulphate could be optimized, given the sensitivity of the culture yeast, and no medium is capable of detecting all wild yeasts (van der Aa Kühle & Jespersen, 1998).

Table 11. Physiological characteristics of possible wild yeast within the brewing industry (Stewart et al., 2017).

Family	Genera	Observations
Saccharomycetaceae (vegetative growth by multilateral budding)	*Debaryomyces*	Weak or no fermentation
	Dekkera	Fermentation (but only under aerobic conditions)
	Issatchenkia	Weak fermentation; forms pseudomycelium and surface film (pellicle)
	Kluyveromyces	Fermentation, usually vigorous
	Pichia	Weak or no fermentation; many form true mycelium or pseudomycelium and a surface pellicle
	Saccharomyces	Vigorous fermentation, no pellicle
	Torulaspora	Vigorous fermentation
	Williopsis	Weak or no fermentation
	Zygosaccharomyces	Vigorous fermentation
Nadsonioideae (vegetative growth by polar budding)	*Hanseniaspora*	Fermentative
	Saccharomycodes	Fermentative
Schizosaccharomycetoideae (vegetative growth by fission or mycelium)	*Schizosaccharomyces*	Fermentative

2.5. Microbiological assessment – quality control

Breweries must strive for a balance between productivity and quality. Moreover, ensuring the microbiological control of beer is essential for consumer quality of the product. For this reason, breweries often apply a food safety management system, HACCP (Pacala et al., 2009), to ensure their objectives. From a microbiology standpoint, the HACCP must take into consideration a few aspects such as: delimitation of critical control points, identification of the contaminant, establish a critical limit, regular monitorization, definition of corrective measures and creation of a historic. Through records it is possible to evaluate the microbiological status over time, throughout the process, that can indicate early on any possible issues.

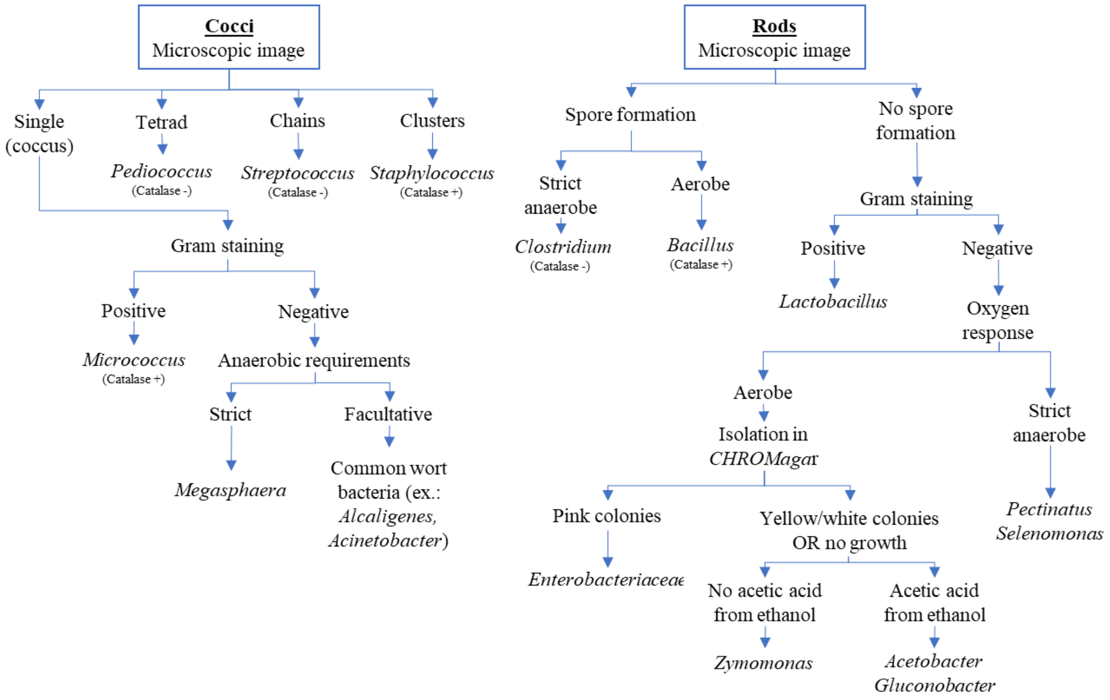

Figure 6. Simplified identification and proper identification of common brewing spoilage microorganisms, based on their morphology (Stewart et al., 2017)

Regarding the microbiological practices, a great deal of breweries still rely on classic microbiology methods for enumeration and identification of (possible) spoilage microorganisms (Turvey et al., 2017). Type of analysis and subsequent maximum limit of CFUs/mL is always dependent of the sample. This analysis involves pouring, spreading onto a solid media or, more commonly, membrane filtration. Incubation of the inoculum can be made under aerobioses or anaerobioses, since it helps to distinguish the microorganisms – those that develop under the lack of oxygen are more prone to cause spoilage. Still, for breweries, the main concern remains with the detection of trace microbial contaminations in the range to 0–100 CFU per 100 mL product (Jespersen & Jakobsen, 1996). This value range is only considered as a general reference, due to the fact that this limit stipulation in different for every brewery (there is no regulation for CFUs in beer). Despite the classic methodologies being able to detect the degree of microbial contamination in a sample, it cannot identify the microorganism present, except when the same was detected in a chromogenic medium. Growth on selective medium and light microscopy can already give an idea of what type of microorganism is present. Further identification can be done using several biochemical assays to narrow down the genera/species of the microorganism (Figure 6) and even commercially available API systems or liquid media. Even if these methods are relatively inexpensive, they have quite some drawbacks, such as the extensive operation time to inoculate and perform multiple identification tests, long incubation times (up to 7 days) and possible tendency for misidentification (Back, 2005). Furthermore, these techniques cannot be used to distinguish or identify non cultivable microorganisms or those that exhibit atypical phenotypic traits.

Table 12. Microbiological flora encountered in the brewery and classification. Wild yeasts found during fermentation capable of competing with or killing brewer's yeasts are considered as a contaminant of utmost urgency, hence they are not considered in this table (Turvey et al., 2017).

Type of microorganism	Definition	Examples
Obligate spoilage	Infection will always lead to spoilage	*Levilactobacillus brevis* *Fructilactobacillus lindneri* *Pediococcus damnosus* *Megasphaera cerevisiae* *Pectinatus frisingensis*
Potential spoilage	Infections can only take hold under specific conditions (ex.: low alcohol content, absence of hops, elevated pH)	*Lactiplantibacillus plantarum* *Lactococcus lactis* *Leuconostoc ssp.*
Indirect spoilage	Organisms that cannot grow in beer but may be present in raw materials and cause off-flavors	*Candida kefyr* *Obesumbacterium proteus*
Indicator flora	Do not cause spoilage but indicate insufficient cleaning, production errors, often coincide with spoilage strains	*Acetobacter pasteurianus* *Klebsiella pneumoniae*
Background (latent) flora	Harmless organisms present in the brewery environment	*Micrococcus ssp.* *Bacillus ssp.* *Clostridia ssp.*
Production flora	Organisms intentionally introduced for beer fermentation or souring processes	*Saccharomyces cerevisiae* *Saccharomyces pastorianus*

Any sample whose analysis originated a CFU concentration greater than the maximum tolerable limit is considered as contaminated, a positive result. Independently of the method used, there should be a valid identification of the microorganism, since this can be correlated with the typical brewery's microflora or possibly trigger corrective measures. A critical evaluation of the results is crucial – understanding if the isolate is coherent with the type of sample and assessing the degree of risk the microorganism poses to the brewery is fundamental. Some breweries even adopt a “green, amber, red” approach (Stewart et al., 2017), where green shows that no corrective action is needed, amber indicates minor microbiological concern (process can continue but there should be an investigation) and red implies a failure to meet the standards and correct actions must be taken. Thus, the microflora can be separated into six categories (Table 12), according with a previous study (Turvey et al., 2017). A more in-depth knowledge of the microbial community across several points of the brewing procedure would allow a better distinction of these categories and could be adapted to each individual brewery (Bose et al., 2021). Additionally, it is possible to know if there could be an issue of biofilm formation if the results (qualitative and quantitative) are analyzed over time and continuously positive (Maifreni et al., 2015); their prevention is related with understanding the ability of the microorganisms to form biofilms and proper hygiene control (CIPs).

2.5.1. New alternatives – molecular methodologies

Beyond the classic methodologies, molecular methods have started to solidify their presence in larger scale breweries (Pringle, 2020). The most popular one is the polymerase chain reaction (PCR), given the low amount of time and sample needed for the analysis and high sensitivity. Fundamentally, using short DNA molecules designed to bind to specific complimentary regions of the genomic DNA (primers), a small region of the microorganism’s genome can be amplified. After the primers are bound, the DNA in that area is synthetized, amplifying the primer-enclosed region of DNA. Afterwards, the amplified DNA can be separated by gel electrophoresis and visualized. Additionally, primers should be designed to bind to a region of genomic DNA that is unique and therefore specific for the microorganism of interest (Asano et al., 2019). A variant of this method is qPCR, which allows the quantification of the initial amount of DNA or RNA, where amplification products are measured as they are produced using a fluorescent label.

Regarding this procedure, to avoid the risk of false negative results, even if it possible to analyze complex mixtures, a pre-enrichment/cultivation is recommended. This method can be very versatile when taking into consideration the own design of a specific primer. However, a considerable diversity of primers is needed in order to encompass a sizeable range of microorganisms, which has a high cost associated plus the need of experience technicians. A valid alternative breweries can employ are the use of commercially available qPCR test kits specifically designed for the purpose of detecting potential spoilers.

Beyond PCR there are other valid molecular methods, such as 16S rDNA sequencing and riboprinting (Barney et al., 2001). The first uses universal primers to amplify the 16S rDNA region by PCR; specific to a particular region conserved amongst all bacterial species. The identification of the microorganism is the results of the comparison between a database of known 16S rDNA sequences with the sequencing of the amplified product. Riboprinting, instead of sequencing the PCR products (similar to the aforementioned method), the amplified DNA is subjected to restriction enzyme digestion. Then, by electrophoresis, the fragments are separated and compared with a database.

Table 13. Comparative of some microbial identification techniques for brewing quality control (Turvey et al., 2017). Key: EP, error prone; O-S, outsourced to external specialist laboratory; S, subjective.

