Microbial Succession in Spontaneously Fermented Grape Must Before, During and After Stuck Fermentation

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The microbial succession in spontaneously fermenting Riesling must was investigated from the beginning (pressing) until the end (sulphuring) of the fermentation in two harvest years (2008 and 2009) at a Moselle winery (Germany). In both years, the fermentation was interrupted by a stuck period. The length of the stuck period varied considerably (20 weeks in 2008 and one week in 2009). Different yeasts (*Candida, Debaryomyces, Pichia, Hanseniaspora, Saccharomyces, Metschnikowia, Cryptococcus, Filobasidium* and *Rhodotorula*) and bacteria (*Gluconobacter, Asaia, Acetobacter, Oenococcus, Lactobacillus, Bacillus* and *Paenibacillus*) were isolated successively by plating. The main fermenting organism was *Saccharomyces uvarum*. 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 concentration differed between the two years and was 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 and 0.12 respectively. The microbiota changed during the stuck period.

INTRODUCTION

During the alcoholic fermentation of grape must, sugars like glucose and fructose are converted to mainly ethanol and CO_2. An often observed phenomenon during spontaneous fermentation is a sluggish or even a stuck fermentation. Different factors have an influence on this situation, e.g. viticultural treatments, harvest conditions, pH, temperature, O_2 concentration, nutrient deficiencies (nitrogen, sugar, vitamins, minerals), glucose/fructose ratio and inhibitory substances (fungicides, killer toxins) (Gafner & Schütz, 1996; Alexandre & Charpentier, 1998; Malherbe *et al*., 2007; 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, winemakers of the upper quality segment avoid these procedures. In their opinion, 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. Interactions between the organisms possibly 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 & 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 the grape must, although 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 fermentation (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 stuck grape must over several months. In contrast to other studies, this has been investigated in the present study from pressing (October) until the end of the fermentation (June and January, respectively). Spontaneously fermented grape must (Riesling) from a Moselle winery in Germany was studied during two harvest periods in which stuck fermentation occurred. Total counts were measured for *S. uvarum*, *H. uvarum* and *C. boidinii*. In addition, some other factors (pH, glucose, fructose, acetate, ethanol) were measured to obtain more information about the circumstances under which the must became stuck.

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MATERIALS AND METHODS

Sampling

Riesling must samples were collected at a Moselle winery (Winningen, Germany) during the whole fermentation period (from pressing until SO_2 addition at the end of the alcoholic fermentation). In the first year, this period was from October 2008 until June 2009, and in the second year it was from October 2009 until January 2010. Samples were collected from one barrel at one position (from a tap at the lower part of the barrel). The must was fermented spontaneously at 12 to 13°C in a 3 000 L stainless steel tank. At day 80 in 2008, during the stuck period, the "same" must from a parallel (also stuck) barrel was combined with our must for practical reasons of the winemaker.

Samples (45 mL) for the isolation of microorganisms were taken from the barrel every two weeks during the entire fermentation period and once a month during the stuck period. After sampling, the must samples were transported to the laboratory in a cooling bag and processed directly after arriving at the laboratory. Samples for the glucose, fructose, acetate and ethanol measurements were taken every two or three days and directly frozen at -18 °C until used further.

Isolation of bacteria and yeasts

Bacteria and yeasts were isolated from the must by plating serial dilutions of must samples on different nutrient media. The media were chosen after preliminary work at the same winery. All media, except tomato juice medium, were adjusted to 1 L with H_2O.

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.

Tomato juice medium (TSM) 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 $MgSO_4 \cdot 7H_2O$, 0.67 g potassium sorbate, and 1 000 mL H_2O. Finally, 333 ml of 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, 25.0 g mannitol, 2.0 g $CaCO_3$, 12.0 g agar.

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_2HPO_4, 2.0 g diammonium hydrogen citrate, 5.0 g sodium acetate, 0.2 g $MgSO_4 \cdot 7H_2O$, 1.0 g Tween-80, 0.05 g $MnSO_4 \cdot H_2O$, 0.67 g potassium sorbate, 12.0 g agar.

Yeast extract peptone agar (YEP) for yeasts:
10.0 g yeast extract, 10.0 g peptone, 5.0 g NaCl, 12.0 g agar.

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.

Potato dextrose agar (PDA) for yeasts:
26.5 g potato dextrose bouillon (Roth, Karlsruhe, Germany), 12.0 g agar.

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.

Tomato juice agar, YPM and TSA were supplemented with 20.0 mg/L cycloheximide after autoclaving to prevent the growth of yeasts, and YPM was supplemented with 20 mL/L ethanol.

