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Vertical distribution of yeast and bacteria in stainless steel tanks during wine fermentation

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The wine fermentation process involves a succession of various microbial populations and at any stage of fermentation a variety of microorganisms are present including those which can spoil the wine. Therefore, it is very important to monitor the microbial population all through the fermentation. The sampling process must ensure obtaining a representative sample. The present work compares four different sampling locations in wine stainless steel tanks of different sizes (1.000 to 10.000 l), during both the alcoholic and the malolactic fermentation. Samples were taken in a sterile manner from the top, the middle, and the bottom of the tanks through the top hatch. Additionally, samples were taken from the sampling valve, after a short forerun. The samples were plated for viable Saccharomyces cerevisiae *yeast and the wine lactic acid bacteria* Oenococcus oeni*. ANOVA (Analysis of Variance) was carried out to determine whether it makes a difference where in the tank a sample is taken. This comparison showed that samples taken from the sampling valve are representative of the tank microflora.*
Key words: wine, yeast, bacteria, sampling, distribution

Vertikale Verteilung von Hefen und Bakterien in Edelstahltanks. *Während der verschiedenen Phasen der Gärung entwickelt sich eine Vielfalt von Mikroorganismen-Populationen, darunter auch solche, die den Wein verderben können. Daher ist es wichtig, diese Mikroorganismen ständig zu kontrollieren. Die Probennahme muss so erfolgen, dass sie repräsentativ ist. In der vorliegenden Arbeit werden vier verschiedene Probennahmeorte in Edelstahltanks unterschiedlicher Größe (1.000 bis 10.000 l) sowohl während der alkoholischen Gärung wie auch während des BSA verglichen. Proben wurden steril durch die obere Tanköffnung vom oberen, dem mittleren und dem unteren Bereich des Tanks entnommen. Zusätzlich wurden Proben nach einem kurzen Vorlauf am Probierhahn genommen. Die Proben wurden hinsichtlich lebender* Saccharomyces cerevisiae*-Hefen und der Milchsäurebakterien* Oenococcus oeni *untersucht. ANOVA (Varianzanalyse) wurde durchgeführt um festzustellen, ob es Unterschiede hinsichtlich der verschiedenen Probennahmeorte gibt. Diese vergleichende Untersuchung zeigte, dass Proben vom Probierhahn repräsentativ für die Mikroflora im Tank sind.*
Schlagwörter: Wein, Hefe, Bakterien, Probennahme, Verteilung

La répartition verticale des lies et des bactéries dans les cuves en inox. *Une multitude de populations de microorganismes, dont ceux susceptibles d'altérer le vin, se développe au cours des différentes phases de la fermentation. Il est donc important de contrôler en permanence ces microorganismes. Le prélèvement doit s'effectuer de la sorte qu'il soit représentatif. Dans le travail présent, quatre différents lieux de prélèvement dans des cuves en inox de taille différente (de 1.000 à 10.000 l), tant au cours de la fermentation alcoolique qu'au cours de la fermentation malolactique, ont été comparés. Les échantillons sont prélevés de manière stérile, à travers l'orifice de la cuve, des parties supérieure, centrale et inférieure de la cuve. En outre, des échantillons ont été prélevés au dégustateur après un court écoulement. Les échantillons ont été examinés en vue de détecter la présence des lies* Saccharomyces cerevisiae *vivants et des bactéries lactiques* Oenococcus oeni*. Une analyse de variance (ANOVA) a été effectuée en vue de déterminer s'il existe des différences entre les lieux de prélèvement des échantillons. Cet examen comparatif a montré*

que les échantillons prélevés au dégustateur sont représentatifs de la microflore dans la cuve.
Mots clés : vin, lies, bactéries, prélèvement d'échantillons, répartition

