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MICROBIOLOGICAL PHASES OF SPONTANEOUSLY FERMENTED BEER

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ABSTRACT

Fermentation is traditionally divided into two types of fermentation bottom and top fermentation. There is a third type of fermentation, which is traditionally used for spontaneously fermented beer. Spontaneous fermentation runs slowly because starts without inoculation. All of the 100 species of yeasts and more than 50 species of bacteria that were obtained from this beer come from the environment. A wide spectrum of microorganisms is discriminated on base of substrate usability, pH and alcohol tolerance, temperature, and speed of growth. So, fermentation is divided into different phases as normal one-organism fermentation. Phases are: the phase of Enterobacteriaceae, the phase of main fermentation, the phase of acidification, and the phase of maturation. Every phase is specific for the mostly obtained microorganism, specific metabolites, or technological processes. During 3 years of fermentation in every case, a unique product with terroir will arise. Understanding this process is necessary to apply its interesting parts to new beverages productions and other fermentation technologies.

Keywords: spontaneous fermentation, beer, lambic, microbiological phases

INTRODUCTION

Throughout history, not only technology of malt-preparing and beer brewing were evaluated. For thousands of years, beer was fermented only with microorganisms from the environment. During those times, fermentation was unpredictable, which preached big failures and losses **(Hornsey, 2003)**. The first pure culture was used in 1883 by Emil Ch. Hansen in Carlsberg brewery labs. This signified new age of brewing, with pure cultures, but without complex aromas and tastes **(Hansen, 1883)**. This new technic exploded around Europe and the increase in lager brewing was significant **(Libkind *et al.*, 2011)** and nowadays highest part of beer production is using *Saccharomyces bayanus* or *Saccharomyces pastorianus* **(Bokulich and Bamforth, 2013)**. But in one region of only roughly 500 square-kilometers area around Brussel and Payottenland in the valley of Senna River, the knowledge of the oldest beer style survived. The terroir of this region provided a fully complex microbiota, which was necessary to produce high quality and constant products, so brewers were not confronted with necessary in the changing brewing process here **(De Keersmaecker, 1996)**. Like in most countries, in Belgium the highest part of production is brewing in large brewing companies, too. Only 2,5 % of Belgium's beer production are Lambic beer, for 20 century the number of small or family-operated brewery shrunk from 3000 to 60. These days about 20 breweries still produce Lambic beer. Some of the breweries were reclassified from breweries to blenders, which are not brewing beer but buying a wort and only fermenting it as a blend of their own product **(Guinard, 1990)**.

SPONTANEOUS FERMENTATION

Fermentation has been running for 3 years **(De Keersmaecker, 1996)**. The principle of spontaneous fermentation is not picking pure yeast culture into the cold wort. Some breweries are using a system of back-slopping, where part of an old batch is added into a new batch, normally it is the bottom of fermented volume, where is a higher concentration of flocculated yeast. Another technique where some part of post-main fermentation species from cask are added into beer (fermented with pure culture) is used too. Any of these techniques are not connected with spontaneous fermentation and this beer will not be possible to call spontaneously fermented beer. But unfortunately, it is used. Spontaneous fermentation must ferment without any targeted inoculation. The main part of the microbiota is obtained during the cooling of wort **(Bamforth, 2005; Spitaels *et al.*, 2014; Van Oevelen *et al.*, 1977)**. New studies show that the microbiota of air is not as important as it was in the past. Nowadays a big part of inoculum comes from casks and attic wood structures. Modern Brussels is not the same place as in 18. or 19. century and "domesticated" inoculum living in an attic is more and more important to produce Lambic. Therefore, the protection of the old brewery is important to protect Lambic production. Air from modern urbanized Brussels is poor to microbiota. Studies talk about low or lost inoculum during the cooling of beer in modern industrial Lambic production and the biggest part of contamination is obtained from casks, which are not sterilized like in pure culture fermented breweries, but only cleaned with water **(De Roos *et al.*, 2019; Spitaels *et al.*, 2014; Spitaels *et al.*, 2015).** Wort must be brewing in cold months when the temperature of the air is so cool to cool the wort at 20 °C for one night because only natural cooling is used. Coolers are constructed in the attic traditionally, cooling running with a low level of wort 10-20 cm and windows in the attic are opened. After cooling wort is transported to old wine wooden casks of red wine and fermented in one step without another transport (like is normal in nowadays brewing systems of CK tanks) for 3 years. The Casks are stored in the cellar where the ambient temperature culminates between 15-25 °C. There are not any coolers or heaters, during summer temperature can attack 25 or more degrees, and during winter temperature shrinks. The studies looked at microbiota and metabolites production and divided fermentation into four phases: Phase of Enterobacteriaceae, phase of the main fermentation, phase of acidification, and phase of maturation **(Esslinger, 2009; Spitaels *et al.*, 2014; Van Oevelen *et al.*, 1977)**.

