Microbial succession in spontaneous fermented grape must before, during and after stuck fermentation

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Abstract

In the present study, the microbial succession in spontaneous fermenting Riesling must from a winery at the Moselle was investigated from the beginning (pressing) until the end (sulphuring) of the fermentation in two harvest years (2008 and 2009). The fermentation period in 2008 was from October until June, in 2009 from October until January. Bacterial strains from different genera (Gluconobacter, Asaia, Acetobacter, Oenococcus, Lactobacillus, Bacillus and Paenibacillus) and yeasts from different genera (Candida, Debaryomyces, Pichia, Hanseniaspora, Saccharomyces, Metschnikowia, Cryptococcus, Filobasidium and Rhodotorula) were successively isolated. The main fermenting organism was Saccharomyces uvarum. In both years of vintage, the alcoholic fermentation was interrupted by a period in which the fermentation stucked, although the length varied considerably (20 weeks in 2008 and one week in 2009). Specific primers were developed for S. uvarum, H. uvarum and C. boidinii, followed by the determination of the total cell counts with qPCR. The initial glucose concentrations were 116 g/l in 2008 and 85,4 g/l in 2009. Also the fructose concentrations were different in both years (114 g/l in 2008 and 77,8 g/l in 2009). The stuck period appeared when the glucose/fructose ratio was 0,34 (2008) and 0,12 (2009). Although S. uvarum was the main fermenting organism, other bacteria and yeasts were present until the end of the fermentation.

Keywords: must, stuck fermentation, spontaneous fermentation, microbial succession, yeasts, bacteria, qPCR, wine making

1. Introduction

During alcoholic fermentation of grape must, sugars like glucose and fructose are converted to mainly ethanol and CO₂. An often observed phenomenon during
spontaneous fermentation, is a sluggish or even a stuck fermentation. Factors which have an influence on this situation are for example: viticultural factors, harvest conditions, pH, temperature, O₂ concentration, nutrient deficiencies (nitrogen, sugar, vitamins, minerals), glucose/fructose ratio and inhibitory substances (fungicides, killer toxins) (Malherbe et al., 2007; Gafner and Schütz, 1996; Alexandre and Charpentier, 1998; Berthels et al., 2008). Although these factors are known, the problem cannot always be solved by the application of classical measures (e.g. temperature adjustment, the addition of nutrients or novel starter cultures). In addition, wine makers of the upper quality segment avoid these procedures, since these measurements could change the characteristic sensory profiles of the individual wines. Therefore, the so far unknown causes have to be studied in more detail. Possibly, interactions between the organisms play an important role, but very little is known regarding these interactions. For a better understanding of the interactions between the different microorganisms, knowledge about the succession of the microbiota in the fermenting must is important. *Saccharomyces cerevisiae* was assigned to play a main role during the fermentation, although other organisms have been isolated from grape must (Du Toit and Lambrechts, 2002; Lopez et al., 2003; Nisioutou et al., 2007; Renouf et al., 2007; Lopandic et al., 2008). In general, non-*Saccharomyces* yeasts start the fermentation (high sugar concentration) and are substituted by *S. cerevisiae* strains when the alcohol concentration increases. Many investigations describe the presence of organisms in grape must, however most studies were restricted to one group of organisms (e.g. yeasts, lactic acid bacteria, acetic acid bacteria) and/or to one stage during the fermentations (Cocolin et al., 2000; Mills et al., 2002; Bae et al., 2006; Renouf et al., 2007). To our knowledge, no studies describe the microbial (yeasts and bacteria) succession in stucked grape must during several months. In contrast to other studies, this has been investigated
in the present study from pressing (October) to the end of the fermentation (June
resp. January). Spontaneously fermented grape must (Riesling) from a winery at the
Moselle in Germany was studied during two harvest periods, in which stuck
fermentation occurred. Total counts were measured for \textit{S. uvarum, H. uvarum} and \textit{C.
bohidinii}. In addition, some other factors (pH, glucose, fructose, acetate, ethanol) were
measured, to obtain more information about the causes of stuck fermentation.