During the process of winemaking, microorganisms are involved at every step (Henick-Kling, 1995). The yeast *Saccharomyces* is essential for the alcoholic fermentation. In addition to transforming sugars into ethanol, the choice of the *Saccharomyces* strain also has a large influence on the taste, flavour and mouthfeel of the final product (Egli et al., 1998; Henick-Kling et al., 1998). After the alcoholic fermentation, red wines and some white wines undergo malolactic fermentation (MLF), where lactic acid bacteria transform malic acid into lactic acid, modify the wine aroma, and texture (Henick-Kling, 1995; Krieger et al., 1993; Henick-Kling and Acree, 1998; Laurent et al., 1994). The preferred lactic acid bacteria for MLF are *Oenococcus oeni*. Here, as in alcoholic fermentations, the strain that carries out the MLF also has a distinct flavour impact. Unfortunately, undesired yeast and bacteria species can grow on the surface of grapes as well as the desired microorganisms, and are carried into the winery with the grape juice. Some of them can add interesting characteristics to the wine, but their flavour contribution is very unpredictable because very little is known about the diversity and physiology of these microorganisms. Most of the time, non-*Saccharomyces* yeasts, acetic acid bacteria, and undesired lactic acid bacteria other than *O. oeni* damage the wine and can cause large financial losses to the wine producer.

Hanseniaspora uvarum (H. uvarum) is the most common yeast in grape must, comprising 50 to 90% of the total yeast population (Gafner et al., 1996). The spoilage yeast *H. uvarum*, called *Kloeckera apiculata* in its asexual form, is capable of fully fermenting the sugar in grape must, but can produce up to 2 g/l of acetic acid during the process (Gil et al., 1996; Romano et al., 1992). Acetic acid undergoes esterification in the presence of alcohol, resulting in esters which can contribute agreeable flavours. Yet if the esterification includes the formation of ethyl acetate which attributes a nail polish remover odour to the wine, a wine can become un-saleable. With the current knowledge about *Hanseniaspora* yeasts, their flavour contribution can neither be controlled nor predicted. Therefore to avoid the risk of damage and even complete loss due to spoilage by *H. uvarum*, winemaking practice has to suppress the growth of *H. uvarum* in grape must and in wine during fermentation.

The yeast *Pichia anomala* can occur in the vineyard even on the surface of healthy berries, in grape musts, during alcoholic and malolactic fermentations and later, during maturation of the wines. Although not a very strong fermenter, *P. anomala* survives and can form up to 500 mg/l ethyl acetate. If *P. anomala* has some oxygen at its disposal, it can grow on the surface of the wine and produce large amounts of acetate, acetate esters, and oxidized flavours (Schnürer, 2003). It is a general food spoiling yeast.

Three different genera of lactic acid bacteria occur on the grapes and in the must (Henick-Kling, 1995). These bacteria are able to perform a malolactic fermentation (MLF), transforming malic acid into lactic acid. To achieve desired aromas and texture, bacteria of *O. oeni* must outgrow the undesired *Pediococcus damnosus, Pediococcus parvulus, Pediococcus pentosaceus, Lactobacillus brevis* and *Lactobacillus hilgardii* which can produce unwanted compounds from the metabolism of sugars that remain in the wine. *L. brevis* strains can produce up to 5 g/l of acetic acid. *P. damnosus* and *L. brevis* can produce large amounts of lactic acid from residual sugar. Strains of *L. hilgardii* and *L. brevis* can produce the unpleasant 'mousy' flavour (Heresztyn, 1986), and *P. damnosus* can produce high concentrations of diacetyl and biogenic amines, as well as ropiness; the wine gets sticky (Lonvaud-Funel, 1999).

