Investigations of Possible Location Dependence of Unique Microflora in Spontaneously Fermented Lambic Beer

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Abstract

Traditionally lambic beer is a spontaneously fermented beverage that relies on successive blooms of microbial species to accomplish attenuation and impart important flavor and aroma profiles. Research shows that consistent blooms are seen in lambic fermentation beginning with wild yeast and enterobacteria, and followed by alcohol production by *Saccharomyces cerevisiae*, lactic acid production by lactic acid producing bacteria, and finally super-attenuation by *Brettanomyces* yeasts. Recent developments in genetic testing have allowed researchers to gain a more sophisticated look at the microbes present in lambic-style beers brewed by American breweries. These results are analyzed and compared to those of Belgian produced lambic beer in an effort to examine differences in species diversity. Research suggests that production of lambic-style beer can be done with good consistency anywhere.

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1. Introduction

Lambic beer is a uniquely fermented beverage whose fermentation relies heavily on microbe species traditionally seen as beer-spoiling organisms. These organisms act in concert over the course of lambic fermentation to produce a beer that is very low in residual sugar and has a pleasant amount of acidic tartness. Conditions surrounding the production, storage during fermentation, and maturation of the product aim to promote the development of these microorganisms' acid producing and super-attenuating capabilities. A certain mystique surrounds lambic beer. Due to the issuance of an appellation controlee, lambic can legally only be produced within a certain radius around Brussels, Belgium. The introduction of modern genetic testing equipment has allowed new research to be done on “lambic-style” beers, and has shown similarities in microflora suggesting that lambic-style beers can be brewed anywhere.

The history surrounding lambic is rich, and is regarded as the oldest surviving commercial beer style (Sparrow, 2005; De Keersmaecker, 1996). The brewing procedures used to produce lambic have remained relatively unchanged in its long evolution. Brewing begins with a mash aimed at producing wort with a high dextrin and protein content. The lack of fermentable sugars produced in the mash will provide a nutrient source for organisms during the long fermentation period. Aged hops are used during the boiling process to limit bitterness in the final product and kill gram-positive bacteria (Sparrow, 2005). After boiling, the wort is pumped to a large, shallow tray called a cool ship. Overnight, the wort is cooled and infected by air circulating over the liquid. The following day, the wort is pumped into wooden casks where it will spend between 1-3 years fermenting between 0 and 25 °C (Van Oevelen, 1977).

Research into lambic fermentation by Van Oevelen (1977) found four overlapping stages each dominated by a unique set of microorganisms. Fermentation begins with an initial drop in pH, and proceeds with a primary alcohol fermentation, a sharp increase in the lactic acid production, and finally a period of super-attenuation to reach a final gravity of less than 1°P. Each set of microorganisms donates a small piece of the overall flavor and aroma profile that gives lambic its complex character.

This paper aims to elucidate the current knowledge surrounding lambic fermentations, the main microbiological species seen in fermentation, and will attempt to determine if
spontaneous lambic beer fermentation is location dependent.

2. Microbiology of Lambic Fermentation

2.1 Cell Physiology

2.1.1 Enterobacteriaceae

The genus Enterobacteriaceae is characterized by Gram-negative, straight, motile and non-motile rods. Members of the genus are facultative anaerobes. Important species related to the brewing industry are catalase-positive and oxidase-negative. These bacteria ferment glucose through two pathways known as the Embden-Meyerhof-Parnas Pathway and the Hexose Monophosphate Pathway to produce acetic acid, lactic acid, succinic acid, ethanol, acetoin, and 2,3-butanediol in varying amounts (Priest, 1987). Broad overviews describing growth of enterobacteria (Priest, 1974; Priest and Campbell, 1987) suggest that pathogenic species like Escherichia coli have never been isolated from brewer’s wort, however, studies performed by Martens (1992) showed nearly 10% of enterobacteria species were identified as Escherichia coli.

2.1.2 Kloeckera

Kloeckera and its sexually reproducing teleomorph (Hanseniaspora) are wild yeasts commonly found in the wine and cider making industries (Zohre, 2002; Fleet, 1984; Cabranes, 1997). Species were shown to be oval, with length and breadth of approximately equal length. Cultures growth showed turbid media after 3 days at 25 ºC (Wiles, 1950). Kloeckera species have been shown to cause detectable levels of acetic acid in wine fermentation (Fleet, 1984). Fermentation trials done in the wine industry showed temperature dependence of K. apiculata in producing levels of esters and higher alcohols (Erten, 2002).