\textbf{2. Materials and Methods}

\textbf{2.1 Sampling}

Riesling must samples were taken at a winery at the Moselle (Winningen, Germany)
during the whole fermentation period (from pressing until SO$_2$ addition at the end of
the alcoholic fermentation). This period was in the first year from October 2008 until
June 2009, in the second year from October 2009 until January 2010. Samples were
taken from one barrel at one position (from the tap at the lower part of the barrel).
The must was fermented spontaneously at 12 - 13°C. At day 80 in 2008, during the
stuck period, the “same” must from a parallel barrel was combined with our must.
Samples (45 ml) were taken every second week during fermentation and once a
month during the stuck period. After sampling, the must samples were transported to
the laboratory in a cooling bag and directly processed after arriving in the laboratory.
Samples for the glucose, fructose, acetate and ethanol measurements were frozen at
-18°C until further use.

\textbf{2.2 Isolation of bacteria and yeasts}

Bacteria and yeasts were isolated from the must by plating serial dilutions of must
samples on different nutrient media.
**Tryptic soya agar (TSA) for bacteria:** 15,0 g tryptone, 5,0 g soya peptone, 5,0 g NaCl, 0,67 g potassium sorbate, 12,0 g agar, ad 1000 ml H\(_2\)O.

**Tomato juice medium for lactic acid bacteria:** 5,0 g peptone, 5,0 g yeast extract, 20,0 g tryptone, 5,0 g glucose, 5,0 g fructose, 3,0 g citric acid, 1,0 g Tween-80, 0,5 g MgSO\(_4\) \(\cdot\) 7H\(_2\)O, 0,67 g potassium sorbate, ad 1000 ml H\(_2\)O. 333 ml centrifuged tomato juice and 16,0 g agar were added after the pH of the medium was adjusted to 6,0.

**Yeast extract peptone mannitol agar (YPM) for acetic acid bacteria:** 5,0 g yeast extract, 3,0 g peptone, 25 g mannitol, 2,0 g CaCO\(_3\), 12,0 g agar, 20 ml ethanol, ad 1000 ml H\(_2\)O

**Man-Rogosa-Sharp agar (MRS) for lactic acid bacteria:** 10,0 g peptone, 10,0 meat extract, 5,0 g yeast extract, 20,0 g glucose, 2,0 g K\(_2\)HPO\(_4\), 2,0 g diammonium hydrogene citrate, 5,0 g sodium acetate, 0,2 g MgSO\(_4\) \(\cdot\) 7H\(_2\)O, 1,0 g Tween-80, 0,05 g MnSO\(_4\) \(\cdot\) HO, 0,67 g potassium sorbate, 12,0 g agar, ad 1000 ml H\(_2\)O

**Yeast extract peptone agar (YEP) for yeasts:** 10,0 g yeast extract, 10,0 g peptone, 5,0 g NaCl, 12,0 g agar, ad 1000 ml H\(_2\)O

**Glucose-peptone-yeast extract agar (GPYA) for yeasts:** 40,0 g glucose, 5,0 g peptone, 5,0 g yeast extract, 15,0 g agar, ad 1000 ml H\(_2\)O

**Potato dextrose agar (PDA) for yeasts:** 26,5 g potato dextrose bouillon (Roth, Karlsruhe, Germany), 12,0 g agar, ad 1000 ml H\(_2\)O

**Glucose-yeast extract-peptone agar (GYP) for yeasts:** 10,0 g yeast extract, 20,0 g peptone, 20,0 g glucose, 15,0 g agar, ad 1000 ml H\(_2\)O

Tomato juice agar, YPM and TSA were supplemented with 20,0 mg/l cycloheximide after autoclaving to prevent growth of yeasts and YPM was in addition supplemented with 20 ml/l ethanol.

After incubation at 20° C, morphological different colonies were picked and transferred to fresh agar plates (triplicate). This last step was repeated several times.
to obtain pure cultures consisting of one strain. To isolate *Oenococcus oeni* strains, 1 ml must was incubated in 10 ml tomato-juice medium (without agar) and incubated at 20° C.