To avoid spoilage from the undesirable yeast and bacteria grape must and wine must be monitored closely, by microbial examination, chemical measurements, and sensory checks. Particularly slow or sluggish fermentations have to be monitored carefully, because stuck fermentations can be caused by spoilage yeasts and some lactic acid bacteria and are very vulnerable to microbial spoilage and chemical oxidation (Bisson, 1999; Phowchinda et al., 1995). Wines with residual sugar are particularly vulnerable to undesired microorganisms. Wines should be checked regularly for progress of fermentation, formation of off-odours, and possible growth of spoilage microorganisms. Early detection of potential spoilage microorganisms allow the winemaker to intervene, remove or inactivate the unwanted microorganisms and avoid the formation of off-odours and spoilage of the wine. Simple plating and microscopic examination can be carried out in many winery laboratories, yet rapid detection and identification of small numbers

of spoilage microorganisms are typically carried out in specialized service laboratories.
Recently several molecular biological methods have been explored for the detection and quantification of wine microorganisms (Bleve et al., 2003; Bujdoso et al., 2001a und b; Egli and Henick-Kling, 2001; Esteve-Zarzoso et al., 1999; Guillamón et al., 1998; Mitrakul et al., 1999; Phister and Mills, 2003; Schütz and Gafner, 1993; Schütz and Gafner, 1994). With the methods available so far wine and must samples might receive a preliminary microscopic check, then the samples are cultured on various nutrient media plates and the culturable yeast and bacteria can be typed with molecular methods.
One part that has been neglected so far is the impact of the sampling method. It is unclear whether it makes a difference where in a tank a sample was taken. Many fermentation tanks are very large and reach several meters in height. It is not practical to stir the wine in the fermentation tanks before a sample is taken. The question that was asked in this project was whether there is a difference in the density of yeasts or bacteria according to the vertical location in a fermentation tank, during alcoholic fermentation through MLF. It is very important for a winemaker to know where in the fermentation tank a sample has to be taken for it to be representative. Before going through the time and expense of testing a sample for microorganisms, a winemaker has to be confident that the sample was representative of the situation of the fermentation.

Materials and Methods

Fermentation assays and microbial strains

Three fermentations in two different wineries were examined from the beginning of the alcoholic fermentation to the end of the malolactic fermentation. All three musts were from 'Müller-Thurgau' grapes (MT1 to MT3).
Two different barrel sizes were investigated. In one winery, 1.700 litre of must were fermented with the *S. cerevisiae* yeast Lalvin W15 (Lallemand, Montreal, Canada) in a 2.000 litre stainless steel tank (trial MT1). After the alcoholic fermentation, 1.000 litre of wine were transferred into another steel barrel and inoculated for malolactic fermentation with *O. oeni* EQ 54 (Lallemand, Montreal, Canada). In another winery, two fermentations were followed: 9.000 (MT2) and 8.000 litre (MT3) must, respectively, were fermented in 10.000 litre stainless steel tanks. Both musts were inoculated with the *S. cerevisiae* yeast Lalvin W27 (Lallemand, Montreal, Canada). The malolactic fermentation was done in 6.000 litre stainless steel tanks both for MT2 and MT3. MT3 did a spontaneous MLF. MT2 did not start malolactic fermentation spontaneously, and was inoculated with *O. oeni* by replacing 10% with another wine which was undergoing malolactic fermentation.

Sampling

Samples were taken, as aseptically as possible, in the top part, the middle, and the lowest part of the tank. Additionally, a sample was taken from the sampling valve after a short forerun. A device was designed for the sterile sampling. A flexible tube was weighted with a stainless steel rod and cut at the appropriate length. The assembly was washed and autoclaved between each sampling. The samples were taken from the hatch at the top of the fermentation tanks with a sterile 50 ml syringe. During alcoholic fermentation, and through MLF, samples were taken regularly (one or more days interval).