After incubation at 20°C, morphologically different colonies were picked and transferred to fresh agar plates. This last step was repeated several times to obtain pure cultures consisting of one species. At least three colonies of the same morphology per medium were picked. To isolate *Oenococcus oeni* species, 1 mL must was incubated in 10 mL tomato juice medium (without agar) and incubated at 20°C.

DNA isolation and amplification from cultured strains

Bacterial genomic DNA 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 a 1% agarose gel (100 V) and purified with a QIAquick PCR purification kit (Qiagen, Hilden, Germany).

The genomic 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 TGA 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.

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 digested (one enzyme per reaction) with *Bsu*RI (5'-GG^CC-3') and *Hpa*II (5'- C^CGG-3'), and the yeasts' rDNA and ITS DNA with *Hha*I (5'-GC^GC-3'), *Hae*III (5'-GG^CC-3') and *Hinf*I (5'-G^ANTC-3'). Two microlitres of DNA was incubated with 1 μL 10 x restriction buffer (MBI-Fermentas, St Leon-Roth, Germany), 6 μL sterile double deionised water and 1 μL enzyme (10 U/μL) (MBI-Fermentas, St. Leon-Rot, Germany) for at least 5 h (*Bsu*RI and *Hpa*II), respectively, and 20 min (*Hha*I, *Hae*III, *Hinf*I) at 37°C. The restricted DNA was separated on a 2% agarose gel (60 V) and, depending on the restriction pattern, the strains were divided into groups. The rDNA of at least one strain of every group was sequenced by Eurofins MWG Operon (Ebersberg, Germany).

Identification

After sequencing of the rDNA (16S or 5.8S and ITS region) of the different strains, the obtained nucleotide sequences were compared with the nucleotide sequences of the identified strains in the NCBI database (http://www.ncbi.nlm.nih.gov) using blastn. 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) had been added. The physiological features were compared with distinguishing data in Bergey's manual of systematic bacteriology (Hammes & Hertel, 2009). The identification of the strain was verified by a specific amplified polymorphic DNA (SAPD)-PCR as described by Pfannebecker (2003).

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 heterodimers were checked as described by Kibbe (2007) and Owczarzy *et al*. (2008). In addition, the 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 under "DNA isolation and amplification from cultured strains". Finally, PCR was carried out with the total DNA isolated from the must and with the DNA isolated from the single cultures previously isolated from the must. PCR purification was followed by a restriction digestion, as described under "Restriction analysis". The obtained pattern of the must sample was compared with the pattern of the previously isolated single cultures.

Quantitative PCR (qPCR)

After the development of specific primers, selected species in the must samples were quantified. DNA was isolated from different must samples from 2008 and 2009 with the Qiagen DNA Blood and Tissue Kit (Qiagen, Hilden, Germany). To be able to calculate cell numbers from the obtained cycle threshold value (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 the Ct values were measured in qPCR.

qPCR was performed in a Mastercycler® ep realplex (Eppendorf, Hamburg, Germany). Each tube contained 1 μL template, 0.5 μL of each primer (0.2 pmol/L), 9 μL RealMasterMix SYBR ROX (5 PRIME GmbH, Hamburg, Germany) and 9 μL H_2O. 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, with a 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).

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 an 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 mmol/L H_2SO_4; oven temperature: 65°C; flow rate: 0.6 mL/mL; injection volume: 5 μL.

Chemicals

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

RESULTS

Isolation and identification

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 became stuck (no visible CO_2 production in the barrel) in both periods, although the length of the stuck fermentation differed considerably (day 45 to 184 in 2008; day 41 to 48 in 2009).

Yeasts

During the fermentation, many different yeasts and bacteria could be isolated successively (Table 1). The yeast strains that were isolated in 2008 belonged to the Saccharomycetales and were represented by mitosporic Saccharomycetales (*Candida*), Saccharomycetaceae (*Debaryomyces*, *Pichia* and *Saccharomyces*), Saccharomycodaceae (*Hanseniaspora, "Kloeckera lindneri"*) 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 species (*Saccharomyces paradoxus,*