2.1.3 Saccharomyces

Brewing industry examples of Saccharomyces fall into two strain varieties: S. cerevisiae used in ale production, and S. pastorianus used in lager production (Barnett, 2008). Saccharomyces strains are able to grow with or without the presence of oxygen, however ester production is inversely proportional to oxygen concentration in growth media (Cowland, 1966). Reproduction is done through an asexual budding mechanism, and 3-4 generations are produced over the course of a single fermentation (Boulton and Quain, 2006). Metabolism of glucose, fructose, sucrose, maltose and maltooltriose via the Embden-Meyerhof-Parnas pathway satisfies the cell’s carbohydrate requirement (Priest, 1987).

2.1.4 Pediococcus

Bacterial isolates from beer from the genus Pediococcus generally fall into the species P. damnosus (Priest, 1987; Coster, 1964). Pediococcus species are Gram positive, catalase negative cocci, and generally appear in non-motile single, pair, or tetrad configurations (Coster, 1964). Species of the genus Pediococcus are facultative anaerobes, and display increased growth rates under anaerobic atmosphere (Coster, 1964). Varieties of P. damnosus show optimal growth temperatures at 22 ºC (Coster, 1964). Growth of Pediococcus species in beer are characterized by large amounts of diacetyl production, lactic acid production, and on occasion the formation of roughness in culture media (Satokari, 2000; Priest, 1987; Van Oevelen, 1977). Appearance of viscous sediment and turbid growth media was seen by Coster (1964).

2.1.5 Brettanomyces

Species belonging to the genus Brettanomyces are generally considered to be wild yeast, and ferment according to Custer’s effect in which glucose fermentation to ethanol is stimulated in the presence of oxygen (Periera, 2012). Growth rates of Brettanomyces species tend to be much slower than S. cerevisiae, studies have shown up to a 6-fold difference between the growth rates of these species (Blomqvist, 2010). Species of this genus can produce large amounts of lactic and acetic acid as by-products of their fermentation, and due to their ability to grow on a large spectrum of fermentable sugars, are seen to play a major role in over attenuation of beer (Kumara, 1993; Uscanga, 2003). Brettanomyces yeasts are known for “sweaty horse blanket” or “mousy” aroma character, these are probably due to esterification of caprylic and capric fatty acids (Spaepen, 1978; Van Oevelen, 1977).

2.1.6 Lactobacillus

Lactobacillus species are Gram-positive, catalase-negative, rod-shaped bacilli. Species of this genus are both homo and heterofermentative (Priest and Campbell, 1987). Nearly all bacteria isolated from a brewery setting matching this description will belong to the Lactobacillus genus and further classification is rarely necessary. Spoilage is seen first as high diacetyl production and the presence of silky turbidity in culture media (Priest and Campbell, 1987). L. brevis and L. lindneri are viewed as the most important beer spoiling species (Suzuki, 2011).

2.2 Stages of Fermentation

Lambic fermentation is characterized by Van Oevelen (1977) as having several overlapping stages, each involving distinct microbiological species. Progression through each new stage of fermentation is facilitated by reaction conditions created during the previous stage such as loss of simple sugar concentration, alcohol concentration increases, or pH decreases.

2.2.1 Enterobacteria and Wild Yeast Fermentation

The first stage of lambic fermentation lasts between 1-2 weeks and is characterized by a small increase in ethanol production and a large drop in pH (Van Oevelen, 1977; Martens, 1991; Martens, 1992). Work performed by Van Oevelen (1977) and Martens (1992) showed the first stage of lambic fermentation was dominated by the Enterobacteriaceae species: Enterobacter cloacae, Klebsiella aerogenes, and Escherichia coli. Table I. presents values for the percent of total isolated enteric species. Increases in bacterial cell density coincide with increases in total acid concentration and reach a maximum of 10^8 cells/mL near the second week of fermentation.
During this period wort pH is dropped quickly by 0.5. This is the result of lactic, acetic, and succinic acid synthesis by enteric species (Van Oevelen, 1977, Martens 1992), and it is the first of several pH decreases seen during the fermentation. Nearly all the acetic acid found in the final product, around 1000 ppm, and around 800 ppm of lactic acid is produced during this stage (Kumara, 1991; Van Oevelen, 1976). A graph depicting the increase in cell density and simultaneous decrease in pH is shown in Figure 1 (Martens, 1991).