Measurements

Each sample was diluted according to an estimated cell number derived from observations under microscopy. The appropriate dilution was spread onto Phytone Yeast Agar plates (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for the yeast counts and on Leuconostoc M5 agar (Scharlau, Barcelona, Spain) for the bacteria. They were incubated, at 25 °C for the yeasts and 30 °C for the bacteria, until colonies of the appropriate size had grown (two to ten days). The viable cell counts (cfu/ml) were determined for both yeast and bacteria. Usually six plates were counted for every sample, three each at two different dilutions, with 2 to 400 colonies each. The wine MT2 could not be tested for *O. oeni* because it had finished the malolactic fermentation too quickly. For each sampling series, the musts or wines were analyzed by HPLC for glucose, fructose, ethanol, malic acid and lactic acid as parameters for the progress of the alcoholic and malolactic fermentation. The samples from the middle part of the fermentation tanks were chosen for the chemical analyses.

Analysis and statistics

The numbers of viable cells obtained from the separate locations of sampling from the tanks were shown in relation to the date of sampling (growth curves). Each growth curve was then compared to the other growth

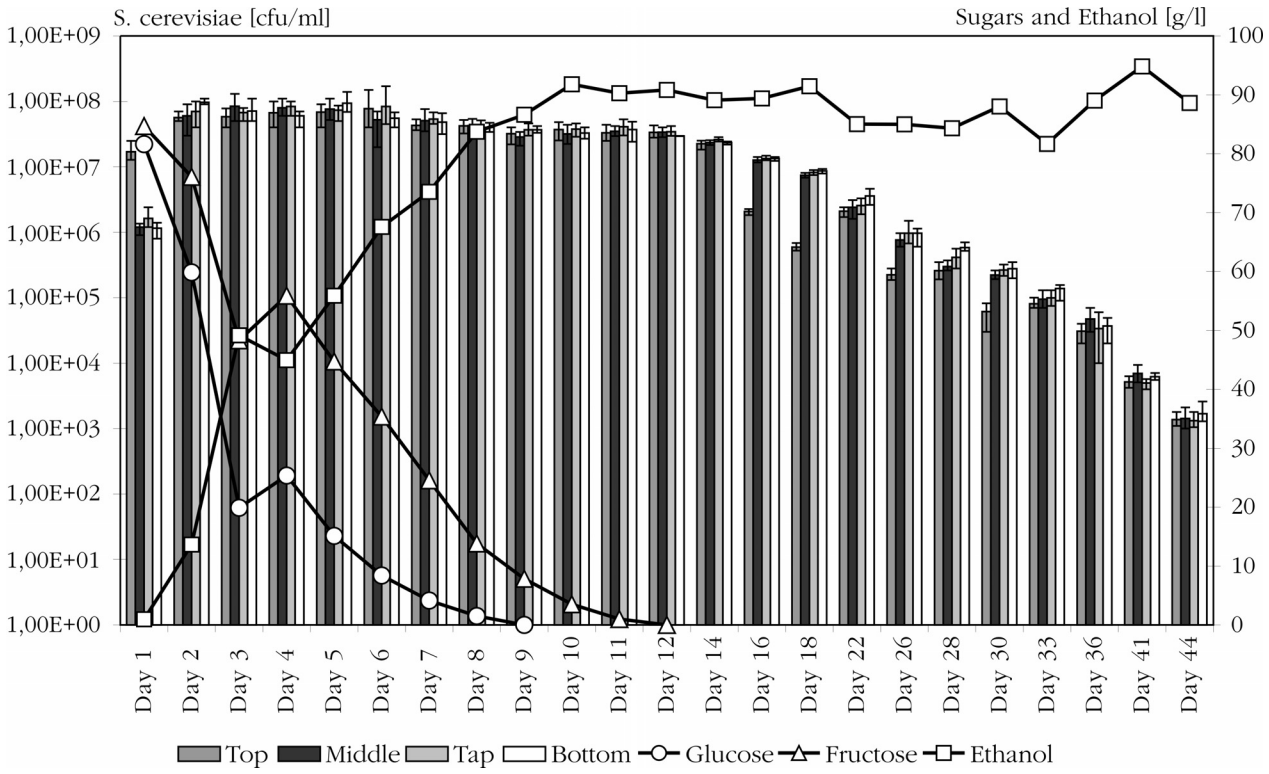

Figure 1: Growth of the yeast from dataset MT1 (2'000 l steel tank): cfu/ml for *S. cerevisiae* (bars), at four locations in the tank (top, middle, tap and bottom). Error bars indicate the minimal and maximal values of cfu. Amount of glucose, fructose and ethanol (lines) to indicate the progress of the alcoholic fermentation. Tap: sampling valve