TABLE 1

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

A	Organism	Strain no.	Medium isolated on	bp	Acc. number	bp compared	%	Day of fermentation									
								21.10.2008	30/10/2008	13/11/2008	26/11/2008	4/12/2008	8/1/2009	12/2/2009	17/3/2009	22/4/2009	17/6/2009
								1	10	24	37	45	80	115	148	184	240
Bacteria	*Acetobacter aceti*	229.12	PDA, TSM, YEP	890	D30768	890/891	100.0								*	x	x
	Acetobacter cibinongensis	1.74	YPM	889	DQ419970	874/888	98.3	x									
	Asaia krungthepensis	184.22	PDA, TSM, TSA, YEP, YPM	952	AB292240	923/943	97.0							x	x	x	
	Uncultured *Acetobacter* sp.	184.24b	PDA, TSA, YEP	953	EU341169	916/929	96.1								x	x	
	Oenococcus oeni	Oeni	TSM[a]	-	-	-	-										x
	Bacillus subtilis	115.36	TSA	581	GQ395246	580/581	99.8							x	x		
	Gluconobacter cerinus	10.46	MRS, PDA, TSM, TSA, YEP, YPM	766	AB178405	759/763	99.0	x	x		x	x		x	x		x
	Gluconobacter frateurii	1.55	MRS, YPM	438	AB470921	436/438	99.5	x									
	Gluconobacter sp.	37.8	MRS	716	AB459531	708/715	98.9				x						
	Lactobacillus paracasei subsp. *paracasei*	A115	MRS	574	HM067019	572/572	99.7							x		x	
	uncultured bacterial clone	148.36	TSA	520	GQ086652	520/520	100.0							*	x		
Yeasts	*Candida boidinii*	80.44	MRS, PDA, TSM, TSA, YEP, YPM, GPYA	668	FJ196791	653/668	96.6						x	x	x	x	x
	Candida friedrichii	229.30	TSA, YPM	616	AB365475	612/614	99.3										x
	Candida oleophila	10.44	MRS, PDA	598	EU541359	594/595	99.3		x					x			
	Candida sp.	10.24	PDA	406	DQ104728	395/395	97.3		x								
	Candida zemplinina	10.15	PDA, GPYA	432	AY372189	427/428	98.8	x	x								
	Debaryomyces hansenii	229.53	YPM	612	EU569039	607/608	99.2										x
	Hanseniaspora uvarum	24.28	MRS, PDA, TSM, YPM, GPYA	714	FJ515178	714/714	100	x	x	x	x	x					
	Kloeckera lindneri	24.31	MRS, YPM	705	AJ512430	637/661	90.4			x		x			x		
	unidentified *Metschnikowia*	1.24	GPYA, PDA	307	AM161118	306/309	99.8	x	x								
	Pichia kluyveri	10.25	PDA	403	FM864201	396/399	98.3		x								
	Pichia membranifaciens	184.4	PDA, TSA, YEP, YPM	451	DQ198964	443/443	98.2								x	x	
	Saccharomyces uvarum	24.3	GPYA, MRS, PDA, TSM, TSA, YEP, YPM	799	EU145772	796/796	99.6			x	x	x	x	x	x	x	x

TABLE 1 (CONTINUED).

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

B	Organism	Strain no.	Medium isolated on	bp	Acc. number	bp compared	%	Day of fermentation					
								14/10/2009	27/10/2009	12/11/2009	1/12/2009	5/1/2009	26/1/2009
								1	14	30	49	84	105
Bacteria	*Acetobacter aceti*	229.12	MRS	890	D30768	890/891	100	x					
	Acetobacter cibinongensis	1.74	YEP	889	DQ419970	874/888	98.3	x					
	Acetobacter malorum	w105.16	GPYA, GYP, PDA, TSA, TSM, YEP, YPM	1254	EU096228	1251/1253	99.8					x	x
	uncultured *Acetobacter* sp.	184.24b	TSM*	953	EU341169	916/929	96.1			x			
	Bacillus horneckiae	w105.33	TSA, YEP	911	EU861362	903/911	98.9			x	x	x	x
	Bacillus patagoniensis	w105.29	TSA, TSM, YEP	849	AY258614	844/849	99.4			x			x
	Bacillus plakortidis	w105.19	TSA	541	AJ880003	541/541	100						x
	Bacillus simplex	w84.30	YPM	894	DQ659140	893/894	99.9				x	x	
	Gluconobacter cerinus	10.46	GYP, PDA, TSA, TSM, YEP, YPM	766	AB178405	759/763	99.0	x	x	x	x	x	x
	Lactobacillus buchneri	w84.23	GYP, TSM	738	AB425940	737/737	99.9		x	x	x	x	x
	Lactobacillus paracasei subsp. *paracasei*	A115	MRS, TSM, TSA, YEP, YPM	574	HM067019	572/572	99.7		x	x	x	x	x
	Paenibacillus humicus	w105.32	TSA	586	GU391497	573/583	97.8						x
	Paenibacillus odorifer	w105.20	TSA, YPM	617	AJ223990	616/617	99.8				x		x
	Paenibacillus sp.	w105.34	TSA, YPM	1181	EF690425	1169/1175	99.0			x		x	x
Yeasts	*Candida bituminiphila*	w105.66	GPYA, GYP, PDA, TSA, YEP, YPM	352	DQ911457	227/259	64.5						x
	Candida boidinii	80.44	MRS, PDA, TSA, TSM, YEP	674	FJ196791	653/669	96.9		x	x	x		
	Candida friedrichii	229.30	MRS, PDA	616	AB365475	612/614	99.3	x	x				
	Candida zemplinina	10.15	GPYA, MRS, PDA, TSA, TSM	432	AY372189	427/428	98.8	x	x	x			
	*Candida*sp.	10.24	PDA, YEP	406	DQ104728	395/395	97.3	x				x	
	Cryptococcus macerans	w1.80	YEP	595	AF444329	588/590	98.8	x					
	Debaryomyces hansenii	229.53	TSA	612	EU569039	607/608	99.2		x				
	Filobasidium floriforme	w1.66	PDA	620	FN400759	610/617	98.4	x					
	Hanseniaspora clermontiae	w1.59	MRS, TSM, YEP	166	AJ512442	165/167	99.4	x	x				
	Hanseniaspora uvarum	24.28	GPYA, MRS, PDA, TSA, TSM, YEP, YPM	714	FJ515178	714/714	100	x	x	x			