	Method	Capital investment	Running costs	Time to results	Information gained
Classic microbiology	Plate counts on selective media	Low	Low	High (days)	Low
	Phenotypic analysis	Low	Low	High (days)	Low, S, EP
Molecular techniques	PCR	High	High	2-3 h	High, binary
	16S sequencing (bacteria)	High/O-S	High	>10 h	High, to species level
	16S riboprinting restriction analysis (bacteria)	High	Med	>8 h	High, to subspecies level
	Ribotyping	High/O-S	Med	High (days)	Subtyping possible
	FISH	Med	Med	2-3 h	Med,
Mass spectrometry	MALDI-TOF MS	High/O-S	Low	<1 h (excluding cultivation)	High, to species/subspecies level

Another emerging technology capable of profiling in the brewing industry is MALDI-TOF MS (Takeuchi et al., 2005), allowing a rapid, accurate and cost-effective detection and identification (up to species level) of microorganisms capable of spoiling beer. This analytical technique involves the extraction of proteins from samples and generation of a mass spectrum profile unique to the microorganism which is then matched/compared with a profile database (van Veen et al., 2010). Despite this technique being more often associated with clinical microbiology, it has been proven to be successful during routine brewery testing practices (Turvey et al., 2016). Some of the major benefits of MALDI-TOF MS are their low running cost, coupled with its reliability and robustness. Still, the initial high investment needed for running the system might scare potential investors; plus, the system is unreliable when dealing with polymicrobial samples, since there is an overlap of spectral patterns or exclusive identification of the dominant strain (Mahé et al., 2014). Such issue could be circumvented if there is a prior isolation of the microorganisms or pre-enrichment, something that breweries already do. Nevertheless, established breweries should take notice of these methodologies (Table 13) if there is a need to decrease the time of analysis, optimize the current methodologies (ex.: identification of the microorganisms) and understand the brewery's microflora.

2.5.2. SBG current methodologies and new perspectives

Currently at SBG most analysis still rely on classic microbiology. Here, proper technicians at the microbiology laboratory are in charge of starting/controlling the propagations, collect some samples, perform various analysis and critically evaluate and report the results; all under aseptic condition while respecting good laboratory practices. At SBG, the focus of each analysis is mostly related with the quantitative aspect, if these respect the maximum CFU/mL value, except in a few selected cases where differential and chromogenic media are used. Since most of the analysis use a generic medium (UBA) adapted for breweries and there is little interest on the identification of the microorganism, some issues might occur, specifically related with false positives. If there is a failure to meet the standards in the final product, the batch is immediately blocked (does not go to the market and the analysis is repeated over a period of time) or in the worst case, destroyed. Thus, time and money are spent, and if there was a case of a false positive such action would be unnecessary if previous tests were done. This process can be applied to every step of brewing, after boiling. Table 14 displays the frequency of unsatisfactory results in the previous year; thus, identifying the isolated and knowing the frequency of false positives are relevant subjects to SBG.

Table 14. Frequency of unsatisfactory results across some sampling points in the brewery during 2022. Sampling was performed under aseptic condition. The analysis includes filtration of 100 mL of the sample into a membrane which is then incubated as 27ºC under aerobic (3 days) or anaerobic conditions (7 days). Data provided by SBG.

Sample	Type of analysis	Total number	Unsatisfactory results	Unsatisfactory results (%)
Beer packaging 1	Aerobiosis	904	51	5,64
	Anaerobiosis	858	2	0,23
Beer packaging 2	Aerobiosis	717	16	2,23
	Anaerobiosis	693	3	0,43
Beer packaging 3	Aerobiosis	360	5	1,39
	Anaerobiosis	355	2	0,56
Beer processing 1	Aerobiosis	256	7	2,73
	Anaerobiosis	656	28	4,27
Beer processing 2	Aerobiosis	263	5	1,90
	Anaerobiosis	263	9	3,42

Another relevant subject is related with false negative results. If there are no colonies on a plate, it does not mean there are zero CFUs; as it could be a matter of viable but nonculturable microorganisms (Liu et al., 2017) or the number of CFUs in the sample could be too low (ex.: only 100 mL of a 20 L keg are analyzed). For this reason, SBG has gained some interest into the possible use of enrichment media and implementation of a qPCR methodology. On a later chapter in this dissertation, there will be a critical review of the potential application of these new methodologies into the routine work at SBG.

3. Materials and methods

3.1. Assessment and identification of positive results

3.1.1. Sampling

For the routine analysis, several sampling points are relevant for quality control; depending on these, some samples are collected every day or periodically. Sampling in the cold and hot block were carried out by specific personnel and delivered to the laboratory; these were periodically – each time a new batch starts. Specific personnel would also be in charge of several sampling points across the process (ex.: beer drive related samples, collection of finished products, final rinsing water – CIP, etc.). Sampling in packaging hall (keg line) were carried out daily by the laboratory technicians (only when the filling line was running).

All the sample were collected into autoclaved Schott flasks or sterile discardable containers, under aseptic conditions. These were secured after the sampling point being sanitized with alcohol (70%) or heated with a vaporizer, and the collection being done in the smallest timeframe possible, if possible, under a flame, always discarding the first jet (between 15 seconds to one minute). When possible, samples were collected through ScandiBrew aseptic designed valves.

3.1.2. Microbiological analysis (classic)

After sampling, the analysis would be performed in the same day in order to prevent any possible alterations in the microbial population present in the mixture. This analysis involves several techniques, such as: membrane filtration, incubation, verify the presence of colonies, identification of the isolate and storage.

3.1.2.1. Membrane filtration

This technique allows the detection and quantification of viable microorganisms in a liquid sample that contains less than 1 CFU/mL (Stewart et al., 2017). This method was utilized since it is the most appropriated for the majority of samples (water and beer) that possess a small concentration of microorganisms; thus, a larger quantity of sample can be tested, when compared to other techniques. The only exception is the analysis of yeast (storage tank) prior to fermentation, where these would be tested for any possible contaminations using the spread plate method.

3.1.2.2. Culture media and incubation

Due to diversity of microorganisms present in a brewery, a few culture mediums are necessary for their detection and isolation. All medium were prepared according with the supplier's information and autoclaved (except the ones that needed an addition of supplements). These could be stored up to three months in the cold (2-8ºC) and when needed, heated in the microwave and shacked until there was a homogenized liquid; then it needed its temperature to stabilize at 45ºC (water bath). Type of sample would determine the media to use, incubation time and conditions (Table 15).

Table 15. List of all relevant samples in the brewing process and their analysis. Anaerobic conditions were achieved in an anaerobic jar, with an oxygen indicator. Key: F, filtration (100 mL); P, pour plate method (1 mL); S, spread plate method (≈ 0,1g).

Sample		**Technique**	**Medium**	**Temp.**	**Period**	**Conditions**
Filling line 1		F	UBA	27ºC	3 days	O_2
		F	UBA	27ºC	7 days	CO_2
Filling line 2	Beer	F	UBA	27ºC	3 days	O_2
		F	UBA	27ºC	7 days	CO_2
	Rinsing water	P	UBA	27ºC	3 days	O_2
Beer type 1		F	UBA	27ºC	3 days	O_2
		F	UBA	27ºC	7 days	CO_2
		F	ABP	27ºC	7 days	CO_2
Beer type 2		F	UBA	27ºC	3 days	O_2
		F	UBA	27ºC	7 days	CO_2
		F	BC	36ºC	1 day	O_2
Beer type 3		F	UBA	27ºC	3 days	O_2
		F	UBA	27ºC	7 days	CO_2
Beer type 4		F	UBA	27ºC	3 days	O_2
		F	UBA	27ºC	7 days	CO_2
Yeast		S	UBA + Actidione	27ºC	3 days	O_2
		S	Taylor	27ºC	3 days	O_2
		S	ABP	27ºC	7 days	CO_2
Wort		P	UBA	27ºC	3 days	O_2

The proper selection of culture media is crucial. Here, UBA is the most generic one and applied to most samples. This media is used specifically for the cultivation of bacteria and yeast specific to breweries; incubation under different conditions allows the differentiation between organisms that can develop in the product. For yeast testing, three different culture mediums were used: yeast and mold agar supplemented with copper sulphate, also known as Taylor medium, used to detect wild yeast. Another is UBA supplemented with cycloheximide (actidione), which suppresses yeast and fungal growth allowing a better isolation of bacterial contaminants (wort bacteria). The last one is UBA supplemented with ABP, which allows the detection of LAB. All the supplements were prepared under aseptic condition, prepared according with the existing SBG methodologies. Furthermore, regarding Taylor medium, the copper sulphate concentration is already adapted to the brewer's yeast, so when applied to other yeasts, negative positive results can occur.

For a further distinction of the isolates, other media could be used, such as BC (*Bacillus cereus* agar), "Brettanomyces Agar Base" or "CHROMagar™ CCA". Such media are selective and chromogenic, which allows a clear identification.

3.1.2.3. Analyses of the results

After incubation, each plate would be inspected to see if there were CFU. These would be counted and the value reported, following the maximum acceptable CFU/mL value; in UBA (aerobiosis), the value could not be higher than 100 CFU (per 100 mL) and in the rest of the analyses it needed to be zero. Additionally, to validate the result, the plate needed to be clear of any external contamination (ex.: mold formation over the entire plate) and no growth could occur outside the membrane (indication of an improper filtration).

3.1.2.4. Identification of the isolates

After a successful positive result, simple phenotypical tests were used to do a preliminary assessment of the type of microorganism. With the resources available at SBG, it could only be possible to perform a few selected tests. When there was a suspicion for *Enterobacteriaceae* these would be isolated in "CHROMagar™ CCA" and test if the isolate could develop in beer. After a prior isolation of a single colony from the original plate, in the same media, five colonies would be each inoculated in two bottles of beer (25 cL). Afterward, these would incubate at room temperature during different times (five and ten days) and analyzed (membrane filtration, same media, both conditions). If there was a suspicion for AAB, a prior isolation of a single colony was needed, followed by the inoculation of five colonies in one bottle of beer (25 cL). After ten days of incubation at room temperature, it would be checked if there was formation of acetic acid by smell.

For the assessment of the characterization of the isolates, a sequential series of techniques were applied, namely microscopical observation (shape, size and morphology), Gram staining, catalase test, oxygen growth in media, and, if needed, *CRHOMagar* specific growth and development in beer and classified according with Figure 6.

Beyond the phenotypical test, colonies were always isolated into another plate (same medium and condition; more than one if the original had different colonies) which then could be store in a fridge for up to one month. Yeast isolated were maintained in slants (UBA) which could be store for up to three months. Furthermore, 15 isolates were selected and sent to an external laboratory (Biocant) for sequencing.

3.1.3. Storage of isolates

Every relevant microorganism (I.e. one of each genera/species) was incubated in liquid media (sterile UBA without agar), at 27ºC, until turbidity occurred. One colony would be inoculated into an Eppendorf tube, volume of media would be 1,0 mL. Once the change in turbidity occurred, 0,5 mL of the broth would be discarded and then, 0,5 mL of glycerol was added (final concentration of glycerol was 25%). Tubes would be vortexed, identified, sealed with parafilm and stored in a box (-20ºC). These were prepared in duplicates and can be stored for one to three years (De Paoli, 2005).