curves of the same fermentation to show whether there are differences in their progression. The trends of these location curves were analyzed (IHAKA and GENTLEMAN, 1996). Differences of trends were assessed by the interaction of measurements with datasets (e.g. MT1 yeasts, MT1 bacteria) and measurements with time, resulting in P-values. For each measurement date, an analysis of variance was done with Microsoft Excel over the four tank locations. When the P-value was significant, another analysis of variance was added, using the three locations that are closest to the mean of the values.

Results

For each fermenter, the number of viable cells per ml is shown in a bar diagram. The four bars at each date represent the different places where the samples were taken: At the top, in the middle, at the bottom, and from the sampling valve (tap). The minimum and maximum values that were counted are indicated as error bars to show the range. Figure 1 shows the concentrations of *S. cerevisiae* in the smaller fermentation tank (2.000 l) in cfu/ml, which corresponds to the fermentation MT1 (yeast). The measured concentrations of glucose, fructose, and ethanol are shown to mark the stage of the alcoholic fermentation. Figure 2 (MT2, yeast) and Figure 3 (MT3, yeast) show the cfu/ml of *S. cerevisiae* for the two larger fermentation tanks (10.000 l), with the same compounds as in Figure 1 as guidelines for the fermentation. In Figure 4, the fermentation MT1 (bacteria) is monitored concerning *O. oeni*. To follow the progress of the malolactic fermentation we show the concentration of malic acid and lactic acid. Figure 5 similarly shows the cfu of *O. oeni* in the fermentation MT2 (bacteria).

The experiment comprised five datasets (Figures 1 to 5), observations at different times, and four measurements (top, middle, and bottom, and sampling valve). Samples were taken with replicate at each sampling time. Graphic displays of the mean measurements, shown with minimal and maximal values, show essentially similar trends for the datasets. Differences in trends were asses-

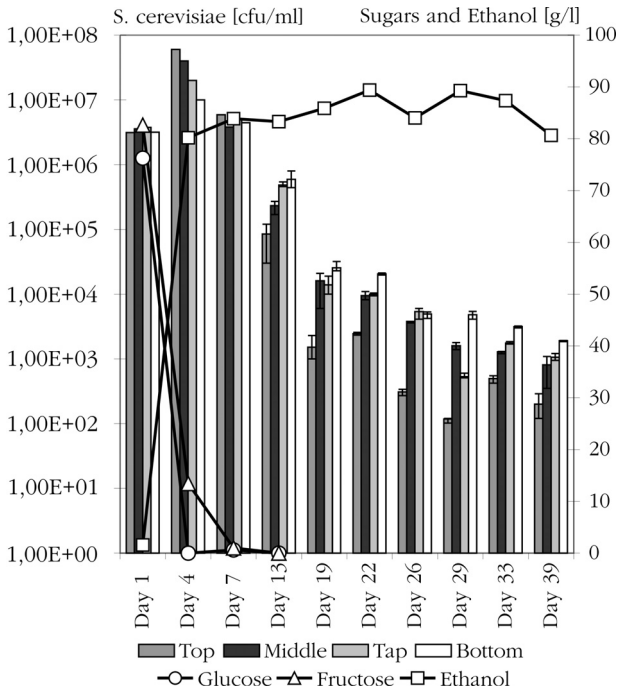

Figure 2: Growth of the yeast from dataset MT2 (10'000 l steel tank): cfu/ml for *S. cerevisiae* (bars), at four locations in the tank (top, middle, tap and bottom). Error bars indicate the minimal and maximal values of cfu. Amount of glucose, fructose and ethanol (lines) to indicate the progress of the alcoholic fermentation. Tap: sampling valve