2.3 DNA isolation and amplification from cultured strains

DNA of bacteria was extracted from the cells with InstaGene™ Matrix (Bio-Rad Laboratories, Hercules, CA, USA) as described by Ultee et al. (2004). One colony was suspended in 100 µl InstaGene™ Matrix. After incubation for 20 min at 56 °C (under shaking), the suspension was vortexed for 10 s, incubated for 10 min at 99 °C (under shaking) and vortexed for 10 s. The samples were centrifuged (1 min, 16 100 g) and the supernatant, containing the DNA, was stored at -20 °C until further use.

The 16S rDNA of the bacteria was amplified by PCR using the universal primers Eubak5 (AGA GTT TGA TCM TGG CT) and C1392R (CCA CGG GCG GTG TGT AC). The PCR was performed in a thermocycler (Techgene; Labtech, Burkhardtsdorf, Germany). The thermal profile consisted of one cycle of 5 min at 95 °C, 1.5 min at 57 °C and 2 min at 72 °C, followed by 30 cycles of 1 min at 95 °C, 1.5 min at 57 °C and 2 min at 72 °C and a final step of 10 min at 72 °C. The PCR products were checked on an 1% agarose gel (100 V) and purified with a QIAquick PCR purification kit (Qiagen, Hilden, Germany).

The DNA of pure yeast cultures was isolated and amplified as described for the bacteria, but with the addition of 10 µl lyticase (3 U/ µl) to the InstaGene™ Matrix to perforate the yeast cell walls. For the DNA amplification ITS4 (TCC TCC GCT TAT GC) and ITS5 (GGA AGT AAA AGT CGT AAC AAG G) primers were used to amplify the ITS1-5.8S-ITS2 region of the rDNA gene. The annealing temperature during the polymerase chain reaction was 54 °C.
2.4 Restriction analysis

To retrieve information about the systematic strain assignment, a restriction fragment length analysis of the amplified rDNA was carried out. The rDNA of isolated bacterial strains was restricted (one enzyme per reaction) with BsuRI (5'-GG^CC-3') and HpaII (5'- C^CGG-3'), the yeasts´ rDNA with Hhal (5'-GC^GC-3'), HaeIII (5'-GG^CC-3') and Hinf (5'-G^ANTC-3'). 2 Ll DNA was incubated with 1 Ll 10x restriction buffer (MBI-Fermentas, St Leon-Roth, Germany), 6 Ll sterile double deionised water and 1 Ll enzyme (10 U/Ll) (MBI-Fermentas, St. Leon-Rot, Germany) for at least 5 h (BsuRI and HpaII) resp. 20 min (Hhal, HaeIII, Hinf 37 °C. The restricted DNA was separated on a 2% agarose gel (60 V) and the strains were, depending on the restriction pattern, divided into groups. The rDNA of at least one strain of every group was sequenced by Eurofins MWG Operon (Ebersberg, Germany).

2.5 Identification

After sequencing of the rDNA (16S or 80S) of the different strains, the obtained nucleotide sequences were compared with the nucleotide sequences of identified strains in the NCBI database (http://www.ncbi.nlm.nih.gov). Not all sequences could be clearly assigned to a certain species. Consequently, the rDNA from strain A115 (Lactobacillus casei/Lactobacillus rhamnosus/Lactobacillus paracasei subsp. paracasei) was also amplified with specific primers for L. casei, L. rhamnosus and L. paracasei as described by (Ward and Timmins, 1999). Strain w84.23 (Lactobacillus buchneri/Lactobacillus parakeferi) was identified by its sugar metabolism. Cells were washed with growth medium (1% peptone, 1% yeast extract) and incubated in 5 ml growth medium to which 5 ml (0,02 g/ml) of different sugars (arabinose, galactose, melezitose, raffinose, saccharose, xylose and glucose) was added. The physiological features were compared with distinguishing data in Bergey´s Manual of Systematic
The identification of the strain was verified by a SAPD-PCR as described by Pfannebecker (2003).

