Lambic and Gueuze are special Belgian beers obtained by spontaneous fermentation. Micro-organisms involved in this fermentation were counted and differentiated using several selective growth media. Micro-organisms were also isolated from samples of Lambic of different age and originating from different casks and beers and identified by classical tests. The following general pattern of microbial development was observed. After 3 to 7 days the fermentation started with the development of wort Enterobacteriaceae and strains of Kloeckera apiculata. These organisms were overgrown after 3 to 4 weeks by strains of Saccharomyces cerevisiae and S. bayanus. These were responsible for the main fermentation, lasting for 3 to 4 months. This was followed by a strong bacterial activity. This period was dominated by the growth of strains of Pediococcus cerevisiae. These reached their maximal numbers during the summer months and were responsible for a five-fold increase in lactic acid concentration. In some casks they caused ropiness. After the main fermentation period Lambic is very sensitive to spoilage by acetic acid bacteria of the genus Acetomonas. The presence of air may be the determining factor for their development. After 6 months a new increase in yeast cells was noted. These belonged now mainly to the genus Brettanomyces bruxellensis and Br. lambicus. They caused a further slow decrease in residual extract and the appearance of special flavours. Oxidative yeasts of the genera Candida, Cryptococcus, Torulopsis and Pichia were also detected and may be responsible for the formation of a film on the beer surface after the main fermentation.

Keywords: Gueuze, Lambic, fermentation.

INTRODUCTION

Spontaneous wort fermentation is used in the production of some special types of Belgian beer, known as Lambic and Gueuze. Wort is cooled in open shallow trays. The cooling takes place overnight and during this period the wort picks up a variety of micro-organisms from the air which is blown over the wort. The next morning the infected wort is run into large casks which are stored at a temperature of between 0°C and 25°C, without any inoculation with yeasts.

The different phases occurring during a two year fermentation period and the occurrence of several fermentation products as a function of fermentation time have been described recently. In that study no attempts were made to isolate or to identify the different types of micro-organism involved in the fermentation stages. We would now like to report on these microbiological aspects of Lambic fermentation and to discuss the importance of several groups of organisms for Lambic and Gueuze production.

EXPERIMENTAL

Sampling

The Lambic fermentation takes place in wooden casks with a content of 200 to 650 litres. The casks have only small apertures, one at the top and two at the front. Only the apertures at the front can easily be reached. It might be expected that, during the fermentation, part of the flora will be found in suspension, part will be settled at the bottom and part will be growing at the wort-air interface. Homogenization of the contents of the casks is not possible. As a consequence the only reasonable sampling place was at the front aperture, at about half the height of the cask. Thus mainly the organisms in suspension, and probably the most active at the time of sampling, were sampled. After cleaning the tap, the first beer was withdrawn and then 250 ml of beer was collected in sterile flasks. These were transported immediately to the laboratory, with cooling in ice-boxes, and submitted to analysis on the same day. About fifty samples of Lambic of different age and originating from different casks and different brews were analysed.

Isolation and enumeration media

Since Lambic is the product of a spontaneous fermentation, almost any micro-organism ever isolated from alcoholic beverages may be expected to occur in this fermentation. Some earlier studies on Lambic mention the presence of Saccharomyces spp., Brettanomyces spp. and bacteria such as Enterobacteriaceae and a ‘Bacillus viscosus bruxellensis’. As large amounts of acetic and lactic acid were found during the previous study, acetate and lactic acid bacteria might reasonably also be expected. On this basis it was decided to use isolation media which could support the growth of most micro-organisms occurring in the brewing industry and media more specific for the detection of yeasts and special bacteria. All media were sterilized by autoclaving at 121°C for 15 min.

Total count.—A medium rich in nutrients and growth factors and known as Universal Beer Agar (UBA) was used for the determination of total counts. The inoculated media were incubated at 25°C and counting was done after 5 and 10 days.

Yeasts.—For total yeast counts, malt extract agar (malt extract of 10° Balling + 1.5% baetoagar) supplemented with 50 ppm streptomycin was used. This medium will be designed as MAS.

For the growth of non-Saccharomyces yeasts, especially Brettanomyces, a medium composed of malt extract (10° Balling), agar (1.5%), CaCO₃ (0.5%), streptomycin (50 ppm), and actidione (10 ppm) was used. This is the MAAKS medium. Most Saccharomyces yeasts are inhibited by 10 ppm actidione, while Brettanomyces still grows at 100 ppm actidione. As Brettanomyces yeasts produce significant amounts of acid, their detection will be facilitated by the inclusion in the medium of CaCO₃ and the formation of zones of CaCO₃ dissolution around their colonies. Other yeasts, however, may also produce some acids, such as members of the genera Candida, Debaryomyces, Hansenula, Pichia, Schizosaccharomyces, Torulopsis and Dekkera. Streptomycin and actidione were prepared at 100× strength as aqueous solution and sterilized by filtration.
Identification of yeasts and bacteria

Yeast colonies isolated from MAS and MAAKS media were subjected to a selection of identification tests according to Lodder, which included the study of appearance in liquid and on solid media, cell morphology, reproduction morphology and the formation of ascospores using Gorodkowa agar, McClay's acetate agar and Fowell's acetate agar. All strains were tested for fermentation of glucose, maltose, lactose, sucrose and galactose, and sometimes raffinose and starch, and for assimilation of the same sugars and also cellobiose, nitrate and ethylamine hydrochloride.