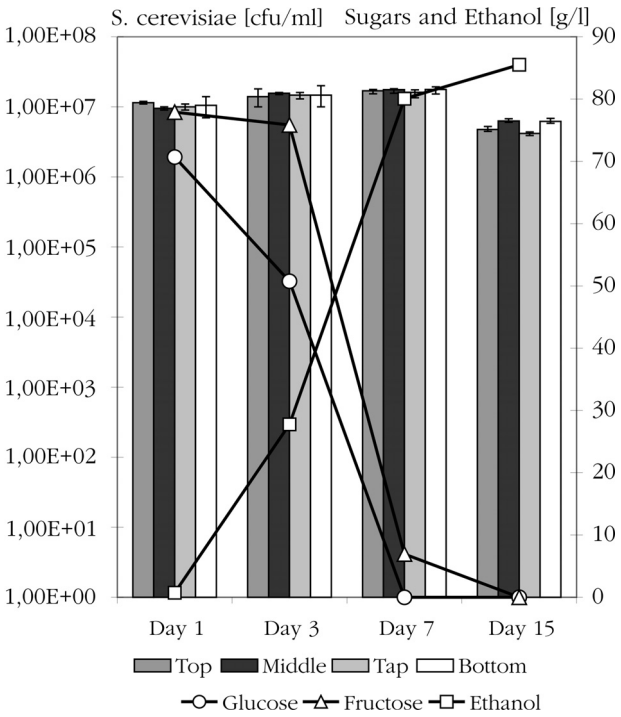

Figure 3: Growth of the yeast from dataset MT3 (10'000 l steel tank): cfu/ml for *S. cerevisiae* (bars), at four locations in the tank (top, middle, tap and bottom). Error bars indicate the minimal and maximal values of cfu. Amount of glucose, fructose and ethanol (lines) to indicate the progress of the alcoholic fermentation. Tap: sampling valve

sed using analysis of variance (ANOVA). Differences of the trends can be determined by the interactions of measurements with datasets and measurements with time. These interactions were not significant: $P > 0.9$ and $P = 0.5$, respectively, over all five datasets.

For each of the five resulting curves (three for the yeasts and two for the bacteria, see datasets), the measurements 'top, middle, bottom, and sampling valve' were compared and the P-values calculated. Additionally, P-values for the interaction between the viable cell number and time were calculated, to determine whether the curves show a trend that is different for each different location (Table 1). For each date, the P-value was calculated to determine whether there was a difference between the locations of sampling. When the differences were significant, ANOVA was performed for the three values which were closest to the overall mean (Table 2). P-values that are significant, meaning $\alpha; \leqslant 5\%$ are printed in bold. Additionally, the ratio of the mean of the values top, middle, and bottom to the value of the sampling valve is shown.

Table 1: P-values for separate fermentations assess the trends for the growth curves at different locations within datasets

Dataset	Micro-organism	P-value[1]
MT1	Yeast	0.9
MT1	Bacteria	0.11
MT2	Yeast	0.31
MT2	Bacteria	0.84
MT3	Yeast	0.77

[1] P–value ≤ 0.05 corresponds to a significant value

Discussion

The results of the experiments clearly show that both bacteria and yeast cell density are very similar in the different parts of the fermentation tanks during the alcoholic and malolactic fermentations. The results for the bacteria are very straightforward. Only four measur-