PHASE OF *ENTEROBACTERIACEAE*

In older studies the *Enterobacteriaceae* phase was described as about 1-month length **(Martens *et al.*, 1991)**, we can say that methods at that time were constructed on different bases. Nowadays studies talk about a shorter phase of about one week of fermentation **(De Roos *et al.*, 2018; De Roos and De Vuyst, 2018)**. *Enterobacteriaceae* are facultative anaerobic bacteria that are using Embden-Meyerhof Pathway to metabolize monosaccharides and carry out mixed-acid fermentation which produces lactic acid, acetic acid, succinic acid, and formic acid. Most influences are lactic and acetic acid because of their impact on taste and pH level **(Van Vuuren and Priest, 2003)**. There was obtained different species of *Enterobacteriaceae* in lambic beers: *Klebsiella pneumonia*, *Enterobacter cloacae*, *Hafnia protea*, *Hafnia alvei*, *Citrobacter freundii*, *Serratia* strains, and *Proteus mirabilis*. In the past Enterobacteriaceae family was neglected without impact on the fermentation process, during times with new methods like chromatography their impact was proven. They can produce off-flavor metabolites like sulfur compounds mainly dimethyl sulfide. Generously, their occurrence relates to phenolic and medical off-flavors in beer and indirectly with diacetyl production. Van Vuuren's team firstly find *Enterobacter agglomerans* **(Martens *et al.*, 1991; Van Vuuren *et al.*, 1979; Vriesekoop *et al.*, 2013). Martens *et al.* (1991)** found the top of *Enterobacteriaceae* in eight days of fermentation, after this (10^7 CFU/mL was detected density in top) population of bacteria slowly shrunk and after 30 – 40 days no one of *Enterobacteria* was