3.2. Practical evaluation of the current qPCR

3.2.1. Samples and enrichment

Commercially available qPCR test kits, “PIKA 4everyone™ Detection Kit LP Screening” and “PIKA 4everyone™ Detection Kit Superattenuator Yeasts”, were used (advised by the supplier) for different samples. Furthermore, the University provided three different strains (*L. plantarum*, *L. casei* and B. *bruxellensis*); all were in a plate (except *L. casei* which came in a broth), with colonies present. The *L. casei* was inoculated in MRS (0,1 mL) and incubated (30ºC, aerobiosis) until there was formation of colonies (≈2 days).

Preparation of stock cultures were made with “PIKA FastOrange® B Bouillon”, for LAB, and “PIKA FastOrange® Wild Yeast Bouillon” for the wild yeast strain. For each, one colony was inoculated into the respected broth until there was a clear color change (27º, aerobioses, ≈ 2days), as indicated by the supplier; negative controls were prepared with just the media.

3.2.2. CFU determination

To determine the real CFU from each stock suspension, serials dilutions were prepared, starting from a single colony, in sterile ringer's solution. Aliquots of 100 µL were plated in two parallels each in either MRS (for LAB) or “Brettanomyces Agar Base” (yeast) and incubated at 30ºC until visible colonies appeared (≈3-5 days). The grown colonies were counted and the CFU/mL of the stock suspension was calculated.

3.2.3. qPCR analysis

From each dilution (and original), a 1,0 mL aliquot was used for qPCR analysis. The “PIKA 4everyone™ Detection Kit LP Screening” and “PIKA 4everyone™ Detection Kit Superattenuator Yeasts” were used according to the manufacturer’s specifications for the DNA extraction and sample preparation, in parallels, on a laminar flow cabinet. The quantitative real-time PCR was performed on a “Chai Open qPCR Dual Channel” (Chai), following the manufacturer’s instructions. Using the Cq value (quantification cycle), the quantity of bacterial DNA present is detected. Further statistical analysis was made in Excel.

4. Results and Discussion

4.1. Identification of microorganisms (phenotypic tests)

Any sample whose analysis originated a CFU concentration greater than the maximum tolerable limit is considered a positive result. Over the period of 9 months, 57 analyzed sample were considered as contaminated (positive result); full description is the following table. The total amount of analyzed samples is difficult to estimate due the high volume of daily samples (≈50-100).

Table 16. Contaminated samples obtained throughout the brewing process; identification of isolates via simple phenotypic tests. For the AAB column, marked results could be *Zymomonas.*

Sample type	Number of positive results	*Yeast*	*Pediococcus*	*Bacillus spp.*	*Enterobacteriacea*	*Staphylococcus or Kocuria*	*Paenibacillus or Clostridium*	*AAB*	*Inconclusive*
Wort	1	1							
Yeast	6	4			1	1			
Beer type 1 and 3	15				6	1	6	1	1
Beer type 2	3	1		2					
Beer type 4	2	1	1						
Filling line 1	14	5			3	1		1*	4
Filling line 2	16	6	4	1		2			3
Total	57								

It should be noted that such identification should be considered as presumptive, due to the limited variety of available tests. Given the variety of samples, any that did not obtain a positive result will not be considered, even if CFUs were formed (below limit). Additionally, every positive result had visually homogenous colonies (possibility for same microorganism); if that was not the case, it would be considered as different positive results.

The classification "*Staphylococcus* or *Kocuria*" is given to the according microorganisms, since its differentiation cannot be done through simple tests. Regarding the AAB classification, depending of the type of sample, further differentiation for *Zymomonas* is difficult. To distinguish between these, one hypothesis was suggested: test the acetic acid formation; here, the isolate would be inoculated in beer (bottle, sealed) and incubate at room temperature for ten days. Afterwards, through sensory analysis, the detection of a vinegar-like aroma would indicate the presence of AAB (Kubizniaková et al., 2021). This method was applied on all relevant samples, which indicated invalid results, since no aroma alteration occurred and no CFU was formed after the analysis of the inoculated beer. Another alternative for the distention would be the use of commercially available test kits, for qPCR, for detection of AAB (*Detection of Acetic Acid Bacteria - 4e™ For everyone Detection Kit B Acetics Screening User Guide*, 2023), which could give more reliable results. The "inconclusive" classification indicated that it was not possible to obtain a proper identification due to a lack of comparison (tests were able to detect a microorganism different from what is expected); these could either been seen as a result of possible errors during tests or as microorganisms not described in the schematic. The "yeast" classification involves any yeast found (disregarding if it constitutes as a wild yeast), independently of the media used; once isolated, it would be tested to confirm if it was a wild yeast. Here, the only reliable method would be the qPCR (wild yeasts), since the use of Taylor medium, if the concentration of copper sulphate was not adapted to the brewer's yeast, could lead to false results (CFUs would be from the brewer's yeast) and further tests would be needed. It should be noted that all yeast strains were able to develop in Taylor media.

Still, regarding these results, due to the limited availability of PCR test kits compatible with the current qPCR, all relevant isolates (yeasts and *Pediococcus*) were tested with the proper kit (in isolates) and all gave negative results. Regarding yeasts, either the isolates were indeed the brewer's yeast or any other yeast unable to spoil beer. As for the isolates suspected as *Pediococcus*, a negative result could indicate an improper identification or a *Pediococcus* with no spoilage ability.

Beyond the identification (phenotypic tests) of all the isolates, these were stored (-20ºC) individually in a broth, mixed with glycerol, for future use, if necessary to test any new culture media. Furthermore, every isolate was recorded (photo of the colonies and microscopic aspect) for future reference at SBG.

4.2. Sequencing results and risk assessment

After the first identification, 15 selected isolates were sent to an external lab for the sequencing of each strain: 16S rRNA sequencing for bacterial identification and ITS rRNA sequencing for fungi (in this case, yeasts). Results can be seen on Table 17. Here, the classification of each isolate follows the descriptions present on Table 12 (obligatory, potential or indirect spoilage, as well as indicator, background or production flora). It should be noted that any microorganisms with a distinct ability to form biofilms and proven have a spoilage ability (to beer) are considered as indicator flora. The overall classification is the result of cross referencing several aspects related with the identified strain. Understanding the physiological characteristics (and metabolism) of these microorganisms, in order to see if they are able to develop in beer, their common environment and checking any previous reports that evidence their presence in breweries is fundamental. Moreover, it is also important to correlate the strain with the sample, since it could influence the classification; one example is related with the yeast strains found, given that the ones found are considered as weak or non-fermenters, if they were detected in the yeast storage tank, these would be seen as obligate or potential spoilers, since it would be possible to occur a competition with the brewer's yeast. Given all this context, an additional category is proposed, false positives; this category includes any isolated microorganism that cannot develop in the sample and was not previously detected in breweries.

Across the 15 isolates, 14 will be considered as one had an unsuccessful identification, so it will not be considered. The external lab stated that the strain was not described in the used database (EzBioCloud 16S).

The identification of the isolate through simple phenotypic tests was successful in 12 samples (86%). Comparing the sequencing results with the phenotypic tests, 12 isolates had a match between both. Such data, validates that these phenotypic tests, as seen on Figure 6, can be used a preliminary identification

Sequencing data revealed that all 14 identified strains are considered as non-beer spoilers and non-pathogenic, as they were not able to normally grow on beer. Furthermore, all of these results imply a new reasoning about the positive results obtained in the laboratory as they might not be a risk to the brewery and its products. This needs a reflection about the need of confirming the positive results obtained in the laboratory. Of course, there is a remark on which these positive results are still important to observe, since it is a clear evidence for hygiene or sampling issues. Such risk, despite not affecting beer, comes from an indication of a poor cleaning procedure, which could lead to a biofilm formation, often associated with spoilage bacteria.

Among these 14 isolates, 3 were considered as false positives (21%), due to the strain present being incoherent with the bibliographic sources and/or possesses no conditions to grow in the product, due to its metabolic characteristics. Still, the origin of each isolate is a tricky topic to tackle, given that, even if the microorganism found is present in the sample, it is difficult to distinguish if it is the result of an improper sampling procedure. An isolate case or a real threat that could jeopardize the procedure. Thus, the current daily/weakly analysis helps to assess the situation. In beer samples, for instance, *Clostridium* sp. was occasionally detected (no pattern observed), however, this might reflect not its presence on the product but being present in the environment and thus giving a false indication of failure in the sampling or microbiological procedure. Due to its characteristics and bibliographic sources, it is considered as background flora.

Posteriorly, all *Enterobacteriaceae* identified strains were test to check if they would be able to develop in beer (5 colonies inoculated in a bottle, incubation at room temperature for 3 and 10 days), of which none were able to, hence not considered as a obligatory spoiler.

All samples recorded, labelled as positive, triggered corrective measures. When this happens to the final product, the batch is immediately blocked and awaits further analysis; in most cases, a resampling is ordered to confirm if the sample is contaminated, before any action is taken (only on those that allow resampling). With the proper identification of the microorganisms, such actions could be avoided, due to fact that these possess no risk. Additionally, the frequency of false positive results exposes possible fragilities or error during sampling or hygiene procedures. One strategy SBG to prevent the detection and report of false positives is a long-term beer stability check. Finished product (only from kegs), under aseptic conditions, 250 mL are stored in a sterile Schott flask for 10 days at room temperature; this allows the development (or death) of any present microorganism. Additionally, a small vial (≈50 mL) of this sample is kept at 25ºC for three months in order to check for any contaminants (only turbidity and particle formation is checked) that would require a long adaptation time, such is the case for *P. frisingensis*. Due to the sheer volume of daily analysis performed at SBG's microbiology laboratory, such methodology is only applied in these sample (keg – finished product), making hard to distinguish these cases on any other samples. These positive results from "Filling line 1", all passed the stability test (three months), which validates a spoiler microorganism free beer.

Table 17. Sequencing results (provided by an external laboratory) of different samples. Classification of each isolate according with the literature and relation with the type of sample.