TABLE 1 (CONTINUED).

B	Organism	Strain no.	Medium isolated on	bp	Acc. number	bp compared	%	Day of fermentation					
								14/10/2009	27/10/2009	12/11/2009	1/12/2009	5/1/2009	26/1/2009
								1	14	30	49	84	105
	unidentified *Metschnikowia*	1.24	GPYA, MRS, PDA; YEP; YPM	307	AM161118	306/309	99.8	x	x	x			
	Pichia fermentans				DQ659348								
	Pichia kluyveri var. kluyveri	w14.8	MRS	383	DQ104732	328/357	85.6		x				
	Pichia fluxuum	w84.20	GYP, GPYA, PDA	444	DQ104734	431/432	97.1					x	
Yeasts	*Pichia kluyveri*	10.25	GPYA, YEP, YPM	403	FM864201	396/399	98.3	x	x				
	Pichia membranifaciens	184.4	GPYA, GYP, MRS, PDA, YPM	451	DQ198964	443/443	98.2	x	x		x	x	
	Rhodotorula glutinis	w1.74	MRS	586	AM160642	583/585	99.4	x					
	Saccharomyces paradoxus	w30.53	YEP	801	D89891	794/794	99.1			x			
	Saccharomyces uvarum	24.3	GPYA, GYP, MRS, PDA, YPM, YEP	799	EU145772	796/796	99.6		x	x	x	x	x
	Saccharomycete sp.	w105.55	GYP, PDA	547	EF121771	480/512	87.7						x

Hanseniaspora clermontiae, Pichia fluxuum and the Basidiomycetes *Crypotococcus macerans, Filobasidium floriforme* and *Rhodotorula glutinis*) were only isolated in 2009. One yeast in 2008 (strain 24.31) and three yeasts in 2009 (strains w105.66, w14.8 and w105.55) could not be assigned to the species level.

Bacteria

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 period until the end of the fermentation. In 2009, *Lactobacillus buchneri* and *Acetobacter malorum* were also isolated, but *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 only very rarely (maximally twice at one sampling period) and they could not grow in the must after isolation (data not shown).

Quantitative determination of *S. uvarum*, *H. uvarum* and *C. boidinii*

Total counts of *S. uvarum* and *H. uvarum* were followed by qPCR (Fig. 1). These two strains were chosen as they are known to be present at higher numbers during (early) fermentation (Bisson & Joseph, 2009; Dittrich & Grossmann, 2011). *S. uvarum* was the main fermenting organism in this study. Since *Candida boidinii* was detected by plating in 2008 from day 80 (stuck period) until the end of the fermentation, its cell counts were investigated as well.

In 2008 (Fig 1A), S. *uvarum* total counts increased rapidly after the beginning of the fermentation, from 5.6 x 10^2 cells/mL to 1.7 x 10^7 cells/mL at day 37. Towards the end of the fermentation, total counts were increased further to 8.7 x 10^7 cells/mL. *H. uvarum* was present at 3.6 x 10^4 cells/mL at the beginning of the fermentation, increased to 5.5 x 10^7 cells/mL at day 37, and then gradually decreased to 1.1 x 10^6 cells/mL at day 80. The final total counts at the end of the fermentation were 7.0 x 10^5 cells/mL. *C. boidinii* counts increased slowly in the first phase of the fermentation, increased to 2.1 x 10^4 cells/mL during the stuck fermentation (day 115), and decreased to the end of the fermentation. Interestingly, all counts had increased by day 115, although no visible CO_2 production was detected at the top of the vessel.