detected. In a newer study **(De Roos and De Vuyst, 2019)** phase of fast-growing and top was very similar, but the phase ended under 2 weeks of fermentation. Fermentation was faster and the equal pH value after 2 weeks was under 4. **Van Vuuren *et al*. (1979)** wrote that *Enterobacteriaceae* are sensitive to pH under 5,5 and alcohol under 2%. In the first view, we can see the difference between these two studies, but in conclusion, we can say, that in both experiments the phase end after the pH value falls under 4, after consumption of all glucose and fructose like in **De Roos *et al.* (2018a)** and when alcohol concentration exceeds 2% this phase end. In old and nowadays studies we can see differences, but first what we need to see are differences in inputs. In old studies wort with pH 5 – 5,5 was used, nowadays brewery adds acids to the wort to shrink the pH volume below 4,5 (4,3 in the study **De Roos *et al*. (2018a)** and pH 4 in the study **Spitaels *et al*., 2015).** Lower pH slows down *Enterobacteriaceae* and the phase of main fermentation starts faster. Breweries use this technique nowadays to reduce the impact of *Enterobacteriaceae* on fermentation because government authorities and European Union made press on breweries to reduce the occurrence indication of fecal contamination during processing. Unfortunately, *Hanseniaspora uvarum* was not detected in beer produced with lactic acid addition. This specie relates to low fermentative capacity but is commonly found during the spontaneous fermentation of wines and cider, where its contribution to flavor complexity is increasingly appreciated **(Bezerra-Bussoli *et al*., 2013; Spitaels *et al*., 2015)**. Acetic acid bacteria are obtained here too, mostly *Acetobacter* and *Gluconobacter* species. In the first part of the fermentation, *Acetobacter orientalis* is the predominant acetic acid bacteria. The spatial analysis shows significant diversity between the concentration of acetic acid during the first phase. More acetic acid was produced in the top part of the beer, where is beer in contact with air. So, more oxygen starts higher acetic acid production in this part (**De Roos *et al*., 2018b).** Meanwhile in American coolship Ale most detected bacteria *Klebsiella oxytoca* and *Enterobacter agglomerans* but also *Enterobacter ludwigii, Enterobacter cloacae, Enterobacter mori, Klebsiella pneumonia*, and *Serratia ureilytica*. Yeasts are *Candida krusei, Pichia fermentans/kluyveri, Cryptococcus keutzingii* a *Rhodotorula mucilaginosa* and predominant *Rhodotorula mucilaginosa* which after the first week of fermentation occupied 40% of obtained strains, so terroir of the different continent has an impact on microbiota during this phase. In American coolship Ale, some *Enterobacteriaceae* were detected over the first 12 weeks, but molecular methods detect them sometimes later, this is big different in opposite to modern Lambic production, where lactic acid is added and detection of *Enterobacteriaceae* stop after 40 days of fermentation **(Bokulich *et al*., 2012).**

PHASE OF MAIN FERMENTATION

Saccharomyces follows the decline of *Enterobacteriaceae,* and they start growing, possibly it is related to relief from competition and acclimation to metabolites of *Enterobacteriaceae* and oxidative yeasts like carboxylic acids. Fermentation run from 2. to 9. week very quickly, after this time 80% of the extract is consumed so there is not any other source of saccharides eatable *to Saccharomyces*. First 3 weeks *Klockera apiculata* is on volume 10^3 CFU/mL, *Kloeckera* is maltose-negative yeast. Nevertheless, it produces a high concentration of secondary metabolites responsible for fruity and floral flavors. After next 2 weeks, *Saccharomyces* is predominant. The most obtained species are *Saccharomyces cerevisiae* and *Saccharomyces bayanus*. Other yeasts which are connected with this phase are *Candida, Cryptococcus, Torulopsis,* and *Pichia*. They are known as oxidative yeasts, and they produce biofilm on the top of beer level like Brettanomyces. This biofilm is necessary as a source of oxygen and it protects the beer from acetic acid bacteria which need oxygen too **(Bokulich and Bamforth, 2017; Sparrow, 2005)**.

Saccharomyces yeasts are commonly popular for their alcohol production which is their primary metabolite but there are other secondary metabolites from Saccharomyces yeast like esters, diacetyl, higher alcohols, and terpenoids with an impact on final beer **(Hirst and Richter, 2018)**. Propanol, isoamyl alcohol, isobutanol, amyl alcohol, 2-phenyl ethanol, and tyrasol are predominant higher alcohol produced by yeasts. Important is the total concentration under 300 mg per l, higher concentration makes unpleasant acrid aromas like solvent. Propanol, butanol, and isobutanol are responsible for an alcoholic aroma, isoamyl alcohol, and amyl alcohol like marzipan or banana. Tyrasol and 2-phenyl ethanol add honey and flora flavors **(Pires *et al.*, 2014)**. Diacetyl in low concentration makes a toasty and nutty aroma, in higher concentration smells like old butter. The biggest producent of diacetyl in Lambic is *Pediococcus*, so the most important are months during summer when the temperature is too high to grow of *Pediococcus* **(Hirst and Richter, 2018).** During the phase of main fermentation, acetic acid production is suppressed. During this phase detection of acetic acid bacteria was under the detection limit in the study by **De Roos *et al*. (2018b).** In American coolship ale production main fermentation starts at 4. week (like in old Lambic production). **(Bokulich *et al*., 2012).**