Sample type	Medium	Description of isolate	Identification (phenotypic tests)	Sequencing result	Observations	Classification
Filling line 1 [11/12/2022]	UBA (aerobiosis)	Yeast	Yeast	*Debaryomyces hansenii*	This species is not regarded as pathogenic. Generally considered to be strictly aerobic, nonfermentive (weak fermenter of maltose and glucose) and ethanol tolerant (up to 15%). Previously associated with surfaces in brewery bottling plants, in mixed biofilms. No records of spoilage associated with beer (Suiker & Wösten, 2022; Wrent et al., 2014).	Indicator flora
Filling line 1 [12/12/2022]	UBA (aerobiosis)	Gram positive bacillus with spores	*Bacillus* spp.	*Lysinibacillus fusiformis*	Non regarded as a pathogenic and can be commonly found is various environments. Facultative anaerobic, previously found in beer (final product), although it is not known if it has any spoilage ability (Munford et al., 2017).	Background flora
Filling line 1 [02/12/2022]	UBA (aerobiosis)	Gram positive coccus	*Staphylococcus or Kocuria*	*Kocuria salsicia*	With the exception of *K. kristinae*. this genus only comprises strict aerobic bacteria, sensible to ethanol, low pH and hops. This species can survive in beer for long periods of time; however, it does not interfere with beer. Common in breweries, negligible spoilage risk (Matoulková & Kubizniaková, 2018).	Background flora
Filling line 2 (beer) [02/02/2023]	UBA (aerobiosis)	Gram positive coccus	*Staphylococcus or Kocuria*	*Kocuria salsicia*		Background flora
Filling line 1 [28/12/2022]	UBA (aerobiosis)	Gram negative bacillus	*Enterobacteriaceae*	Non-defined strain	Non-defined strain.	Non-defined strain

Filling line 2 (beer) [02/02/2023]	UBA (anaerobiosis)	Gram positive coccus	*Staphylococcus or Kocuria*	*Staphylococcus warneri*	Despite being facultative anaerobic microorganisms, these microorganisms are susceptible to alcohol. No records were found of this species present in the final product; however, it was previously isolated in brewery surfaces, as it shows a strong ability to form biofilms. Other species within this genus have a high ability to spoil beer (Maifreni et al., 2015).	Indicator flora
Filling line 1 [20/01/2023]	UBA (anaerobiosis)	Gram negative bacillus	*Enterobacteriaceae*	*Enterobacteriaceae* (biggest similarity with *Scandinavium* sp.; *Enterobacter* sp.; *Cedecea sp.*)	Enterobacteria usually cannot grow in finished beer. Their presence serves as a hygiene indicator throughout the brewing process. Still, growth during the initial stages of the brewing process may cause unwanted off-flavors in the final product. No records of such genera found in finished beer (P. Ashtavinayak & H. A. Elizabeth, 2016; Matoulková et al., 2018; Prest et al., 1994).	False positive (strain dependent)
Beer type 2 [08/02/2023]	UBA (anaerobiosis)	Yeast	Yeast	*Saccharomyces cerevisiae*	Brewer’s yeast; cross contamination or a different strain present.	Production flora
Beer type 2 [28/01/2022]	BC agar (aerobiosis)	Gram positive coccus	*Bacillus cereus*	*Cellulosimicrobium* sp.	The identified genus is pleomorphic (initial phase is presented as bacillus and later on as coccus). Stick aerobic microorganism; no records found of its appearance in breweries or beer, except during fermentation of some Lambic beers (De Roos et al., 2018)	False positive
Yeast [30/01/2023]	ACT (aerobiosis)	Gram negative bacillus	*Bacillus* spp.	*Sphingomonas echinoides*	*Sphingomonas* are strict aerobic and alcohol sensitive. Records indicate their detection in natural mineral water and during fermentation. It is not known if these affect beer quality; still, some strains were found in microbrewery surfaces, with a moderate to strong ability to form biofilms (Maifreni et al., 2015; Takahashi et al., 2015)	Background flora

Filling line 2 (beer) [03/02/2023]	UBA (aerobiosis)	Yeast	Yeast	*Candida sake*	Such aerobic yeasts are considered as weak fermenter that could proliferate during the initial phases of fermentation or in final beer in cases of oxygen ingress. Still, records have shown that this microorganism could appear in brewery surfaces or pipping and is regarded as a harmless contaminant (Sohlberg et al., 2022; Suiker & Wösten, 2022)	Indicator flora
Filling line 1 [05/02/2023]	UBA (anaerobiosis)	Gram negative bacillus	*Enterobacteriaceae*	*Serratia marcescens*	Facultative anaerobic microorganism that can reduce nitrates to nitrites. Moreover, *S. marcercens* is an opportunistic pathogen for hospitalized patients and can cause urinary infections. Its occurrence and activity are limited to the beginning of fermentation; no records found of its appearance in finished beer (Matoulková et al., 2018)	False positive
Beer type 1 [07/02/2023]	UBA (anaerobiosis)	Gram positive bacillus with spores	*Clostridium*	*Clostridium* sp.	Endospores of *Clostridia* are able to survive the conditions of mashing and wort boiling, but cannot germinate in the following phases of beer production (inhibited by bitter hop substances and acidic pH). Their presence in the early stages of brewing may damage the finished product. Pathogenic strains are unable to survive in the final product (Brozova et al., 2018c)	Background flora
Beer type 1 [20/02/2023]	UBA (anaerobiosis)	Gram positive bacillus with spores	*Clostridium*	*Clostridium* sp.		Background flora
Wort [09/02/2023]	UBA (aerobiosis)	Yeast	Yeast	*Saccharomyces cerevisiae*	Brewer's yeast; cross contamination or a different strain present	Production flora

4.3. Enrichment media application and CFU/mL determination

The feasibility evaluation of the qPCR methodology within SBG can be done when looking at the current procedure and its capabilities. For this purpose, two different scenarios were explored: testing the performance on spoilage isolates and on real samples.

Due to the variety of availability of qPCR test kits, SBG opted to focus on only two; both are oriented for the detection of beer spoiling microorganism, one for LAB and another for superattenuating yeasts (yeasts that can ferment beer beyond what the brewer's yeast is capable – considered as wild yeasts). In order to evaluate the practical detection limits with qPCR compared to CFU, microorganisms compatible with the kits in used were needed. All previous isolates, suspected of being LAB or yeasts, underwent qPCR (one colony, in duplicates) and all gave negative results. In order to advance with the study, "Universidade Católica Portuguesa – ESB (Porto)" provided three different strains that would be detected by the system. Stock cultures were made using the advised enrichment media by the supplier (Figure 7). The broth allowed a clear visual evaluation after three days (change of color and/or turbidity increase).

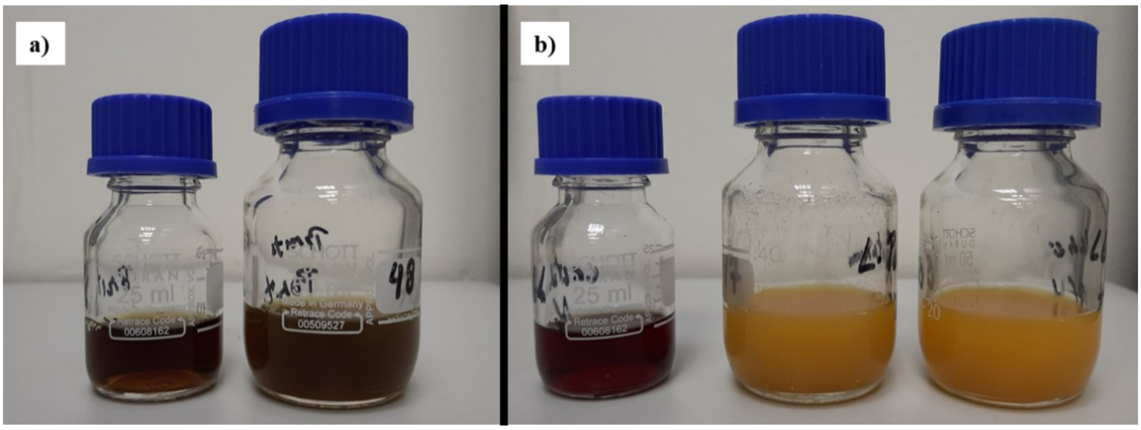

Figure 7. Photograph of the stock cultures (taken on 05/05/2023) – 20 mL volume of broth, incubation at 27ºC, 3 days. **a)** *B. bruxellensis* stock culture (with negative control), turbidity increase and slight color change; **b)** *L. plantarum* (middle) and *L. casei* (right) stock cultures (with negative control), drastic color change with deposit formation.

Determination of the real CFU/mL was calculated according with the number of grown colonies on the plates (Table 18). It should be noted that the results related with the *L. casei* are omitted beyond this point due to a lack of results; qPCR was preformed in two different occasions (with duplicates) on the original stock suspension and always lead to no detection. One possible explanation to this, supported by the supplier, it the different strain present. A negative detection indicates that the strain is in fact not a beer spoiler (PCR primers only detect the real beer spoilers) and the change in color on the stock suspension confirms the presence of a *Lactobacillus*; due to these characteristics it is suspected that the obtained strain could be *L.paracasei*, which is phylogenetically and phenotypically closely related to *L. casei* (Huang et al., 2018).

Table 18. CFU determination and calculation of CFU/mL of the original stock suspension from the first dilution with less than 300 CFU.

Sample	Average CFU (*B. bruxellensis*)	Average CFU (*L. plantarum*)
Original stock culture	>300	>300
1º dilution (10^1)	>300	>300
2º dilution (10^2)	>300	>300
3º dilution (10^3)	>300	>300
4º dilution (10^4)	156	>300
5º dilution (10^5)	15	>300
6º dilution (10^6)	1	199
7º dilution (10^7)		21
CFU/mL	$1,56 \times 10^6$	$2,00 \times 10^8$

4.4. qPCR - quantitative and data analysis

Quantitative real-time PCR implies a PCR amplification of DNA in real time, measured by a fluorescent probe, most commonly an intercalating dye or a hydrolysis-based probe, which enables the quantification of the PCR product (Adams, 2020). Detection of the qPCR cycle results in an amplification curve with initiation, exponential and plateau phases. Low fluorescence levels at the beginning of amplification are used to establish the baseline fluorescence level. As the reaction progresses into the exponential growth, fluorescence reaches a level which is significantly higher than the baseline; this is referred to as the threshold level (Adams, 2020). Whenever a fluorescence value reaches the quantification threshold, the number of cycles needed are recorded; this is defined as the quantification cycle or Cq. The lower the Cq value is, the higher quantity of target sequence is present in the sample. Another relevant factor in quantitation is the use of an IPC, which are reference genes, that have sequences different from the target, that allows to demonstrate the variability resulting from imperfections of the technology used and preparatory procedures (Kozera & Rapacz, 2013). Ideally, amplification of the IPC should occur with similar efficiency, similar Cq values, in all samples and controls, in order to ensure the quality of the qPCR reaction. On Figure 8 is presented the respective amplification curves for each trial; the first one appears to be successful ("S shaped" curves) while the second appears to have a jagged signal throughout amplification plot on some samples. This could be explained by a poor amplification or weak probe signal.