2.7 Quantitative determination of selected strains

Primer development

To obtain information about the total counts of *Saccharomyces uvarum*, *Hanseniaspora uvarum* and *Candida boidinii*, specific primers were developed (Table 2) as described by Rozen and Skaletsky (2000). Primer specificity was firstly checked by NCBI Blasts (http://www.ncbi.nlm.nih.gov). Unintended self complimentary and hetero dimers were checked as described by Kibbe (2007) and Owczarzy *et al* (2008). In addition, developed primers were checked for their specificity to a variety of wine yeasts and bacteria from our institute's strain collection in a PCR as described above. Finally, PCR was carried out with the total DNA isolated from must and with the DNA isolated from the single cultures previously isolated from the must. After PCR purification, a restriction digestion followed as described above. The obtained pattern was compared with the pattern of the previously isolated single cultures.

Quantitative PCR (qPCR)

After the development of specific primers, selected strains in must samples were quantified. DNA was isolated from different must samples of 2008 and 2009 with the Qiagen DNA Blood and Tissue Kit (Qiagen, Hilden, Germany). To be able to calculate cell numbers from obtained Ct-values, fresh cultures (grown in GYP-medium) were counted with a Blaubrand® (Brand GmbH + Co KG, Wertheim, Germany) counting chamber. After the preparation of serial dilutions, the DNA of these cultures was isolated with a Qiagen DNA Blood and Tissue Kit (Qiagen, Hilden, Germany) and Ct-values were measured in qPCR.
qPCR was performed in a Mastercycler® ep realplex (Eppendorf, Hamburg, Germany). Each tube contained 1 Ll template, 0.5 Ll of each primer (0.2 pM), 9 Ll RealMasterMix SYBR ROX (5 PRIME GmbH, Hamburg, Germany) and 9 Ll H$_2$O. The initial step was 2 min at 95 °C, followed by 40 cycles of 20 s at 95 °C, 20 s at 60 °C and 20 s at 68 °C. The final elongation was 15 s at 68 °C and final denaturation for 15 s at 95 °C. The fluorescence (520 nm) was measured after every cycle. The Ct value was measured at a fluorescence of 92 (a.u.). All samples were measured in duplicate. The efficiency coefficient was calculated as described by Higuchi et al. (1993).

2.6 Determination of selected must contents

Must samples (1 ml) were centrifuged (5 min, 16 100 g) and the supernatant was diluted (1 to 40 fold) with double deionised water, depending on the concentration of the substances. Glucose, fructose, acetate and ethanol concentrations were measured using a HPLC system: Shimadzu DIL-10ADVP auto injector (Shimadzu, Kyoto, Japan), Shimadzu LC-6A pump, Shimadzu SCL-6B system controller, refractive index detector 156 (Beckman, Krefeld, Germany); column heater ERC Gecko 2000 (Gynkotek HPLC, Germany); HPLC column: HPX 87H 300 x 8,8 mm (Biorad, München, Germany) connected with a precolumn of the same packing. Mobile phase: 6.5 mM H$_2$SO$_4$; oven temperature: 65°C; flow rate: 0.6 ml/ml; injection volume 5 Ll.

2.7 Chemicals

All primers were purchased from Eurofins MWG Operon (Ebersberg, Germany), Taq polymerase and nucleotides from PeqLab (Erlangen, Germany).
3. Results and Discussion