In 2009 (Fig 1B), counts of *S. uvarum* (77 cells/mL) were lower compared to 2008 at the beginning of the fermentation. As in 2008, highest counts at the beginning were for *H. uvarum* (1.7 x 10^7 cells/mL in 2009), although these counts did not increase further. Again, *S. uvarum* counts increased rapidly after the fermentation started and were 3.0 x 10^7

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

Organism	Primer	Sequence	Length	T_m (°C)	Product size	Comments
Saccharomyces uvarum	CSP2-F	ATCGAATCTTTGAACGCACATTG	23	57.1	173	modified from Hierro *et al.* (2007)
(24.3)	SCER-R	CGCAGAGAAACCTCTCTTTGGA	22	60.3		
Hanseniaspora uvarum	Hans-F2	GCACATTGCGCCCTTGAGCAT	21	61.8	173	
(37.4)	Hans-R2	ATCACAGCGAGAACAGCGTCTC	22	62.1		
Candida boidinii	80.44-1F	GTGGTGATGAACGACACTTTCG	22	60.3	135	
(80.44)	80.44-1R	AGGCAAAGCCCATAACTCCAAC	22	60.3		

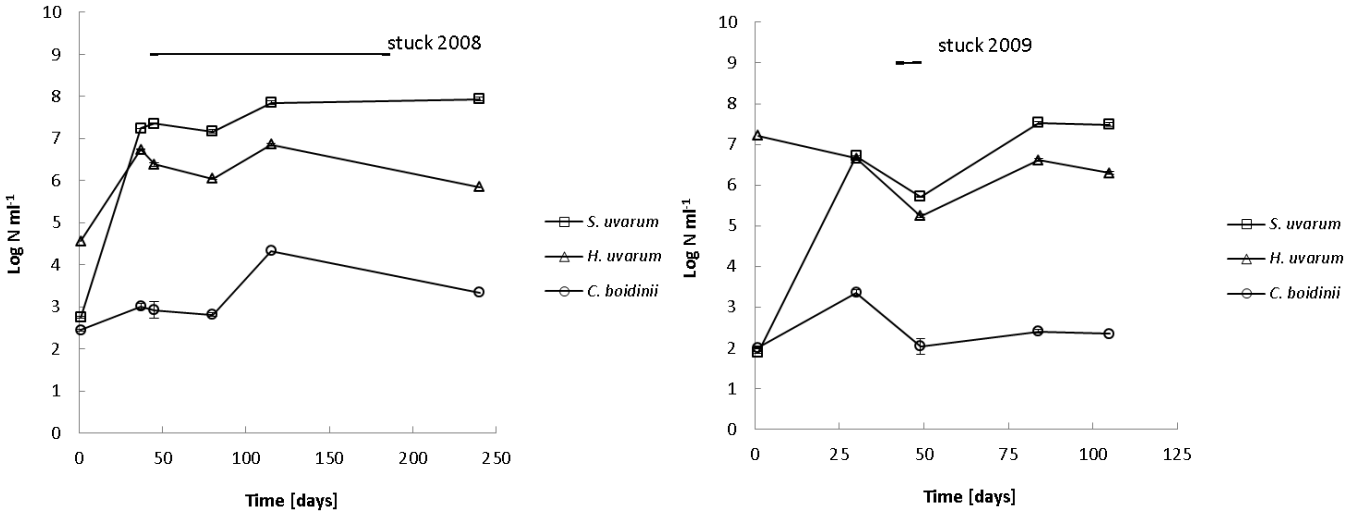

FIGURE 1
Total counts of *S. uvarum*, *H. uvarum* and *C. boidinii* in 2008 (A) and 2009 (B). The stuck period in 2008 was from day 45 to day 184, while in 2009 it was from day 41 to day 48. Standard deviations are calculated from duplicate qPCR measurements.

cells/mL at the end of the fermentation. From day 30 these counts were higher than those of *H. uvarum. C. boidinii* counts increased at the beginning of the fermentation until day 30, after which they decreased again. From day 49 these counts stayed constant at around 2.2 x 10^2 cells/mL.

Determination of selected must contents

Glucose and fructose

The sugar concentration in the grape must (Fig. 2A and 2B) was considerably higher in 2008 than in 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 fermentation, the glucose and fructose concentrations decreased rapidly from day 20 in 2008 and day 10 in 2009. At a glucose concentration of 15 g/L in 2008 and 3.2 g/L in 2009, and a fructose concentration of 44 g/L in 2008 and 27 g/L in 2009, the fermentation became stuck (day 45). By day 80 in 2008, two "identical" barrels were combined and this caused an increase in the sugar concentration.