Bacterial colonies from VRB and UBAP were divided into three groups according to the gram stain, the catalase reaction, the growth on VRB agar and the morphology.

Gram-positive cocci.—Gram-positive, catalase-negative cocci were submitted to the Hugh and Leifson test as described by Skerman. Homo- or heterofermentatvity was tested by the method of Gibson & Abd-el-Malek. Oxygen requirements were studied by incubating isolated bacteria, aerobically or anaerobically, in Universal Beer Broth (UBB). Growth was estimated by visual inspection and the time necessary to obtain growth was noted. Optimum growth temperature was studied by incubating the isolates in UBB at 22°C, 30°C or 37°C. Growth in UBB at pH of 4.2 was followed using a method of Gibson & Abd-el-Malek. Oxygen requirements were submitted to the Hugh and Leifson test as described by Skerman. Homo- or heterofermentative bacteria were identified as gram-positive, non-motile, catalase-negative cocci. They were homofermentative and occurred as single cells, in pairs or in tetrads. These were identified as Pediococcus spp. Some present in equal amounts. The lactose non-fermenting organisms were slightly more resistant to the rise in ethanol concentration and the lowering of the pH. During the first month of fermentation the counts on VRB agar coincided with the counts on aerobic UBAP, indicating that most bacteria were wort Enterobacteriaceae. These bacteria disappeared completely in later stages, while the counts on aerobic UBAP became very variable, differing from cask to cask. After about 4 months the counts on anaerobic UBAP rose considerably, becoming maximal after 6 to 8 months. Later these bacteria seemed to settle out and the counts became irregular. With respect to yeasts there was a first period, dominated by acetidione-negative and apiculate forms. These disappeared and became dominated by acetidione-sensitive strains after about 14 days. After 7 to 8 months counts on MAS and on MAAKS were similar, indicating that most of the yeasts present were again of the acetidione-resistant type.

Identification of microorganisms

From the different media several colonies were isolated and submitted to further characterization.

Yeast.—From MAS and MAAKS about 100 colonies were isolated and 42 colonies were studied further. The morphological characteristics and some physiological data of these isolates are given in Table I. It is clear that most isolates belonged to the genus Saccharomyces and to the genus Brettanomyces.

Acetic acid bacteria.—At a period when no bacteria were detected on VRB-agar, 27 colonies, consisting of gram-negative rods, were isolated from aerobic UBAP. They were unable to grow on VRB-agar and they were catalase-positive. They all produced acetic acid from ethanol. About 10% of the isolates oxidized acetic acid to CO₂ and H₂O and transformed lactate to carbonate. These bacteria were identified as Acetobacter spp. About 90% of the isolates were identified as Acetomonas spp. (no further oxidation of acetic acid, no transformation of lactate).

Lactic acid bacteria.—38 isolates of bacteria growing on anaerobic UBAP were identified as gram-positive, non-sporeulating, non-motile, catalase-negative cocci. They were homofermentative and occurred as single cells, in pairs or in tetrads. These were identified as Pediococcus spp. Some
**TABLE 1. Morphological and physiological characteristics of yeasts isolated from Lambic.**

<table>
<thead>
<tr>
<th>Yeast Species</th>
<th>Number of Strains</th>
<th>Growth at 22°C</th>
<th>Ascorbic Acid</th>
<th>Pseudomonomylucose/Myzostatin</th>
<th>Fermentation: glucose</th>
<th>Maltoose</th>
<th>Lactose</th>
<th>Saccharose</th>
<th>Galactose</th>
<th>Raffinose</th>
<th>Starch</th>
<th>Assimilation: glucose</th>
<th>Maltoose</th>
<th>Lactose</th>
<th>Saccharose</th>
<th>Galactose</th>
<th>Raffinose</th>
<th>Starch</th>
<th>Cellobiose</th>
<th>Nitrate</th>
<th>Ethylamine</th>
<th>Hydrochloride</th>
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<td>Brettanomyces globosus</td>
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<td>Brettanomyces uvarum</td>
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<td>Kloeckera spiculata</td>
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<tr>
<td>Pichia sp.</td>
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<td>Saccharomyces bayanus</td>
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<td>Saccharomyces uvarum</td>
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<td>Torulopsis sp.</td>
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<td>Torulopsis sp.</td>
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</table>

- **weak reaction.**
- **S:** slow reaction.
- **C:** raffinose is completely fermented.
- **1/3:** raffinose is fermented only for 1/3.

shorter lag period. These two strains did not grow at pH 4-2, nor did they produce acid from dextrin or arabinoise.