This study provides an overview of the succession of the culturable yeasts and bacteria in a fermenting grape must which stucked during the fermentation period. In general, the variety of the organisms on the grapes and consequently in the must are influenced by the region and climate, the grape variety, the disease pressure, the level of damage of the grapes and the vineyard practises (Bisson and Joseph, 2009). The fermentation period (from pressing until sulphuring) was from October until June (240 days) in 2008 and from October until January (105 days) in 2009. Fermentation stucked (no visible CO$_2$ production in the barrel) in both periods, although the length of the stuck differed considerably (day 45 to 184 in 2008; day 41 to 48 in 2009). During the fermentation many different yeasts and bacteria could be isolated successively (Table 1). The yeast strains which were isolated in 2008, belonged to the Saccharomycetales (except “Kloeckera lindneri”) and were represented by mitosporic Saccharomycetales (Candida), Saccharomycetaceae (Debaryomces and Pichia), Saccharomycodaceae (Hanseniaspora, Saccharomyces) and Metschnikowiaceae (Metschnikowia). At the beginning of the fermentation, Candida oleophila, Candida zemplinina, Pichia kluyveri, Hanseniaspora uvarum and Metschnikowia pulcherrima were isolated. The main fermenting organism, Saccharomyces uvarum, was isolated from day 24 until the end of the fermentation in June. Candida boidinii could be detected from day 80 until the end and Candida friedrichii, Debaryomyces hansenii and Pichia membranifaciens appeared at day 148 and day 240 respectively. In contrast, Candida friedrichii was isolated at the beginning of the fermentation period in 2009 and some other strains (Cryptococcus macerans, Filosbasidium floriforme, Hanseniaspora clermontiae, Pichia fluxuum,
Rhodotorula glutinis and Saccharomyces paradoxus) were only isolated in 2009. One yeast in 2008 ("Kloeckera lindneri") and two yeasts in 2009 ("Candida bituminiphila" and "Saccharomyces sp.") could not be assigned to the species level.

The fermentation started with the wild yeasts Candida, Pichia, Hanseniaspora, Metschnikowia, Cryptococcus, Filobasidium and Rhodotorula, although the last three genera were not isolated in 2008. Either they were absent or their titre was too low to detect them in the grape must by serial dilutions and subsequent plating. The Basidiomycetes of the genera Cryptococcus and Rhodotorula are weak fermenters and known to appear on the grapes during the early stage of ripening, followed by the Ascomycetes Hanseniaspora, Candida and Metschnikowia as dominant grape surface flora as the grapes ripen (Bisson and Joseph, 2009). Yeasts of these genera appearing at the beginning of the fermentation, are normally repressed by Saccharomyces strains, due to lowered sugar and higher alcohol concentration at progressive fermentation (Dittrich and Grossmann, 2011). Interestingly, not S. cerevisiae (often isolated from musts), but S. uvarum was isolated in this study. This could be explained by the relative low temperature of the wine cellar (11 – 13 °C). S. uvarum is more cryotolerant than S. cerevisiae (Englinton, 2004).

Total counts of S. uvarum and H. uvarum were studied by qPCR (Fig. 1). Since Candida boidinii was detected in 2008 by plating from day 80 (stuck period) until the end of the fermentation period, its cell counts were investigated as well. S. uvarum was responsible for the main fermentation and its cell counts increased rapidly after the beginning of the fermentation. In 2008 (Fig. 1A) H. uvarum was present at higher cell counts than S. uvarum at the beginning of the fermentation, but from day 37, the counts of S. uvarum were higher. It is interesting, that with plating, H. uvarum was
not detected after day 45, but with PCR this strain was detected until the end of the fermentation. With plating, only viable cells are quantified, with PCR death and viable cells are counted and lower cell counts can be detected. Total counts of *C. boidinii* increased during the stuck fermentation, however, these counts stayed below $2,2 \cdot 10^4$ cells/ml total counts did not change during stuck fermentation until day 115 and decreased thereafter. Total counts of *S. uvarum* were constant (appr. 2 $\cdot 10^7$ cells/ml) during the first period of the stuck (day 37 to day 80) and thereafter increased to $8,7 \cdot 10^7$ cells/ml at the end of the stuck period.

In 2009 (Fig 1B) the counts of the three tested strains decreased after day 30 and increased again after day 49. As in 2008, highest counts at the beginning were for *H. uvarum* ($1,7 \cdot 10^7$ cells/ml) and counts of *S. uvarum* were low (77 cells/ml). Again, *S. uvarum* counts increased rapidly after the fermentation started and were higher than *H. uvarum* counts from day 30. However, in 2009 the stuck period was too short to investigate changes in cell counts. The decrease of the cell counts just before the stuck fermentation could be explained by sedimentation of the cells. Ascending gas bubbles of CO$_2$ lead to a mixing of the must. When CO$_2$-production is decreasing, there is less mixture and the cells are sedimenting.