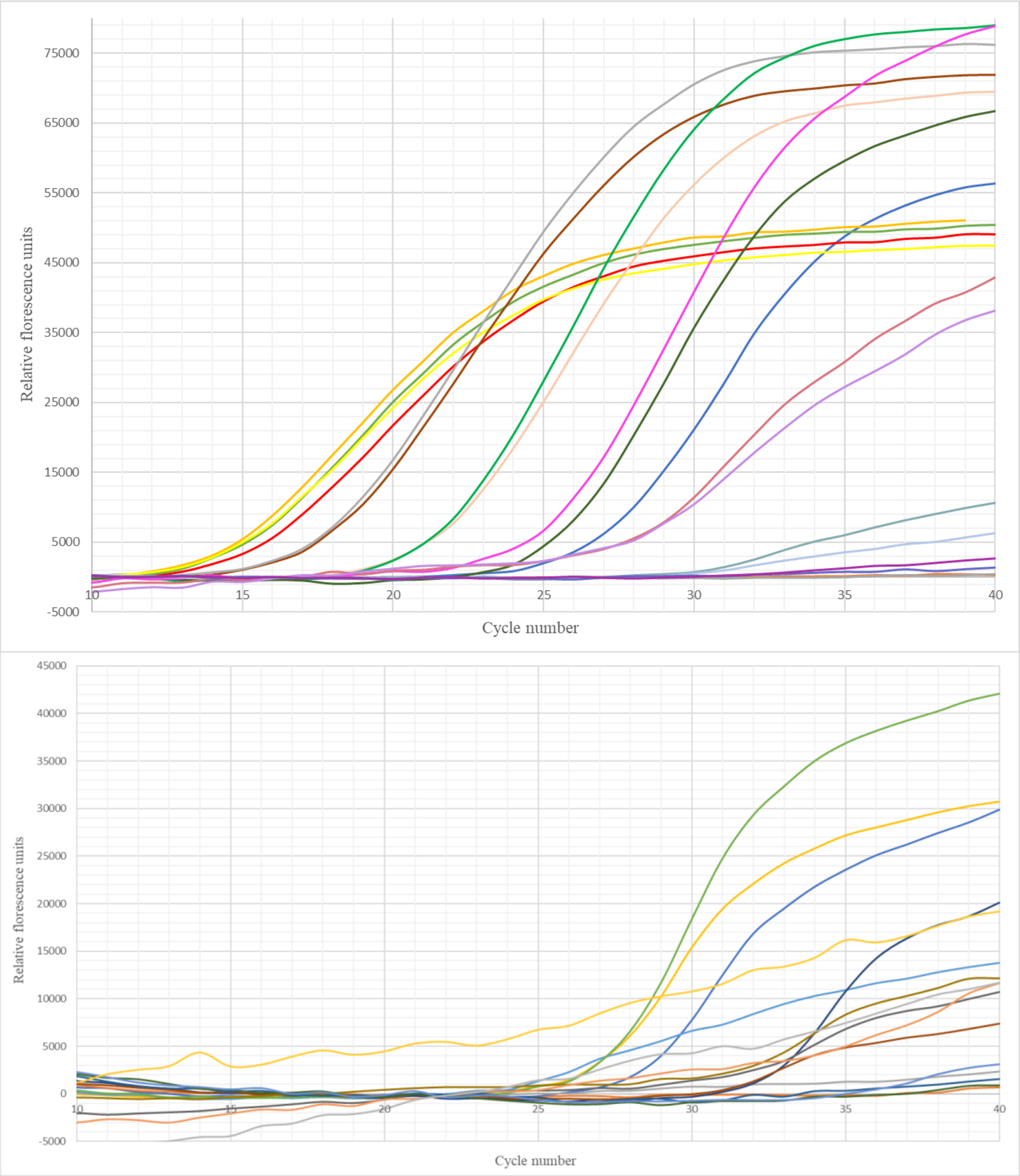

Figure 8. Generated amplification curves (baseline subtracted) for the *L. plantarum* trial (top) and *B. bruxellensis* (bottom) trials.

Regarding the data analysis, there are two types of quantitation methods to choose from, absolute and relative. The first one requires a standard of known concentration for the IPC and the gene of interest (GOI) which will generate a standard curve using the log concentrations and the Cq values. Therefore, a comparison between the standard curve and the Cq values from the samples, allows the determination of the molecular amount or concentration of the target. The second approach involves the analysis of changes in the gene expression in a given sample relative to another reference sample, using the ratio between the IPC and the GOI. The Cq of the IPC is removed from the GOI Cq, to remove any errors in sample loading. This generates a ΔCq values for all samples, which is then compared back to a control sample to generate a ΔΔCq value (Adams, 2020).

Table 19. Cq obtained values per sample (including IPC) and the associated calculated log CFU/mL, related with *L. plantarum*.

	Cq (duplicates)				Average	
Sample	1º GOI	1º IPC	2º GOI	2º IPC	GOI Cq	Log CFU7mL
Positive control	26,90	30,45				
Negative control	0	30,14				
Original broth	0	29,41				
Original stock culture	15,44	0	15,27	32,14	15,35	8,30
1º dilution	14,70	31,37	14,53	32,04	14,61	7,30
2º dilution	17,98	33,56	17,98	33,28	17,98	6,30
3º dilution	21,68	36,25	21,82	0	21,75	5,30
4º dilution	25,54	0	25,11	32,34	22,59	4,30
5º dilution	27,10	27,27	26,51	0	26,81	3,30
6º dilution	30,41	27,64	10,14	27,49	28,93	2,30
7º dilution	0	27,05	0	27,76	0	1,30

Table 20. Cq obtained values per sample (including IPC) and the associated calculated log CFU/mL, related with *B. bruxellensis.*

	Cq (duplicates)				Average	
Sample	1º GOI	1º IPC	2º GOI	2º IPC	GOI Cq	Log CFU/mL
Positive control	28,27	20,89				
Negative control	0	29,37				
Original broth	0	28,83				
Original stock culture	26,67	32,00	26,68	32,01	26,67	6,19
1º dilution	24,59	30,21	27,14	35,07	25,87	5,19
2º dilution	32,61	28,47	31,22	28,00	31,91	4,19
3º dilution	30,97	28,14	29,64	28,20	30,31	3,19
4º dilution	0	28,34	0	28,36	0	2,19
5º dilution	36,24	28,30	29,68	28,77	32,56	1,19
6º dilution	24,47	28,87	0	29,37	24,47	0

Regarding the system in use, all the parameters are set to default (ex.: threshold value) and the relation between the Cq and quantity is already established: >30 is considered low, between 30 and 25 is medium and <20 is high. Commercial test kits have included positives and negative controls and if no Cq value is generated in the positive control (or a Cq generation in the negative control) the experiment is invalid. Tables 19 and 20 present each obtained Cq value (and the associated log CFU/mL). Whenever an IPC is not detected, the GOI result is invalid. This could be the result of a very high amount of target DNA in the reaction tube at the start. The target DNA will start to amplify faster than the IPC and therefore all reagents included in the reaction tube will be used up before the amplification of the IPC will start. However, since the duplicates on the *L. plantarum* trial have nearly identical Cq values, this should not be an issue. Furthermore, some Cq values are unexpected: both first dilutions had a higher value than the original stock culture (unknown cause) and the last dilution from the yeast trial gave the highest value (discrepant value, considered as an outlier).

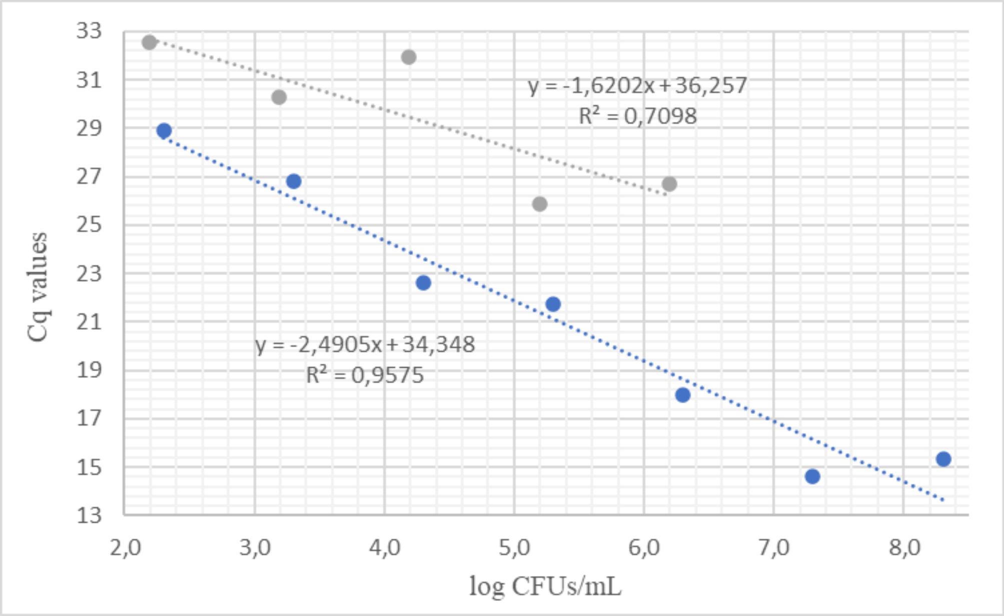

Figure 9. qPCR curves relating the Cq value to log CFFU/mL for L. plantarum (blue) and *B. bruxellensis* (grey).

As it was expected, a higher concentration value corresponds to a lower Cq value; such correlation is presented in a linear regression (Fig. 9), which, in theory, would allow the calculation of the expected CFU/mL in a sample (Ilha et al., 2016; Ruiz-Villalba et al., 2021). However, the presented model is quite limited due to the small number of different concentrations and the low number of replicates (only two), despite one of the R^2 values being greater than 0,95. Furthermore, with the data available, it was not possible to verify the limits of detection of the system; for this purpose, other protocols are suggested (Forootan et al., 2017), however it is worth to note that the relation between

Cq and suspension count can be related, opening the possibility for having a "incubation + time + Cq" method and specification for semi-quantitative quality control in the future.