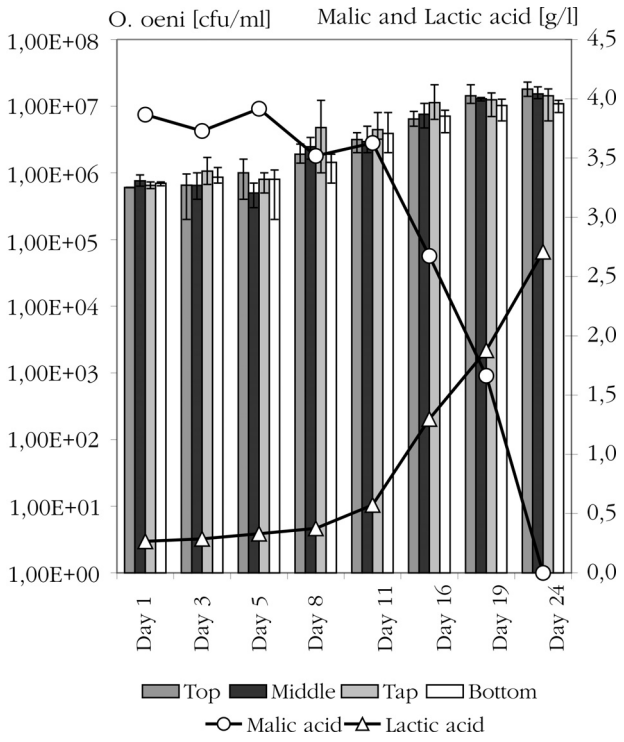

Figure 4: Growth of the bacteria from dataset MT1 (1'000 l steel tank): cfu/ml for *O. oeni* (bars), at four locations in the tank (top, middle, tap and bottom). Error bars indicate the minimal and maximal values of cfu. Amount of malic acid and lactic acid (lines) to indicate the progress of the MLF. Tap: sampling valve

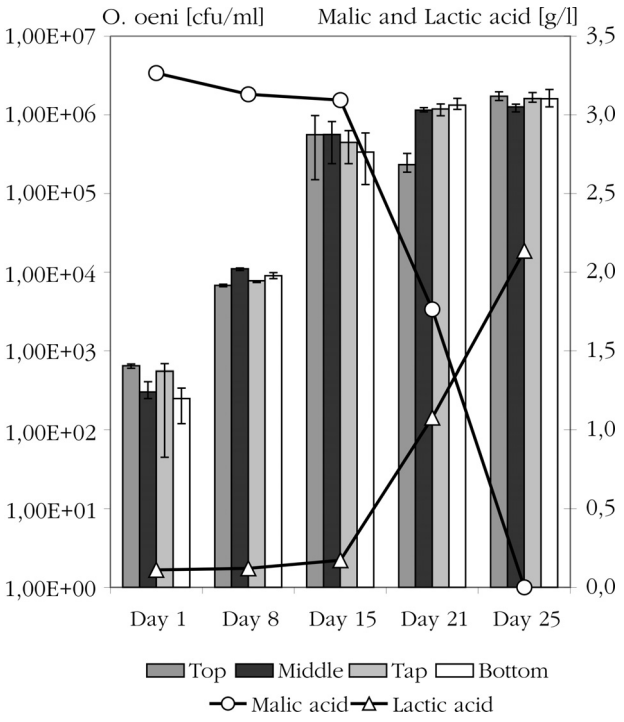

Figure 5: Growth of the bacteria from dataset MT2 (6'000 l steel tank): cfu/ml for *O. oeni* (bars), at four locations in the tank (top, middle, tap and bottom). Error bars indicate the minimal and maximal values of cfu. Amount of malic acid and lactic acid (lines) to indicate the progress of the MLF. Tap: sampling valve