In general, cell counts of the three investigated organisms were lower in 2009 compared to 2008. This could be explained by lower sugar concentrations in 2009, which will be discussed later.

*Candida zemplinina* is able to grow in must with high sugar and high ethanol concentration and at low temperatures. However, the temperature tolerance does not make this strain as a competitor of *S. uvarum* in must fermentations, since the ethanol concentration is less inhibitory to *Saccharomyces* species than to *C. zemplinina* (Sipiczki, 2003). This was also observed in the present study, since *C. zemplinina* was not isolated anymore after 24 (2008) resp. 49 (2009) days. Although
most yeast isolates disappeared during the fermentations, some yeasts were isolated until the end of the fermentation, e.g. *C. oleophila*. This strain is known for its lytic activity, as it produces cell wall degrading enzymes like exo-ß-1,3-glucanase, chitinase and protease (Bar-Shimon et al., 2004), which could play a role during stuck fermentation. In earlier studies, *Candida* sp. showed to be able to complete the fermentation (Bisson and Joseph, 2009).

In 2008, the isolated bacterial species belonged to two main groups: Proteobacteria and Firmicutes. The Proteobacteriaceae could be assigned to three genera, namely *Gluconobacter*, *Asaia* and *Acetobacter*. The Firmicutes group was represented by Lactobacillaceae (*Lactobacillus* and *Oenococcus*) and Bacillaceae (*Bacillus*).

*Gluconobacter cerinus* could be isolated during the whole fermentation period, *Acetobacter cibinongensis* and *Gluconobacter frateurii* only at day 1, *Gluconobacter* sp. at day 37 and the other bacteria during the stuck until the end of the fermentation. In 2009 also *Lactobacillus buchneri* and *Acetobacter malorum* were isolated, however, *Gluconobacter frateurii*, *Asaia krungthepensis* and *Oenococcus oeni* were not found. Interestingly, *Acetobacter aceti* was isolated at the end (from day 148) of the fermentation period in 2008, but at the beginning of the fermentation (day 1 only) in 2009. In addition, different species of the families Bacillaceae and Paenibacillaceae were isolated in 2009. However, they were isolated very rarely (maximally twice at one sampling period) and they could not grow in must after isolation (data not shown). In both years, different acetic acid bacteria could be isolated during almost the whole fermentation period. Acetic acid bacteria are strictly aerobic, although they can survive in the absence of oxygen (Bartowsky and Henschke, 2008). They have a high tolerance to ethanol and oxidise it to acetic acid. They are often found in sugar rich media like must (Guillamón and Mas, 2009).
Growth of acetic acid bacteria has been observed in grape musts or during stuck fermentations when exposed to oxygen (Bartowsky and Henschke, 2008).

Interestingly, in both years *Gluconobacter cerinus* was isolated during the whole fermentation period. Its presence was also described in botrytized wines by Barbe et al. (2008). Lactic acid bacteria are known to be found in must and wine due to their tolerance to acidic conditions and ethanol. They can cause stuck fermentations by inhibiting *Saccharomyces* species (Huang et al., 1996). They can metabolise acids in must like tartrate, malate and citrate. Especially *Oenococcus oeni* (isolated in 2008) has a high tolerance to acid and ethanol and is therefore often used as a starter culture for the malolactic fermentation. Most of the lactic acid bacteria weakly grow or even disappear during the alcoholic fermentation (König and Fröhlich, 2009).

*Oenococcus oeni* could not be isolated in 2009. *Lactobacillus buchneri* was only detected in 2009. In our study *Lactobacillus paracasei* subsp. *paracasei* was isolated during the stuck period in 2008, in 2009 also before and after the stuck period. This strain has rarely been found in must or wine before (Dicks and Endo, 2009), but it is possible that it is responsible for the malolactic fermentation together with the other lactic acid bacteria found during this study. The relation between the presence of lactic and acetic acid bacteria in stuck fermentation has to be studied in more detail together with their ability to inhibit growth of *S. uvarum*.