4.5. qPCR – current application and evaluation

Currently, at SBG, the qPCR methodology and enrichment media are only used in a few, more sensible cases. According with the brewery, this method is only applied on specific yeast samples, prior to pitching, when there are time constraints. Over the period of the internship, this analysis was performed on 8 occasions (in parallel to the classic analysis) for the detection of wild yeasts and LAB. All classic analysis had no CFU growth and all qPCR analysis gave negative results. It should be noted that on two instances the qPCR indicated a Cq value greater than 35; the use of enrichment media was needed for a repeat, of which both gave negative results. According with the manufacturer, the limits of detection are 15 and 35 and if the detected value is close to the limits, the use of enrichment media is advised. On a side note, the low frequency of samples in need to be analyzed via qPCR, can also be seen as a hurdle, from an economic standpoint. Kits not used effectively (ex.: only two sample per run) will need extra reagents/controls in the long run, increasing the running cost.

Due to the daily high volume of samples needed to be analyzed, plus all the other responsibilities in need to be taken cared, the implementation of a qPCR methodology for most samples seems implausible. An investment into a more robust system (multiplex qPCR) would allow the detection of a wider variety of spoilage inducing microorganisms and analyze more samples per run. The use of the described enrichment media it is unreliable for two reason: the visual evaluation can be deceptive, color change might occur but a beer spoiler might not be present (such was the case for *L.paracasei*) and liquid media is very susceptible to contamination when not handled properly, plus it is always depended of qPCR confirmation. The employment of such media in order to reduce the inoculation time during stability tests can be seen as irrelevant due to the fact that, regardless of the analysis time, the finished product stays a long period of time in the warehouse, prior to quality control release.

Nevertheless, the implementation of this media and qPCR on selected sample has been proven as being a lifesaver whenever time constrains exist, due to its precise and consistent results.

5. Conclusion

The main purpose of this work consisted on a critical analysis of the current methodologies applied, at SBG, regarding the microbial quality control. Currently the samples are only analyzed from a quantitative standpoint; whether if they are in line with the respective maximum tolerable CFU/mL limit. The work presented demonstrated that a qualitative aspect (identification of microorganisms) must be taken into consideration as well. Thereby, a proper identification and classification would allow a more thorough quality control assessment, that would prevent the unnecessary blockage of product. The risk assessment presented has been proven to be relevant for the brewery.

The implementation of enrichment media, along with qPCR, has been labelled as successful and is being currently implemented as an indispensable tool for the analysis of certain samples and confirmation of beer spoilers. Through this work, despite the limited data, it was possible to understand the correlation between CFUs/mL and Cq. However, one of the stipulated objectives was not met, consisting in the verification of the detection limits of the system, due to the lack of data. Therefore, the acquired results lay the groundwork for a future study of a new possible method, focusing on the correlation between Cq, CFUs/mL and incubation time, which is beneficial to SBG.

Overall, SBG should consider to routine control all positives (plate count) with a confirmation methodology that the Author recommends to be a group of phenotypic tests followed by qPCR and, if needed, external sequencing.

6. Final remarks and future perspectives

Throughout the period of internship, the Author had an enlightening experience which allowed the development of personal/technical skills within the routine work of a microbiology laboratory at SBG. Knowledge of the brewing process and the current applied methodologies allowed the Author to acquire a critical view, regarding the microbiology quality control parameters.

Currently, the microbiological quality control practices at SBG are enough, although some attention should be drawn to the qualitative aspect of each analysis; even it all practices follow a maximum CFU/mL limit. An implementation of such methodology could provide be another layer of information for the brewer, which could reveal certain issues of fragilities within the brewing procedure, as it was the case in this dissertation. Data suggest that there are fragilities within sampling practices, due to the frequency of false positives (21%), no obligatory or indirect spoilage microorganisms were found and no evidence of biofilm formation was detected. Due to the fact that samples, which had no spoilage microorganisms, had their correspondent batch locked for a period of time, exhibits a need to further improve quality control measures.

One possible solution to this issue was assessing the feasibility for the implementation of the current qPCR into routine analysis. Such methodology, coupled with enrichment media, is advantageous whenever there are time constraints, however, its broad application across all samples is impractical, due to high cost and time needed. Ideally, via qPCR, multiple genera could be identified and quantified in a complex matrix (beer), but with the current system, this is unachievable. The use of enrichment media is always dependent of an qPCR confirmation and can only be used for the detection of wild yeasts and LAB. Given the high volume of daily samples (which increases the need of the media, and subsequently, the running cost) and the lack of a time constraints, currently, there is no need for its application. For a future study, it would be relevant for SBG to understand the minimum required time of incubation (enrichment media with a spiked beer) for the detection of reliable results.

Overall, it is advised to SBG the adoption of more specific molecular methodologies (ex.: sequencing, more robust qPCR system, MALDI-TOF MS, etc.) for the differentiation of the detected microorganisms; such methods could be applied in-house or exported to an external laboratory. Nevertheless, these would still be all culture dependent methods. Such identification would allow the association between the microorganism and the risk it possess to the process. From malt production up until the retail side (ex.: dispensers at a restaurant), many microorganisms can be detected. Despite the fact that a plethora of beer spoilage microbes have been identified and characterized, the mechanisms of contamination and persistence still remain as an open are of study. With this in mind and all the data acquired, a future study of the typical microbial communities in some key areas within the brewery would provide insight about the microorganisms to expect and refine this risk assessment.

References

17 - Microbiology. (2004). In D. E. Briggs, C. A. Boulton, P. A. Brookes, & R. Stevens (Eds.), *Brewing* (pp. 606-649). Woodhead Publishing. https://doi.org/https://doi.org/10.1533/9781855739062.606

2023 Brewers Association Beer Style Guidelines. (2023). Brewers Association. Retrieved 02/04/2023 from https://www.brewersassociation.org/edu/brewers-association-beer-style-guidelines/#88

Adams, G. (2020). A beginner's guide to RT-PCR, qPCR and RT-qPCR. *The Biochemist*, *42*(3), 48-53. https://doi.org/10.1042/bio20200034

Asano, S., Shimokawa, M., & Suzuki, K. (2019). PCR Analysis Methods for Detection and Identification of Beer-Spoilage Lactic Acid Bacteria. *Methods Mol Biol*, *1887*, 95-107. https://doi.org/10.1007/978-1-4939-8907-2_9

Ashtavinayak, P., & Elizabeth, A. (2016). Review: Gram Negative Bacteria in Brewing. *Advances in Microbiology*, *06*, 195-209. https://doi.org/10.4236/aim.2016.63020

Ashtavinayak, P., & Elizabeth, H. A. (2016). Review: Gram Negative Bacteria in Brewing. *Advances in Microbiology*, *Vol.06No.03*, 15, Article 64826. https://doi.org/10.4236/aim.2016.63020

Back, W. (2005). *Colour Atlas and Handbook of Beverage Biology*. Carl. https://books.google.pt/books?id=U7rGAAAACAAJ

Barney, M., Volgyi, A., Navarro, A., & Ryder, D. (2001). Riboprinting and 16S rRNA gene sequencing for identification of brewery Pediococcus isolates. *Appl Environ Microbiol*, *67*(2), 553-560. https://doi.org/10.1128/aem.67.2.553-560.2001

Bartmańska, A., Wałecka-Zacharska, E., Tronina, T., Popłoński, J., Sordon, S., Brzezowska, E., Bania, J., & Huszcza, E. (2018). Antimicrobial Properties of Spent Hops Extracts, Flavonoids Isolated Therefrom, and Their Derivatives. *Molecules*, *23*(8). https://doi.org/10.3390/molecules23082059

Batt, C. A. (2014). LACTOBACILLUS | Introduction. In C. A. Batt & M. L. Tortorello (Eds.), *Encyclopedia of Food Microbiology (Second Edition)* (pp. 409-411). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-384730-0.00176-2

Behr, J., & Vogel, R. F. (2009). Mechanisms of hop inhibition: hop ionophores. *J Agric Food Chem*, *57*(14), 6074-6081. https://doi.org/10.1021/jf900847y

Bokulich, N. A., & Bamforth, C. W. (2013). The microbiology of malting and brewing. *Microbiol Mol Biol Rev*, *77*(2), 157-172. https://doi.org/10.1128/mmbr.00060-12

Bose, N., Auvil, D. P., Moore, E. L., & Moore, S. D. (2021). Microbial Communities in Retail Draft Beers and the Biofilms They Produce. *Microbiology Spectrum*, *9*(3), e01404-01421. https://doi.org/doi:10.1128/Spectrum.01404-21

Brozova, M., Kubizniaková, P., & Matoulková, D. (2018a). Brewing Microbiology - Bacteria of the Genera Bacillus, Brevibacillus and Paenibacillus and Cultivation Methods for their Detection - Part 1. *Kvasny Prumysl*, *64*, 50-57. https://doi.org/10.18832/kp201813

Brozova, M., Kubizniaková, P., & Matoulková, D. (2018b). Brewing microbiology - bacteria of the genera Bacillus, Brevibacillus and Paenibacillus and cultivation methods for their detection - part 2. *Kvasny Prumysl*, *64*, 233-241. https://doi.org/10.18832/kp201829

Brozova, M., Kubizniaková, P., & Matoulková, D. (2018c). Brewing microbiology - bacteria of the genus Clostridium. *Kvasny Prumysl*, *64*, 242-247. https://doi.org/10.18832/kp201830

Cattoor, K., Dresel, M., De Bock, L., Boussery, K., Van Bocxlaer, J., Remon, J. P., De Keukeleire, D., Deforce, D., Hofmann, T., & Heyerick, A. (2013). Metabolism of hop-derived bitter acids. *J Agric Food Chem*, *61*(33), 7916-7924. https://doi.org/10.1021/jf300018s

De Paoli, P. (2005). Bio-banking in microbiology: from sample collection to epidemiology, diagnosis and research. *FEMS Microbiol Rev*, *29*(5), 897-910. https://doi.org/10.1016/j.femsre.2005.01.005

De Roos, J., Vandamme, P., & De Vuyst, L. (2018). Wort Substrate Consumption and Metabolite Production During Lambic Beer Fermentation and Maturation Explain the Successive Growth of Specific Bacterial and Yeast Species [Original Research]. *Frontiers in Microbiology*, *9*. https://doi.org/10.3389/fmicb.2018.02763

Degremont. (1979). *Water treatment handbook, 5th edition*. John Wiley. https://books.google.pt/books?id=uxMmcgAACAAJ

Detection of Acetic Acid Bacteria - 4e™ For everyone Detection Kit B Acetics Screening User Guide. (2023). Retrieved 05/01/2023 from https://static.chaibio.com/spree/products/160/PIKA_4e_Acetics_Screening_Kit_Manual.pdf?1534623624

Dobson, C. M., Deneer, H., Lee, S., Hemmingsen, S., Glaze, S., & Ziola, B. (2002). Phylogenetic analysis of the genus Pediococcus, including Pediococcus claussenii sp. nov., a novel lactic acid bacterium isolated from beer. *Int J Syst Evol Microbiol*, *52*(Pt 6), 2003-2010. https://doi.org/10.1099/00207713-52-6-2003