An important factor regarding stuck fermentation is the glucose/fructose ratio. Stuck fermentation appeared at a glucose/fructose ratio of 0.34 in 2008 (Fig. 2C). The ratio increased to 0.5 after the combination of the two barrels and then remained constant during the stuck period. After the fermentation started again, it decreased rapidly to 0.07. In 2009, the glucose/fructose ratio was 0.12 when the fermentation became stuck. It did not change during the stuck period and then decreased to 0.08 at the end of the fermentation.

Ethanol

Ethanol was produced to a final concentration of 12.8% in 2008 and 11% in 2009 (data not shown). In 2008, a rapid increase in the ethanol concentration was observed from day 24 to day 45. During the stuck period, the ethanol concentration remained stable. After the restart of the fermentation, the concentration increased quickly to 12.8% at the end of the fermentation. In 2009, the ethanol concentration at the beginning of the stuck period was 10% (day 41). As was observed in 2008, the concentration did not change during the stuck period.

pH and acetate

The pH increased slowly 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. In 2008, acetate could be measured before the stuck period appeared. Its concentration changed from 0.11 to 0.25 g/L from day 24 to the end of the fermentation (data not shown). In 2009, acetate was only detected after the stuck period (day 71) and increased to 0.33 g/L at the end of the fermentation.

DISCUSSION

This study provides an overview of the succession of the culturable yeasts and bacteria in a spontaneously fermenting grape must which became stuck during the fermentation period. No special measures (e.g. the addition of starter cultures) were taken to restart the stuck fermentations at the winery concerned. The must stayed in the barrels until the fermentation was finished, which normally took months.

In general, the variety of organisms on the grapes and consequently in the must is influenced by the region and climate, the grape variety, the pressure from disease, the level of damage of the grapes and the vineyard practices (Bisson & Joseph, 2009).

Yeasts

The fermentation started with the yeasts belonging to the genera *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 for them to be detected 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 of *Hanseniaspora*, *Candida* and *Metschnikowia* as dominant grape-surface yeast microbiota as the grapes ripen (Bisson & Joseph, 2009). Yeasts of these genera appearing at the beginning of the fermentation are normally repressed by *Saccharomyces* species, due to the lower sugar and higher alcohol concentration of progressive fermentation (Dittrich & Grossmann, 2011). Interestingly, it was not *S. cerevisiae* (often isolated from musts), but *S. uvarum*, that was isolated in this study. This could be explained by the relatively low temperature of the wine cellar (11 to 13°C), since *S. uvarum* is more cryotolerant than *S. cerevisiae* (Eglinton *et al*., 2000). Earlier investigations of Riesling musts at this winery showed that *S. uvarum* and not *S. cerevisiae* was the main fermenting organism (data not shown).

S. *uvarum* was responsible for the main fermentation and its cell counts increased rapidly after the beginning of the fermentation. *H. uvarum* was present at higher cell counts (3.6 x 10^4 cells/mL) than *S. uvarum* at the beginning of the fermentation, but, from day 37, the counts of *S. uvarum* were higher. Interestingly, *H. uvarum* was not detected by plating after day 45, but this species was detected with PCR until the end of the fermentation. Only viable cells are quantified with plating, but dead and viable cells are counted with qPCR. In addition, lower cell numbers can be detected with qPCR.

At the start of the stuck period, cell counts no longer increased. The *S. uvarum* and *C. boidinii* counts stayed