The presence of *Bacillus* sp. and *Paenibacillus* sp. was surprisingly. Although Bacilli have been found in wines before (Gigi and Vaughn, 1962), no studies about wine are known which describe the presence of those *Bacillus* and *Paenibacillus* species isolated in this investigation. However, probably only spores were present, since the vegetative cells were not able to grow in the must of our study (data not shown).
The sugar concentration in the grape must (Fig. 2A and B) was considerably higher in 2008 compared to 2009 (glucose: 122 g/l (2008) and 85 g/l (2009); fructose: 119 g/l (2008) and 78 g/l (2009)). After a slow metabolism of the sugars during the first days of the must fermentation, the glucose and fructose concentration rapidly decreased from day 20 (2008) resp. day 10 (2009) of the fermentation. At a glucose concentration of 15 g/l (2008) resp. 3,2 g/l (2009) and a fructose concentration of 44 g/l (2008) resp. 27 g/l (2009), the fermentation stucked. Gafner and Schütz (1996) describe that stuck fermentations were mainly observed when approximately 80% of the sugars have been converted. In our study 86% (2008) and 81% (2009) of the sugars (glucose and fructose) were metabolised, which is in accordance with Gafner and Schütz (1996). Although the glucose concentration did not change during the stuck in 2009, it increased again at the beginning of the stuck period in 2008. This was due to a combination of two barrels. The second barrel had apparently a higher sugar concentration than our investigated barrel. Although the sugar concentration did not change during the stuck period, cell counts of \textit{C. boidinii} increased during the stuck period. This could be caused by lysis of yeast cells. Yeasts store glycogen in their cells (Pérez et al, 2002). This could leak from death cells and be converted to glucose which can be used by \textit{C. boidinii} for growth.

An important parameter to predict stuck fermentation is not only the sugar concentration itself, but also the ratio of glucose to fructose (Fig. 2C). Fermentation stuck appeared in 2008 at a glucose/fructose ratio of 0,34. As the stuck started, the ratio increased again to 0,52, due to the addition of must from another barrel and remained constant during the stuck period. Then it decreased rapidly to 0,07 after fermentation started again. In 2009, the glucose/fructose ratio was 0,12 when the
fermentation stuck. It did not change during the stuck period and then decreased to 0.08 at the end of the fermentation.

In 2008 and 2009 the metabolisation rate of glucose and fructose was the same at the beginning of the fermentation as is shown by a constant glucose/fructose ratio during the first two weeks. This can be explained by the presence of the wild yeasts, which have the same preference for glucose as for fructose (Dittrich and Grossmann, 2011). After approximately 3 weeks, glucose was metabolised much faster than fructose resulting in a rapid decrease of the glucose/fructose ratio to 0.34 (2008) resp. 0.12 (2009). As was shown in many studies, *Saccharomyces* has a higher preference for glucose as for fructose, leading to a decrease of the glucose/fructose ratio (Gafner and Schütz, 1996; Dittrich and Grossmann, 2011). During the decrease of the glucose/fructose ratio *S. uvarum* was isolated as main fermenting organism, which could explain the faster metabolism of glucose compared to fructose. Gafner and Schütz (1996) showed that they could induce stuck fermentation by decreasing the glucose/fructose ratio below 0.1. As long as the glucose/fructose ratio was above 0.5, no stuck appeared. This has also been observed in our study.

Ethanol was produced to a final concentration of 12.8 % (101.12 g/l) in 2008 and 11.7 % (92.30 g/l) in 2009. In 2008, a rapid (5 %) increase of the ethanol concentration was observed from day 24 to day 45. During the stuck period, the ethanol concentration stayed constant, followed by a rapid increase after the stuck to 12.8 % at the end of the fermentation. In 2009, the ethanol concentration was increased rapidly from day 16 to 10% at the beginning of the stuck period (day 41). During the stuck, the concentration did not change. It is interesting that the higher ethanol concentration at the beginning of the stuck (2009) was associated with a shorter stuck period.
The pH slowly increased from 3.01 at the beginning to 3.34 at the end of the fermentation in 2008 and from 3.12 to 3.25 in 2009. This indicated a net reduction of the acid concentration in the must.