Farber, M., & Barth, R. (2019). *Mastering Brewing Science: Quality and Production*. Wiley. https://books.google.pt/books?id=SuacDwAAQBAJ

Forootan, A., Sjöback, R., Björkman, J., Sjögreen, B., Linz, L., & Kubista, M. (2017). Methods to determine limit of detection and limit of quantification in quantitative real-time PCR (qPCR). *Biomol Detect Quantif*, *12*, 1-6. https://doi.org/10.1016/j.bdq.2017.04.001

Gänzle, M. G. (2015). Lactic metabolism revisited: metabolism of lactic acid bacteria in food fermentations and food spoilage. *Current Opinion in Food Science*, *2*, 106-117. https://doi.org/https://doi.org/10.1016/j.cofs.2015.03.001

Gomes, R. J., Borges, M. F., Rosa, M. F., Castro-Gómez, R. J. H., & Spinosa, W. A. (2018). Acetic Acid Bacteria in the Food Industry: Systematics, Characteristics and Applications. *Food Technol Biotechnol*, *56*(2), 139-151. https://doi.org/10.17113/ftb.56.02.18.5593

Guillamon, J., & Mas, A. (2011). Acetic Acid Bacteria. In (pp. 227-255). https://doi.org/10.1016/B978-0-12-375021-1.10009-8

Haakensen, M., & Ziola, B. (2008). Identification of novel horA-harbouring bacteria capable of spoiling beer. *Can J Microbiol*, *54*(4), 321-325. https://doi.org/10.1139/w08-007

Haikara, A. (1985). Detection of Pectinatus Contaminants in Beer. *Journal of the American Society of Brewing Chemists*, *43*(1), 43-46. https://doi.org/10.1094/ASBCJ-43-0043

Haikara, A., & Helander, I. (2006). Pectinatus, Megasphaera and Zymophilus. In M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, & E. Stackebrandt (Eds.), *The Prokaryotes: Volume 4: Bacteria: Firmicutes, Cyanobacteria* (pp. 965-981). Springer US. https://doi.org/10.1007/0-387-30744-3_32

Harrison, M. A., & Albanese, J. B. (2017). Beer/Brewing. In. https://doi.org/10.1016/B978-0-12-809633-8.13014-6

Hill, A. E. (2015). Introduction to brewing microbiology. In A. E. Hill (Ed.), *Brewing Microbiology* (pp. xxvii-xxix). Woodhead Publishing. https://doi.org/https://doi.org/10.1016/B978-1-78242-331-7.02001-3

Hornsey, I. S. (2003). *A History of Beer and Brewing*. The Royal Society of Chemistry. https://doi.org/10.1039/9781847550026

Huang, C.-H., Li, S.-W., Huang, L., & Watanabe, K. (2018). Identification and Classification for the Lactobacillus casei Group [Hypothesis and Theory]. *Frontiers in Microbiology*, *9*. https://doi.org/10.3389/fmicb.2018.01974

Iijima, K., Suzuki, K., Asano, S., Kuriyama, H., & Kitagawa, Y. (2007). Isolation and Identification of Potential Beer-Spoilage Pediococcus inopinatus and Beer-Spoilage Lactobacillus backi Strains Carrying the horA and horC Gene Clusters. *Journal of the Institute of Brewing*, *113*(1), 96-101. https://doi.org/https://doi.org/10.1002/j.2050-0416.2007.tb00262.x

Ilha, E. C., Scariot, M. C., Treml, D., Pereira, T. P., Sant′Anna, E. S., Prudêncio, E. S., & Arisi, A. C. M. (2016). Comparison of real-time PCR assay and plate count for Lactobacillus paracasei enumeration in yoghurt. *Annals of Microbiology*, *66*(2), 597-606. https://doi.org/10.1007/s13213-015-1137-7

Jenson, I. (2014). BACILLUS | Introduction. In C. A. Batt & M. L. Tortorello (Eds.), *Encyclopedia of Food Microbiology (Second Edition)* (pp. 111-117). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-384730-0.00018-5

Jespersen, L., & Jakobsen, M. (1996). Specific spoilage organisms in breweries and laboratory media for their detection. *International Journal of Food Microbiology*, *33*(1), 139-155. https://doi.org/https://doi.org/10.1016/0168-1605(96)01154-3

Jevons, A. L., & Quain, D. E. (2021). Draught beer hygiene: use of microplates to assess biofilm formation, growth and removal. *Journal of the Institute of Brewing*, *127*(2), 176-188. https://doi.org/https://doi.org/10.1002/jib.637

Kim, S., & Goodfellow, M. (2015). Bergey's Manual of Systematics of Archaea and Bacteria. In (pp. 1-30). https://doi.org/10.1002/9781118960608.gbm00186

Kozera, B., & Rapacz, M. (2013). Reference genes in real-time PCR. *J Appl Genet*, *54*(4), 391-406. https://doi.org/10.1007/s13353-013-0173-x

Kubizniaková, P., Kyselová, L., Brožová, M., Hanzalíková, K., & Matoulková, D. (2021). The role of acetic acid bacteria in brewing and their detection in operation. *Kvasny Prumysl*.

Kubizniaková, P., & Matoulková, D. (2016). Brewing microbiology - lactic acid bacteria and cultivation methods for their detection – Part II. *Kvasny Prumysl*, *11-12*, 335-345.

L'Anthoën, N. C., & Ingledew, W. M. (1996). Heat Resistance of Bacteria in Alcohol-Free Beer. *Journal of the American Society of Brewing Chemists*, *54*(1), 32-36. https://doi.org/10.1094/ASBCJ-54-0032

Laing, H., Bull, S., Joyce, S., & O'Malley, C. (2021). Investigating cleaning in place (CIP) chemical, water, and energy use: A comparative study of standard operating procedure (SOP) for UK North East microbreweries. *Journal of Brewing and Distilling*, *10*, 17-28. https://doi.org/10.5897/JBD2021.0055

Lee, K. Y., Park, J. M., Kim, T. Y., Yun, H., & Lee, S. Y. (2010). The genome-scale metabolic network analysis of Zymomonas mobilis ZM4 explains physiological features and suggests ethanol and succinic acid production strategies. *Microb Cell Fact*, *9*, 94. https://doi.org/10.1186/1475-2859-9-94

Liu, J., Li, L., Li, B., Peters, B. M., Deng, Y., Xu, Z., & Shirtliff, M. E. (2017). Study on spoilage capability and VBNC state formation and recovery of Lactobacillus plantarum. *Microb Pathog*, *110*, 257-261. https://doi.org/10.1016/j.micpath.2017.06.044

Lynch, K. M., Zannini, E., Wilkinson, S., Daenen, L., & Arendt, E. K. (2019). Physiology of Acetic Acid Bacteria and Their Role in Vinegar and Fermented Beverages. *Compr Rev Food Sci Food Saf*, *18*(3), 587-625. https://doi.org/10.1111/1541-4337.12440

Mahé, P., Arsac, M., Chatellier, S., Monnin, V., Perrot, N., Mailler, S., Girard, V., Ramjeet, M., Surre, J., Lacroix, B., van Belkum, A., & Veyrieras, J.-B. (2014). Automatic identification of mixed bacterial species fingerprints in a MALDI-TOF mass-spectrum. *Bioinformatics*, *30*(9), 1280-1286. https://doi.org/10.1093/bioinformatics/btu022

Maifreni, M., Frigo, F., Bartolomeoli, I., Buiatti, S., Picon, S., & Marino, M. (2015). Bacterial biofilm as a possible source of contamination in the microbrewery environment. *Food Control*, *50*, 809-814. https://doi.org/https://doi.org/10.1016/j.foodcont.2014.10.032

Market Insights - Beer - Worldwide. (2023). statista. Retrieved 25/03/2023 from https://www.statista.com/outlook/cmo/alcoholic-drinks/beer/worldwide

Matoulková, D., & Kubizniaková, P. (2014). Brewing microbiology - strictly anaerobic bacteria Megasphaera, Pectinatus, Selenomonas and Zymophilus and methods for their detection. *Kvas. Prum.*, *60*, 285-295. https://doi.org/10.18832/kp2014028

Matoulková, D., & Kubizniaková, P. (2018). Brewing Microbiology - Kocuria (Micrococcus) and Cultivation Methods for their Detection - Part 1. *Kvasny Prumysl*, *64*, 10-13. https://doi.org/10.18832/kp201804

Matoulková, D., Vontrobová, E., Brozova, M., & Kubizniaková, P. (2018). Microbiology of brewery production - bacteria of the order Enterobacterales. *Kvasny Prumysl*, *64*, 161-166. https://doi.org/10.18832/kp201824

McGovern, P. E., Zhang, J., Tang, J., Zhang, Z., Hall, G. R., Moreau, R. A., Nuñez, A., Butrym, E. D., Richards, M. P., Wang, C. S., Cheng, G., Zhao, Z., & Wang, C. (2004). Fermented beverages of pre- and proto-historic China. *Proc Natl Acad Sci U S A*, *101*(51), 17593-17598. https://doi.org/10.1073/pnas.0407921102

Menz, G., Aldred, P., & Vriesekoop, F. (2011). Growth and survival of foodborne pathogens in beer. *J Food Prot*, *74*(10), 1670-1675. https://doi.org/10.4315/0362-028x.Jfp-10-546

Michel, M., Cocuzza, S., Biendl, M., Peifer, F., Hans, S., Methner, Y., Pehl, F., Back, W., Jacob, F., & Hutzler, M. (2020). The impact of different hop compounds on the growth of selected beer spoilage bacteria in beer. *Journal of the Institute of Brewing*, *126*(4), 354-361. https://doi.org/https://doi.org/10.1002/jib.624

Munford, A. R. G., Alvarenga, V. O., Prado-Silva, L. d., Crucello, A., Campagnollo, F. B., Chaves, R. D., Oteiza, J. M., & Sant'Ana, A. S. (2017). Sporeforming bacteria in beer: Occurrence, diversity, presence of hop resistance genes and fate in alcohol-free and lager beers. *Food Control*, *81*, 126-136. https://doi.org/https://doi.org/10.1016/j.foodcont.2017.06.003

Munford, A. R. G., Chaves, R. D., & Sant'Ana, A. S. (2020). Inactivation kinetics of beer spoilage bacteria (Lactobacillus brevis, Lactobacillus casei, and Pediococcus damnosus) during acid washing of brewing yeast. *Food Microbiology*, *91*, 103513. https://doi.org/https://doi.org/10.1016/j.fm.2020.103513

National Beer Sales & Production Data. (2022). Brewers Association. Retrieved 28/03/2023 from https://www.brewersassociation.org/statistics-and-data/national-beer-stats/

Nuñez, M. (2014). Micrococcus. In C. A. Batt & M. L. Tortorello (Eds.), *Encyclopedia of Food Microbiology (Second Edition)* (pp. 627-633). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-384730-0.00206-8

Pacala, M. L., O, T., Begea, M., & Sirbu, A. (2009). HACCP study and validation by QMSFSM for brewing process – application for training of students from food technologies. *Proceedings of the5th Balkan Region Conference*, *II*, 447-452.