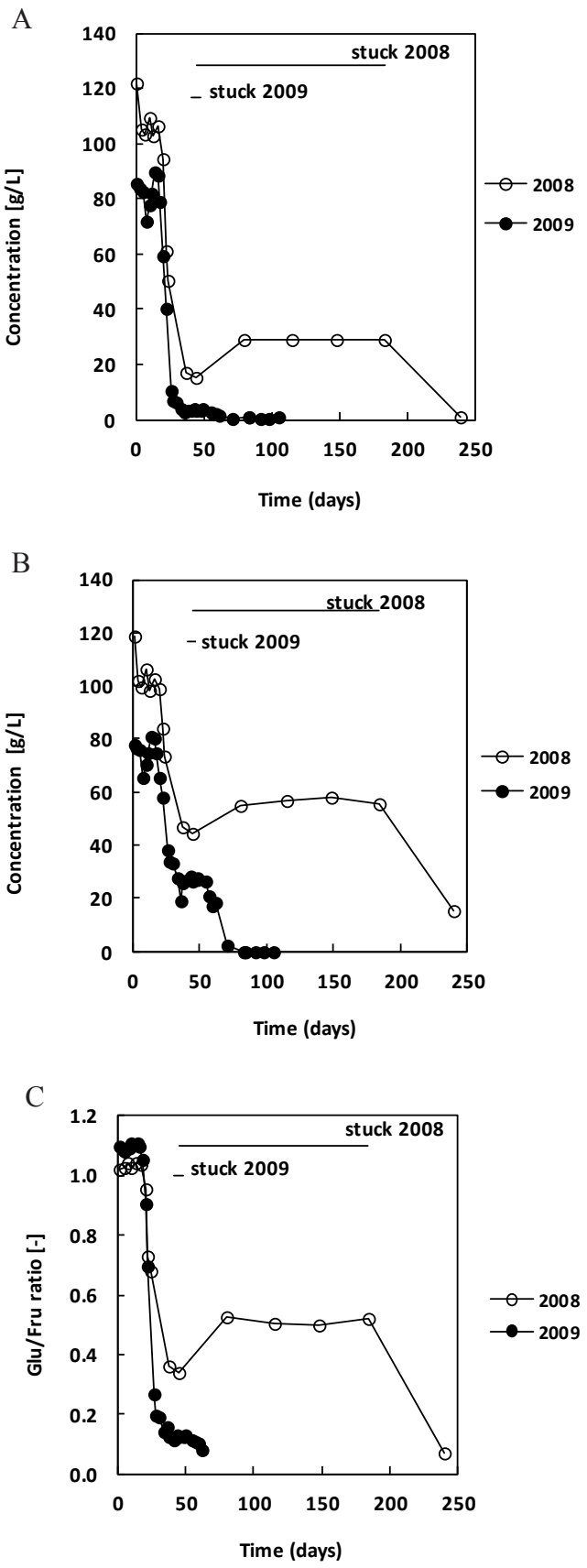

FIGURE 2

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 it was from day 41 to day 48.

more or less constant, while *H. uvarum* counts decreased. A decrease in cell counts at the beginning of 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 no CO_2 is produced, the cells slowly sediment. By day 115, all the counts increased again, although the fermentation was still stuck (no visible CO_2 production was detected at the top of the vessel).

A similar effect was observed in 2009. As in 2008, the highest counts at the beginning were for *H. uvarum*, while the counts of *S. uvarum* were low. The *S. uvarum* counts increased rapidly after the fermentation started, and were higher than *H. uvarum* counts from day 30. In general, the cell counts of the three investigated organisms were lower in 2009 compared to 2008. This could be explained by the lower sugar concentrations in 2009, which will be discussed below. The counts of the three tested species decreased after the beginning of the stuck period and increased again thereafter (day 49).

Candida zemplinina is able to grow in must with a high sugar and a high ethanol concentration and at low temperatures. However, the temperature tolerance does not make this species 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 no longer isolated after 24 and 49 days in 2008 and 2009 respectively. Although most yeasts disappeared during the fermentation, some yeasts were isolated until the end of the fermentation, e.g. *C. oleophila*. This species is known for its lytic activity, as it produces cell wall-degrading enzymes like exo-β-1,3-glucanase, chitinase and protease (Bar-Shimon *et al*., 2004), which could play a role during stuck fermentation. In earlier studies, *Candida* spp. were shown to be able to complete the fermentation (Bisson & Joseph, 2009).

Bacteria

In both years, different acetic acid bacteria could be isolated during almost the entire fermentation period. Acetic acid bacteria are strictly aerobic, although they can survive in the absence of oxygen (Bartowsky & Henschke, 2008). They have a high tolerance of ethanol and oxidise it to acetic acid. They are often found in sugar-rich media, like must (Guillamón & Mas, 2009). Growth of acetic acid bacteria has been observed in grape musts or during stuck fermentations when exposed to oxygen (Bartowsky & Henschke, 2008). Interestingly, in both years *Gluconobacter cerinus* was isolated during the whole fermentation period. Its presence was also described in botrytised wines by Barbe *et al*. (2001). Lactic acid bacteria are known to be found in must and wine due to their tolerance of acidic conditions and ethanol. They can cause stuck fermentations by inhibiting *Saccharomyces* species (Huang *et al*., 1996), and can metabolise acids like tartrate, malate and citrate in must. *Oenococcus oeni* (isolated in 2008) in particular has a high tolerance of acid and ethanol and therefore is 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 & Fröhlich, 2009). *Oenococcus oeni* was not 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, and before as well as after the stuck period in 2009. This species has rarely been found in must or wine before (Dicks & Endo, 2009), but it is possible that it is responsible for the malolactic fermentation together with the other lactic acid bacteria found in this study. The relationship between the presence of lactic and acetic acid bacteria and stuck fermentation has to be studied in more detail, together with their ability to inhibit the growth of *S. uvarum*.