Additional studies by Martens and Verachtert (1991) describe the early metabolism of simple wort sugars in K. apiculata in the early metabolism of simple wort sugars. Cell densities of K. apiculata reach their maximum level of 10⁸ cells/mL after one to two weeks (Van Oevelen, 1977). After 30 days of fermentation, the ethanol concentration of the solution is approximately 2 g/100 mL (Martens, 1991). Enterobacteria have been shown to be ethanol sensitive above 2.0% abv, and pH sensitive below 5.5 (Priest, 1974). The small rise in ethanol concentration, and the drop in pH during this stage is enough to kill off all enterobacteria cells in suspension, and after approximately 4 weeks of fermentation no remaining viable cells remain (Van Oevelen, 1977; Martens, 1991).

2.2.2 Alcohol Fermentation and Lactic Acid Production
The wild yeast K. apiculata seen in the first stage of fermentation is soon outcompeted by Saccharomyces cerevisiae. Cell densities of S. cerevisiae reach a maximum of 5x10⁶ cells/mL between 3 and 4 weeks after inoculation (Van Oevelen, 1977). This stage of fermentation takes between 4-8 months and corresponds with the main alcohol production; attenuating the beer from 12 °P to around 3 °P. The resulting lambic has an ethanol content of about 5.5% abw (Kumara, 1991).

Studies on lambic fermentation by Van Oevelen (1977) show that a second period of acid producing bacterial growth involving Pediococcus spp. occurs 3-4 months after inoculation. The third stage of fermentation occurs concurrently with the S. cerevisiae fermentation and represents an important acidification step in developing the character of the final product. Pediococcus cell densities reach their maximum with the aid of warm summer temperatures, approximately 7 months after inoculation (Van Oevelen, 1977; Bokulich, 2012). Increases in lactic acid concentration can reach between 5000-7000 ppm by the end of the first year of fermentation (Van Oevelen, 1977; Martens 1991). Several isolated strains cultured from industrial lambic show tendencies towards “ropiness” in casks, and this increase in viscosity may be important to later metabolism by Brettanomyces species (Van Oevelen, 1977; Spaepen, 1982). Work done by Martens (1991) and Kumara (1991) indicate that there may be a symbiosis that occurs between Pediococcus and Brettanomyces species. Cultures growth with both bacteria and yeast species show a more rapid and complete attenuation than when Brettanomyces grows in pure culture.

2.2.3 Brettanomyces Super Attenuation
Approximately 8 months after inoculation, the appearance of Brettanomyces yeast strains correspond with a period of super attenuation, below 1 °P, as characterized by Van Oevelen (1977). Kumara (1991) isolated 9 B. bruxellensis strains and 15 B. lambicus strains from a sample of commercially produced lambic. With the help of antibiotics, it was shown that Brettanomyces yeasts and not Saccharomyces yeasts are able to survive in conditions resembling a 1-year-old lambic beer. Data from this experiment is shown in Table II.

Brettanomyces plays a large role in the development of the key esters seen in lambic beers: ethyl lactate, ethyl acetate, iso-amyl acetate, and phenethyl acetate (Spaepen, 1982). Interesting experiments have been done (Spaepen, 1982) to show that chemical esterification is not responsible for the formation of ethyl lactate and ethyl acetate in any relevant time scale, proving that microbial synthesis is essential to the unique character of lambic. Other studies (Spaepen, 1982; Spaepen 1978) show that lambic beers are high in caprylic (C₆) and capric (C₈) fatty acids and their synthesis is probably due to Brettanomyces and Saccharomyces yeasts. These fatty acids play an important role in the formation of ethyl caproate and ethyl caprate, two esters responsible for a “goaty” flavor characteristic of some Brettanomyces strains (Engan, 1974).

### Table I. Enterobacteriaceae in Fermenting Lambic

<table>
<thead>
<tr>
<th>Isolated Species</th>
<th>Isolated Colonies (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. cloacae</td>
<td>48.3</td>
</tr>
<tr>
<td>K. aerogenes</td>
<td>22.7</td>
</tr>
<tr>
<td>E. coli</td>
<td>8.7</td>
</tr>
</tbody>
</table>

*Data adapted from Martens (1992)