Acetic acid normally occurs in wine at a concentration ranging from 0.2 to 0.6 g/l (Vilela-Moura et al., 2011). High acetate concentrations could cause stuck fermentation by inhibiting yeast growth (Alexandre and Charpentier, 1998). Acetate can be produced by yeasts (e.g. Hanseniaspora, Candida, Pichia, Saccharomyces), lactic acid bacteria and acetic acid bacteria (Dittrich and Grossman, 2011). Due to the presence of these organisms during the whole sampling period, acetate concentrations were measured (data not shown). In 2008, acetate could be measured before the stuck appeared. Its concentration varied from day 24 to the end of the fermentation between 0.11 and 0.25 g/l. In 2009, acetate was only detected after the stuck period (day 71) and varied between 0.15 and 0.33 g/l. Since acetic acid did not reach concentrations higher than 0.33 g/l, it is expected that acetic acid was not the cause of the stuck fermentation.

In this study the glucose/fructose ratio in 2008 did not change during the stuck after a constant value of 0.5 was reached. As the value was still 0.5 at the moment the fermentation started again and acetate did not reach limiting levels, other factors must play a role during the stuck period. An interaction between the organisms could be possible. Not much is known about these interactions. Killer yeasts, for example Hanseniaspora uvarum and Pichia kluvyeri, but also strains of Debaryomyces, Candida, Cryptococcus, Kluyveromyces and Metschnikowia which inhibit growth of Saccharomyces are known (Vagnoli et al., 1993; Radler et al., 1990). Bar-Shimon et al. (2004) described the biocontrol of yeasts by a lytic activity of Candida oleophila.
Pichia membranifaciens is known for its antifungal activity, probably due to the excretion of lytic enzymes such as chitinase and ß-1,3-glucanase (Cao et al., 2010). It also produces a killer toxin against Saccharomyces cerevisiae (Santos et al., 2005). In this study acetic acid bacteria were present during the whole fermentation period. Interactions between these bacteria and S. uvarum have to be studied in detail as well as the interaction between the isolated lactic acid bacteria and S. uvarum. Currently, studies are carried out which investigate interactions between S. uvarum and the isolated yeasts and bacteria. Specific primers for all isolated yeasts and bacteria are being developed. When the mechanisms of these interactions are known, it will be easier to take precautions to avoid stuck fermentations.

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Table 1: Closest relatives (NCBI database) of the isolated bacteria and yeasts in Riesling must samples in 2008 (A) and 2009 (B). *: strain was isolated after enrichment. Strain number: number which was assigned to the isolate after isolation; bp: amount of sequenced base pairs; acc. number: accession number of the closest relative in the NCBI-database, bp compared: identical basepairs to compared basepairs, % of identical basepairs to sequenced basepairs.

Table 2: Specific primers for the quantification of S. uvarum, H. uvarum and C. boidinii in must samples.

Fig. 1: Total counts of S. uvarum, H. uvarum and C. boidinii in 2008 (A) and 2009 (B). The stuck period in 2008 was from day 45 to day 184 and in 2009 from day 41 to day 48. Standard deviations are calculated from triplicate measurements.

Fig. 2: The glucose concentration(A), fructose concentration (B) and the glucose/fructose ratio (C) in Riesling must during the fermentation in 2008 (open symbols) and 2009 (closed symbols). The stuck period in 2008 was from day 45 to day 184 and in 2009 from day 41 to day 48.
Fig. 1

Graph showing the logarithm of the population density (Log N/mL) over time for different species:
- **S. uvarum**
- **H. uvarum**
- **C. boidinii**

The graphs depict two distinct time periods:
- **Stuck 2008**
- **Stuck 2009**

The x-axis represents time in days, ranging from 0 to 300 for the first graph and from 0 to 120 for the second graph.
Fig. 2.1

A

B
Fig. 2.2

The graph shows the Glu/Fru ratio over time (days) for two years, 2008 and 2009. The data points indicate a trend where the ratio decreases over time, with a notable increase in the Glu/Fru ratio in 2008 before decreasing significantly. The graph also highlights that the system appears to be 'stuck' in both years, with a significant change in 2009.
Tabelle 1.1

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