PHASE OF ACIDIFICATION

In new studies **(Bokulich *et al*., 2012; De Roos *et al*., 2018a; Spitaels *et al.*, 2014; Spitaels *et al*., 2015)** are predictions, that the phase of acidification and the phase of maturation are the same, so we can talk about one long phase of maturation with predominant species *Brettanomyces bruxellensis* and *Pediococcus damnosus*. In the prediction of American coolship Ale is the phase of acidification more expressive, any acetic acid is added here so there is a potential free field to produce it. After 2 weeks of fermentation LAB start growing and being predominant *Leuconostoc* spp. and with measurable contingents of *Lactococcus lactis, Lactococcus garviae, Streptococcus* sp., *Lactobacillus delbreuckii, Lactobacillus curvatus, Lactobacillus brevis,* and *Lactobacillus kunkeei*. This is different in opposite to Lambic, where *Lactobacillus* aren't the predominant because big batches of aged hops are added to beer boil. Beta acids from hop have an antimicrobial activity to *Lactobacillus*, so different brewing processes maybe make different conditions for growing. After 4 weeks of fermentation predominant family is *Pediococcus* (more than 80%) and the second predominant is *Lactobacillus* sp. **(Bokulich *et al*., 2012, Vriesekoop *et al*., 2013).** Acetic acid is produced in this phase too, after the main fermentation press to other species is lower. The new phase of acetic acid acidification starts with the new predominant specie *Acetobacter pastorianus*. In this phase, more microbial activity and more acetic acid production were detected too. Differences start after supplementing casks with another lambic, because of losses of evaporation. Mixing beer homogenizes beer and adds some new oxygen, so after this step, acetic acidification runs constantly in all volume. Change in predominant specie is probably connected with better alcohol and acetic acid toleration of *Acetobacter pastorianus*. Gene analysis shows a higher level of copies of genes with acetic and ethanol tolerance effects. (**De Roos *et al*., 2018b).**

Brettanomyces are non-conventional, wild yeasts, which play a big role in spontaneous fermentation, impact of these yeasts is not ambigenous, *Brettanomyces* are popular like one of the biggest spoiled microorganisms in wine and they produce off-flavor compounds, which can evocate burnt plastic, barnyard, medicinal, horse sweat, and leather amongst some other unpleasant flavors **(Licker *et al*., 1998; Colomer *et al*., 2019).** *Brettanomyces* combinate high alcohol tolerance and feature high fermentation capacity – they can reduce maltotetraose and maltopentaose, and can reach cell counts of 10^4 –10^5 cells per ml. So, they have a big potential and a long time to produce the typical *Brett flavor* **(Steensels *et al*., 2014; Crauwels *et al*., 2015, Kumara and Verachtert, 1991)**. Cells of *Brettanomyces* are smaller than cells of *Saccharomyces*, they can survive casks cleaning in wooden pores and secondary contaminate the beer. In the wine, some low concentrations of *Brett* secondary metabolites are requested **(Crauwels *et al*., 2015)**. Mousy taints are most contradicted in wine infected by *Brettanomyces* or lactic acid bacteria. Responsible compounds are 2-acetyl-3,4,5,6-tetrahydropyridine, 2-acetyl-1,2,5,6-tetrahydropyridine, and 2-ethyl-3,4,5,6-tetrahydropyridine which are the result of pyridines synthesized from lysine and ethanol **(Snowdon *et al*., 2006)**. Volatile phenolic compounds are most frequently associated with *Brett*-flavor, mostly 4-vinyl guaiacol, 4-vinyl phenol, 4-ethyl guaiacol, and 4-ethylphenol. They are connected with barnyard, clove, horsy, leathery, medicinal, spicy, and smoky aromas. Proportions of production of volatile phenols are close correlated with substrate specificity. Hydroxycinnamic acids are precursors of the production of volatile phenols and the strain of yeasts is important too, strains in beer did not produce 4-ethyl guaiacol a 4-ethylphenol above the detection limit. The concentration of 4-ethyl guaiacol is higher than the concentration of 4-ethylphenol in beer in wine it is upside down (**Oelofse *et al*., 2009)**. Another study ascribes the impact of different concentrations of phenols on different concentrations of cumaric and ferulic acids **(Kheir *et al*., 2013)**. *Brettanomyces* produce a high volume of isovaleric acid (volatile fatty acids), which is connected with an unpleasant rancid odor. It is typical for a young beer where fatty acids are not esterified yet **(Gamero *et al*., 2014)**. An important group of aromatic compounds are volatile esters, they are responsible for a fruity and flower character in beer. *Brettanomyces* produce a high concentration of ethyl acetate, ethyl lactate, ethyl caprate, and ethyl caprylate. During production, they can reduce isoamyl acetate than is responsible for banana aroma **(Verachtert, 1992)**. And finally, *Brettanomyces* can add another flavor to beverages, they use B-glucosidase enzymes and can add locked flavors that are locked in complex glycosidically bound sugars **(Daenen, 2008)**.