Patel, A. K., Singhania, R. R., Pandey, A., Joshi, V. K., Nigam, P. S., & Soccol, C. R. (2014). ENTEROBACTERIACEAE, COLIFORMS AND E. COLI | Introduction. In C. A. Batt & M. L. Tortorello (Eds.), *Encyclopedia of Food Microbiology (Second Edition)* (pp. 659-666). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-384730-0.00096-3

Prest, A. G., Hammond, J. R., & Stewart, G. S. (1994). Biochemical and Molecular Characterization of Obesumbacterium proteus, a Common Contaminant of Brewing Yeasts. *Appl Environ Microbiol*, *60*(5), 1635-1640. https://doi.org/10.1128/aem.60.5.1635-1640.1994

Priest, F. G., & Barker, M. (2010). Gram-negative bacteria associated with brewery yeasts: reclassification of Obesumbacterium proteus biogroup 2 as Shimwellia pseudoproteus gen. nov., sp. nov., and transfer of Escherichia blattae to Shimwellia blattae comb. nov. *Int J Syst Evol Microbiol*, *60*(Pt 4), 828-833. https://doi.org/10.1099/ijs.0.013458-0

Pringle, A. (2020). A Brewer's Guide to Applying PCR in a Quality Assurance Program. *Technical Quarterly*, *57*, 190-199. https://doi.org/10.1094/TQ-57-3-1011-01

Rachon, G., Rice, C. J., Pawlowsky, K., & Raleigh, C. P. (2018). Challenging the assumptions around the pasteurisation requirements of beer spoilage bacteria. *Journal of the Institute of Brewing*, *124*(4), 443-449. https://doi.org/https://doi.org/10.1002/jib.520

Raihofer, L., Zarnow, M., Gastl, M., & Hutzler, M. (2022). A short history of beer brewing: Alcoholic fermentation and yeast technology over time: Alcoholic fermentation and yeast technology over time. *EMBO Rep*, *23*(12), e56355. https://doi.org/10.15252/embr.202256355

Relatório de gestão e sustentabilidade 2021. (2022). SBG. https://superbockgroup.com/management-reports/?lang=en

Ruiz-Villalba, A., Ruijter, J. M., & van den Hoff, M. J. B. (2021). Use and Misuse of C(q) in qPCR Data Analysis and Reporting. *Life (Basel)*, *11*(6). https://doi.org/10.3390/life11060496

Sakamoto, K., & Konings, W. N. (2003). Beer spoilage bacteria and hop resistance. *Int J Food Microbiol*, *89*(2-3), 105-124. https://doi.org/10.1016/s0168-1605(03)00153-3

Smith, K. A. (2012). Louis pasteur, the father of immunology? *Front Immunol*, *3*, 68. https://doi.org/10.3389/fimmu.2012.00068

Smith, N. A. (1994). CAMBRIDGE PRIZE LECTURE NITRATE REDUCTION AND N-NITROSATION IN BREWING*. *Journal of the Institute of Brewing*, *100*(5), 347-355. https://doi.org/https://doi.org/10.1002/j.2050-0416.1994.tb00835.x

Sohlberg, E., Sarlin, T., & Juvonen, R. (2022). Fungal diversity on brewery filling hall surfaces and quality control samples. *Yeast*, *39*(1-2), 141-155. https://doi.org/https://doi.org/10.1002/yea.3687

Spitaels, F., Wieme, A. D., Janssens, M., Aerts, M., Van Landschoot, A., De Vuyst, L., & Vandamme, P. (2015). The microbial diversity of an industrially produced lambic beer shares members of a traditionally produced one and reveals a core microbiota for lambic beer fermentation. *Food Microbiol*, *49*, 23-32. https://doi.org/10.1016/j.fm.2015.01.008

Stewart, G. G., Russell, I., & Anstruther, A. (2017). *Handbook of Brewing*. CRC Press. https://doi.org/10.1201/9781351228336

Storgårds, E. (2000). Process hygiene control in beer production and dispensing. *VTT Publications*.

Suiker, I. M., & Wösten, H. A. B. (2022). Spoilage yeasts in beer and beer products. *Current Opinion in Food Science*, *44*, 100815. https://doi.org/https://doi.org/10.1016/j.cofs.2022.100815

Sun, S., Wang, X., Yuan, A., Liu, J., Li, Z., Xie, D., Zhang, H., Luo, W., Xu, H., Liu, J., Nie, C., & Zhang, H. (2022). Chemical constituents and bioactivities of hops (Humulus lupulus L.) and their effects on beer-related microorganisms. *Food and Energy Security*, *11*(2), e367. https://doi.org/https://doi.org/10.1002/fes3.367

Super Bock Group. (2023). https://www.superbockgroup.com/en/detail/about-the-group/

Suzuki, K., Iijima, K., Sakamoto, K., Sami, M., & Yamashita, H. (2006). A Review of Hop Resistance in Beer Spoilage Lactic Acid Bacteria. *Journal of the Institute of Brewing*, *112*. https://doi.org/10.1002/j.2050-0416.2006.tb00247.x

Takahashi, M., Kita, Y., Kusaka, K., Mizuno, A., & Goto-Yamamoto, N. (2015). Evaluation of microbial diversity in the pilot-scale beer brewing process by culture-dependent and culture-independent method. *J Appl Microbiol*, *118*(2), 454-469. https://doi.org/10.1111/jam.12712

Takeuchi, A., Iijima, K., Suzuki, K., Ozaki, K., & Yamashita, H. (2005). Application of Ribotyping and rDNA Internal Space Analysis (RISA) for Assessment of Microflora in Brewery Environments. *Journal of the American Society of Brewing Chemists*, *63*(2), 73-75. https://doi.org/10.1094/ASBCJ-63-0073

Turvey, M. E., Weiland, F., Keller, E. J., & Hoffmann, P. (2017). The changing face of microbial quality control practices in the brewing industry: Introducing mass spectrometry proteomic fingerprinting for microbial identification. *Journal of the Institute of Brewing*, *123*(3), 373-387. https://doi.org/https://doi.org/10.1002/jib.428

Turvey, M. E., Weiland, F., Meneses, J., Sterenberg, N., & Hoffmann, P. (2016). Identification of beer spoilage microorganisms using the MALDI Biotyper platform. *Appl Microbiol Biotechnol*, *100*(6), 2761-2773. https://doi.org/10.1007/s00253-016-7344-8

Ueki, A., Watanabe, M., Ohtaki, Y., Kaku, N., & Ueki, K. (2014). Description of Propionispira arcuata sp. nov., isolated from a methanogenic reactor of cattle waste, reclassification of Zymophilus raffinosivorans and Zymophilus paucivorans as Propionispira raffinosivorans comb. nov. and Propionispira paucivorans comb. nov. and emended description of the genus Propionispira. *Int J Syst Evol Microbiol*, *64*(Pt 10), 3571-3577. https://doi.org/10.1099/ijs.0.063875-0

van der Aa Kühle, A., & Jespersen, L. (1998). Detection and identification of wild yeasts in lager breweries. *Int J Food Microbiol*, *43*(3), 205-213. https://doi.org/10.1016/s0168-1605(98)00113-5

van Veen, S. Q., Claas, E. C., & Kuijper, E. J. (2010). High-throughput identification of bacteria and yeast by matrix-assisted laser desorption ionization-time of flight mass spectrometry in conventional medical microbiology laboratories. *J Clin Microbiol*, *48*(3), 900-907. https://doi.org/10.1128/jcm.02071-09

Vaughan, A., O'Sullivan, T., & Van Sinderen, D. (2005). Enhancing the Microbiological Stability of Malt and Beer — A Review. *Journal of the Institute of Brewing*, *111*(4), 355-371. https://doi.org/https://doi.org/10.1002/j.2050-0416.2005.tb00221.x

Vriesekoop, F., Krahl, M., Hucker, B., & Menz, G. (2012). 125th Anniversary Review: Bacteria in brewing: The good, the bad and the ugly. *Journal of the Institute of Brewing*, *118*(4), 335-345. https://doi.org/https://doi.org/10.1002/jib.49

Wang, B., Shao, Y., & Chen, F. (2015). Overview on mechanisms of acetic acid resistance in acetic acid bacteria. *World J Microbiol Biotechnol*, *31*(2), 255-263. https://doi.org/10.1007/s11274-015-1799-0

Wrent, P., Rivas, E. M., Gil de Prado, E., Peinado, J. M., & de Silóniz, M. I. (2014). Debaryomyces. In C. A. Batt & M. L. Tortorello (Eds.), *Encyclopedia of Food Microbiology (Second Edition)* (pp. 563-570). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-384730-0.00081-1

Yanase, H. (2014). Zymomonas. In C. A. Batt & M. L. Tortorello (Eds.), *Encyclopedia of Food Microbiology (Second Edition)* (pp. 856-863). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-384730-0.00365-7

Yang, S., Pappas, K. M., Hauser, L. J., Land, M. L., Chen, G. L., Hurst, G. B., Pan, C., Kouvelis, V. N., Typas, M. A., Pelletier, D. A., Klingeman, D. M., Chang, Y. J., Samatova, N. F., & Brown, S. D. (2009). Improved genome annotation for Zymomonas mobilis. *Nat Biotechnol*, *27*(10), 893-894. https://doi.org/10.1038/nbt1009-893

Zheng, J., Ruan, L., Sun, M., & Gänzle, M. (2015). A Genomic View of Lactobacilli and Pediococci Demonstrates that Phylogeny Matches Ecology and Physiology. *Appl Environ Microbiol*, *81*(20), 7233-7243. https://doi.org/10.1128/aem.02116-15

Zheng, J., Wittouck, S., Salvetti, E., Franz, C., Harris, H. M. B., Mattarelli, P., O'Toole, P. W., Pot, B., Vandamme, P., Walter, J., Watanabe, K., Wuyts, S., Felis, G. E., Gänzle, M. G., & Lebeer, S. (2020). A taxonomic note on the genus Lactobacillus: Description of 23 novel genera, emended description of the genus Lactobacillus Beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae. *Int J Syst Evol Microbiol*, *70*(4), 2782-2858. https://doi.org/10.1099/ijsem.0.004107