### Table II. Viability of Yeast Growth in Simulated 1-Year-Old Lambic

<table>
<thead>
<tr>
<th>Yeast Species</th>
<th>Control</th>
<th>2 days</th>
<th>5 days</th>
<th>99</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. cerevisiae</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>99</td>
</tr>
<tr>
<td>B. lambicus</td>
<td>95</td>
<td>98</td>
<td>75</td>
<td>95</td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>70</td>
<td>97</td>
<td>83</td>
<td>94</td>
</tr>
<tr>
<td>Lactic Acid</td>
<td>89</td>
<td>97</td>
<td>83</td>
<td>94</td>
</tr>
<tr>
<td>Lactic Acid+Acetic</td>
<td>72</td>
<td>95</td>
<td>0</td>
<td>92</td>
</tr>
</tbody>
</table>

*Numerical values are percent viable cells. 100 mL solutions containing 10⁶ cells/mL were incubated at 28 °C without or with addition of ethanol (5 vol %), acetic acid (800 ppm), or lactic acid (7000 ppm), or combination.*

**Data adapted from Kumara (1991)**
that a three-phase fermentation profile exists for ACA. Due to the absence of "brewing seasons," a clear distinction was seen during the second phase when this occurred in spring versus in winter. Similar microbial species are involved with the first stage of ACA fermentation: domination by Enterobacteria and wild yeast, followed by the appearance of Saccharomyces. The second phase of ACA fermentation involves a rise in Lactobacillus and Pediococcus species. Finally, similar to traditional lambic fermentation, the long maturation and super-attenuation period of ACA is seen immediately after Pediococcus dominance and is associated with Brettanomyces bruxellensis growth.

The significant differences between this ACA study and those examining traditional lambic fermentation are mainly related to yeast and bacterial species diversity during the first and second weeks of fermentation. During this early time, a greater diversity of yeast species was observed, shown in Table III. \textit{S. cerevisiae} became the dominant yeast species after week 4. Brettanomyces became the dominant yeast species during week 12.

<table>
<thead>
<tr>
<th>Yeast Species Diversity in ACA Fermentation</th>
<th>Approx. Relative Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Saccharomyces cerevisiae}</td>
<td>~60%</td>
</tr>
<tr>
<td>\textit{Rhodotorula mucilaginosa}</td>
<td>~40%</td>
</tr>
<tr>
<td>\textit{Pichia} spp.</td>
<td>Sporadic Minor Contributions</td>
</tr>
<tr>
<td>\textit{Candida} spp.</td>
<td>Sporadic Minor Contributions</td>
</tr>
<tr>
<td>\textit{Cryptococcus} spp.</td>
<td>Sporadic Minor Contributions</td>
</tr>
</tbody>
</table>

*Data adapted from Bokulich (2012)*

Enrichment of a resident microflora has been shown to be important for batch-to-batch consistency in the wine industry (Blanco, 2011). The same principles can be applied to lambic and ACA industries. The reuse of barrels provides an established micro-biome for consistent flavor and aroma development. This appears to only be one component of consistency in spontaneously fermented beers, as beers brewed into new barrels show similar microbial profiles to those brewed into reused barrels (Bokulich, 2012). Aerial contamination consistency is probably due in part to air flowing over the walls and ceiling of the brewhouse already "seeded" with appropriate microbiota. The method of aerial contamination by adjacent environments has been shown to be a risk factor in production of fruits and vegetables (Suslow, 2003).

4. Concluding Remarks

Lambic beer has an extremely complex flavor and aroma profile produced from an equally complex fermentation. Many different microbial species traditionally seen as a nuisance, come together in concert to produce a beer with a delicate balance of acidic tartness, dryness, and fruit flavor.

Because of legal restrictions protecting lambic beer brewed in Belgium, beers brewed to mimic lambic beer are designated as "lambic-style" beers. Several examples produced in the breweries of the United States display similar flavor and aroma profiles as their European counterparts, however until recently, microbiological identification had not pin-pointed actual differences between the two beers. New genetic testing methods have allowed researchers to compare the "fingerprints" of each stage involved in American coolship ale and traditional lambic
fermentations. Results show similarities in microbiological species diversity; however, more genetic testing must be done on lambic beer brewed in Belgium in order for an accurate comparison to be made. One can speculate that a more sophisticated comparison between the two would show very similar micro-biomes due to the widespread presence of spoilage microbes in the global brewing environment. It would seem that over time the natural selection involved with pH, ethanol concentration, and restrictive nutrient profiles in fermentation would produce an appropriate micro-biome for the development of lambic-style beer regardless of the brewing location.

5. References


Bokulich NA, Bamforth CW, Mills DA (2012) Brewhouse-Resident Microbiota Are Responsible for Multi-Stage Fermentation of American Coolship Ale. PLOS One 7