PHASE OF MATURATION

Maturation is the last phase of beer production, in new studies, authors did not see any big differences between the phase of acidification and the phase of maturation **(Bokulich *et al*., 2012; De Roos *et al*., 2018a; Spitaels *et al.*, 2014; Spitaels *et al*., 2015)** but this is only microbiological aspect. Technological aspects are different, Lambic beer ferment in open fermenters, so beer has a low CO2 level and is sensory dull. For carbonization, two ways are used. The first way is mixing two Lambic, one old Lambic two or three years old any young one-year-old Lambic. The second way is mixing old Lambic with new contaminated wort, this beverage is called Gueuze. So Gueuze is more refermented with higher alcohol volume in the range of 6-7% and lambic is lower carbonated and lower alcoholic about 5-6% **(Verachtert and Derdelinckx, 2014)**. Storage of gueuze bottles can last for ten years. Besides preservation, other purposes ascribed to the bottle refermentation and maturation of lambic beers are the improvement of the aroma, taste, and mouthfeel **(Bongaerts *et al*., 2021)**. Traditionally other sugar sources are

used for refermentation. In years with a good harvest, big portions of fruit were added to beer. Fruit is rich not only in sugar but organic acids too. Analogous to other lactic acid bacteria in wine production, *Pediococcus damnosus* can perform a malolactic fermentation during fruit refermentation in spontaneous fermentation. This process degrades tart malic acid from fruit (apples or grapes) and changes it to lactic acid which is sweeter and softer **(Versari *et al*., 1999; Zhang and Lovitt, 2006)**. Refermentation is connected with *Brettanomyces* and *Pediococcus* mostly. The concentration of 4-ethylphenol and 4-ethyl guaiacol grow as well as lactic acid and ethyl lactate. Degradation of isoamyl acetate continues. Gueuze beer is stable for 10 years **(Spitaels *et al*., 2015)**.

CONCLUSION

The traditional way of beer fermentation is a complicated and not always successful process. A long time of production, big losses, and inconsistent products are the biggest challenges here, but the final product is unique and highly valuable. Nowadays, when we understand mechanisms in the processing, we can apply parts of these techniques or use specific microorganisms which are responsible for accepting metabolites. Part of wild yeast obtained from spontaneous fermentation has a small fermentation capacity and is possible to use for low-alcohol or non-alcoholic beer production. *Brettanomyces* species live in renaissance nowadays, new techniques and knowledge of their behavior enable them to produce different beverages with a new dimension of taste and flavors. B-glucosidase was isolated and now is using the food industry, this is only one of the potential applications of spontaneous fermentation in the modern food industry.

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