Although the genus *Pediococcus* has been the subject of many studies,14-21,24,25,31,38 it is still the least known group of homofermentative coccal lactic acid bacteria. In the 1974 edition of Bergey's Manual,4 the gram-positive, non-endospore forming cocci are grouped in three families, the genus *Pediococcus* being a member of the family Streplococcaceae. Many disagreements exist among authors concerning the classification of species within the genus. Günther & White12,13 and Coster & White16 distinguished four species: *P. cerevisiae, P. parvulus, P. damnosus* and *P. halophilus*. All brewery strains are grouped in the species *P. damnosus*. They have a low optimal growth temperature (22°C) and they fail to grow at 37°C or in the presence of 4% NaCl. They are divided into three sub-species: *P. damnosus var. damnosus*, var. *diastaticus* and var. *ilmusos*. Nakagawa & Kitahara22 proposed 5 species: *P. cerevisiae, P. acidilactici, P. pentosaceus, P. halophilus* and *P. urinae et. equi*. In this classification *P. cerevisiae* is characterized by its ability to grow in hopped wort and beer, a low optimal temperature, a requirement for acid media and CO₂, a preference for anaerobic conditions. In the opinion of Coster & White6 these are the characteristics of *P. damnosus*. As Bergey's Manual has adopted the classification of the Japanese workers, our isolated strains from Lambic should belong to the genus *P. cerevisiae*. The growth of one of the isolates on different media is shown in Fig. 2. Tomato Juice Broth is an excellent medium, with optimal growth at pH 5. Although the organism was isolated from Lambic, almost no growth occurred in sterile Lambic or Gueuze. In Gueuze supplemented with 1% glucose, very slow growth occurred. Growth in a medium with 1% yeast extract as source of nitrogen and essential nutrients was also very poor.
Fig. 3. Evolution of some important parameters of spontaneous Lambic fermentation: 1 = ethanol; 2 = lactic acid; 3 = ethyl-lactate; 4 = pH; 5 = real extract content; 6 = acetic acid.
become apparent. The very fast growth of wort Enterobacteriaceae and of Kl. apiculata results in a decrease of the pH from 5.1 to 4.6. This coincides with the synthesis of amounts of acetic acid, of the same order of magnitude as found in the final product. Important amounts of lactic acid are also made. During this period, the density of the wort is only slightly diminished, since neither of the micro-organisms mentioned can ferment maltose or maltotriose. The mixed acid fermentation is responsible for the formation of foam at this stage of the fermentation, as CO₂ and H₂ are formed. As wort Enterobacteriaceae are known to produce several sulphur compounds, carbonyls and phenols, in Lambic fermentation experiments have shown that some early products may disappear during later phases of fermentation. Saccharomyces yeasts are responsible for the main alcoholic fermentation. The large increase in lactic acid concentration coincides with the presence of Pediococcus cerevisiae. Lactobacillus spp. could never be isolated. The growth of Brettanomyces coincides with a further slow decrease in residual extract. Many isolates, when grown in pure culture, developed a strong 'mousy' taste. This taste, although very bad and objectionable in high concentrations, is also found in the final product, Gueuze, where it contributes to the typical flavor of the product. This may already indicate that Brettanomyces is important for the final flavor characteristics of Gueuze. Acetic acid bacteria may be found during the whole secondary fermentation period, in variable but normally low numbers. In our opinion they represent a constant danger since, whenever they find favorable conditions for growth, they produce up to 4000 ppm of acetic acid and a Lambic which is called 'hard'. Wort infected normally at the brewery and transported to the laboratory in 50 litre glass bottles, closed with cotton plugs, fermented normally during the first months, but then became overgrown by acetic acid bacteria. Dissolved oxygen and factors controlling it may be very important for their development.

Conclusions

From this study it seems apparent that the main microbial groups active in Lambic fermentation are wort Enterobacteriaceae, yeasts such as Kl. apiculata, Saccharomyces and Brettanomyces and bacteria such as Pediococcus. Many problems remain to be solved such as

(a) where do the different organisms find their origin?
(b) can the development of acetic acid bacteria be controlled?
(c) which organisms cause ropeyness when it occurs and can it be controlled?
(d) what is the nature of the mousy smell produced by Brettanomyces?
(e) are the events occurring in other Lambic breweries similar?
(f) can Lambic be made with pure cultures?
(g) are there other unknown fermentation products in Lambic?

Acknowledgement.—The technical assistance of Mrs Knaepen was highly appreciated. Eng. P. De Neve is thanked for the practical information given and the opportunity to follow the fermentations at brewery level. The support of this work by a grant from the N.F.W.O. Foundation, Brussels, is gratefully acknowledged.

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