The presence of *Bacillus* spp. and *Paenibacillus* spp. was surprising. Although Bacilli have been found in wines before (Gigi & Vaughn, 1962), no studies about wine are known that describe the presence of the *Bacillus* and *Paenibacillus* species that were 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).

Glucose/Fructose

The sugar (glucose and fructose) concentration was higher in 2008 than in 2009. In this study, the shortest stuck period was observed in the must with the lower sugar concentration at the beginning of the stuck period. According to Gafner & Schütz (1996), stuck fermentations are observed mainly when approximately 80% of the sugars have been converted. In our study, 86% and 81% of the sugars (glucose and fructose) were metabolised in 2008 and 2009 respectively, which is in accordance with Gafner and Schütz (1996). Although the glucose concentration did not change during the stuck period in 2009, it increased again at the beginning of the stuck period in 2008. This was due to the combination of two barrels. The second barrel apparently had a higher sugar concentration than the barrel we were investigating when they were combined. Although the sugar concentration did not change during the stuck period, cell counts of *C. boidinii* and *S. uvarum* increased during the second half of the stuck period. This could be caused by lysis of other yeast cells. Yeasts store glycogen in their cells (Pérez-Torrado *et al*., 2002). This could leak from dead cells and be converted into glucose, which can then be used by *C. boidinii* and *S. uvarum* for growth.

An important parameter to predict stuck fermentation is not only the sugar concentration itself, but also the ratio of glucose to fructose. In 2008 and 2009, the rate of metabolism of glucose and fructose was the same at the beginning of the fermentation, as is shown by the constant glucose/fructose ratio during the first two weeks. This can be explained by the presence of wild yeasts, which have the same preference for glucose and fructose (Dittrich & Grossmann, 2011). After approximately three weeks, glucose was metabolised much faster than fructose, resulting in a rapid decrease in the glucose/fructose ratio to 0.34 and 0.12 in 2008 and 2009 respectively. As has been shown in many studies, *Saccharomyces* has a higher preference for glucose than for fructose, leading to a decrease in the glucose/fructose ratio (Gafner & Schütz, 1996; Dittrich & Grossmann, 2011). During the decrease of the glucose/fructose ratio, *S. uvarum* was isolated as the 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 fermentation appeared. This was also observed in our study.

Ethanol, pH, acetate

It is interesting that the higher ethanol concentration at the beginning of the stuck formation in 2009 was associated with a shorter stuck period. However, the higher sugar concentrations in 2008 caused a higher final ethanol concentration, which was reached at the end of the fermentation.

The increase in the pH during the whole fermentation period indicated a net reduction in the acid concentration of 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 & Charpentier, 1998). Acetate can be produced by yeasts (e.g. *Hanseniaspora*, *Candida*, *Pichia* and *Saccharomyces*), lactic acid bacteria and acetic acid bacteria (Dittrich & Grossman, 2011). Due to the presence of these organisms during the whole sampling period, it was decided to measure acetate concentrations. 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 did not change during the stuck fermentation in 2008 after a constant value of 0.5 had been 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. One possibility could be an interaction between the organisms. Not much is known about these interactions. *Hanseniaspora uvarum* and *Pichia kluyveri*, but also species of *Debaryomyces*, *Candida*, *Cryptococcus*, *Kluyveromyces* and *Metschnikowia,* which inhibit the growth of *Saccharomyces*, have been described as killer yeasts (Radler *et al*., 1990; Vagnoli *et al*., 1993). Bar-Shimon *et al*. (2004) showed 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 β-1,3-glucanase (Cao *et al*., 2010). It also produces a killer toxin against *Saccharomyces cerevisiae* (Santos *et al*., 2005). Interactions between the yeasts and bacteria isolated in the present investigation and *S. uvarum* have to be studied in detail. Currently, studies are being carried out to investigate interactions between *S. uvarum* and the isolated yeasts and bacteria. Specific primers for all the 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 without using starter cultures. This is preferred by some winemakers, as they believe the final wine will acquire a more specific sensory profile when the must is fermented spontaneously.

CONCLUSION

This study provides an overview of the succession of the microbiota during the stuck fermentation of a spontaneously fermenting must, together with cell counts and glucose/fructose concentrations. Although *S. uvarum* was the main fermenting organism, other bacteria and yeasts could be isolated until the end of the fermentation. During the stuck period the microbiota changed. Other factors than the glucose or fructose concentration and the acetate concentration must play a role at the onset of the stuck period. It is expected that interactions between the different organisms could have an influence on the appearance of stuck fermentations.

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