ements show significant differences. Although these differences are mathematically significant, they do not impact the validity of the statement that samples can be taken from the sampling valve to obtain a representative sample of the bacteria. The ratio 'mean of three measurements top, middle and bottom' divided by 'tap' (tap: sampling valve) lies between 68% and 115%. If the cells were perfectly uniform, this ratio would be 100%. *S. cerevisiae* showed differences, particularly between the top measurements and the others. In the fermentation MT1, the measurement from the top part of the tank is markedly higher than that of the others on the first day. The reason for that is that the yeast culture was inoculated from the top hatch, and took a day to distribute evenly throughout the tank. The alcoholic fermentation took 11 days to finish, and 13 days later, the wine was transferred into the smaller stainless steel tank for the MLF. During that time, the yeasts began to settle. They started to settle again after inoculation with the bacteria, because the MLF did not start immediately. In both cases, the measurements from the top part of the stainless steel tanks dropped compared to the other locations. MT2 and MT3 were fermented in much larger stainless steel tanks, reaching six meters in height. For MT2, the measurements from the top part of the tank started dropping as soon as the alcoholic fermentation was finished. Even leaving the top part of the tank out of the equation, the measurements middle, tap and bottom were still significantly different from each other. But again, the ratio 'mean of three measurements top, middle and bottom' divided by 'tap' stays within reasonable limits, at 56% to 183%. The ratio was higher in only two cases, up to 409%. MT3 started MLF quickly, and so the yeast did not have time to settle before its end, and no data is available for the bacteria.

Samples taken from the sampling valve of stainless steel fermentation tanks, after a short forerun, are representative for the population of microorganisms that are active in musts and wines during alcoholic and malolactic

Table 2: Statistical analysis for all fermentations, F- and P-values for each sampling date (ANOVA)

Dataset	Ferm. Day	P-Value[1]				Mean/Tap[2]
		Overall	Without Top	Without Middle	Without Tap	
MT1	1	**5.40E-10**	**0.024**			*3.96*
Yeast	2	0.207				1.02
	3	0.515				1.06
	4	0.624				0.83
	5	0.237				1.08
	6	0.247				0.74
	7	0.418				0.88
	8	0.866				1.05
	9	**0.047**	**0.018**	0.296		*0.88*
	10	0.551				0.9
	11	0.417				0.85
	12	0.531				0.94
	14	0.327				0.88
	16	**1.80E-06**	0.691			*0.70*
	18	**5.30E-07**	0.152			*0.68*
	22	0.388				1.05
	26	0.067				0.67
	28	**0.01**	0.209			*0.92*
	30	0.054				0.71
	33	**0.015**	0.478			*1.06*
	36	0.695				1.13
	41	0.651				1.23
	44	0.722				1.13
MT2	1	0.687				0.88
Yeast	4	n. d.				1.83
	7	0.391				0.92
	13	**8.00E-05**	**0.014**			*0.63*
	19	**0.003**	0.088			1.03
	22	**1.60E-11**	**1.30E-05**			*1.09*
	26	**2.80E-06**	0.105			0.56
	29	**4.10E-12**	**7.00E-08**			*4.09*
	33	**1.10E-10**	**6.70E-07**			*0.93*
	39	**7.20E-10**	**6.80E-06**		**2.00E-05**	*0.91*
MT3	1	0.337				1.05
Yeast	3	0.393				1.01
	7	0.707				1.08
	15	**0.001**			**0.012**	*1.38*
MT1	1	0.386				1.06
Bacteria	3	**0.038**			**0.012**	*0.68*
	5	0.584				0.96
	8	0.629				0.41
	11	0.34				0.75
	16	0.116				0.62
	19	0.332				1.00
	24	0.123				1.03
MT2	1	**0.003**	**0.031**			*0.72*
Bacteria	8	**0.005**	0.522			*1.15*
	15	0.299				1.09
	21	**2.00E-04**		0.144		*0.76*
	25	0.771				0.95

[1] *P*-values for all four sampling locations (overall), and for the three values closest to the mean of all values (without top, without middle, without tap). Significant values ($P \leq 0.05$) are shown in **bold**.
[2] Ratio of mean cfu of the measurements (top, middle, bottom) and tap. Ratios of data points with significant P-values are in *italic*. A ratio of 1 means that the cfu/ml of the micro-organism is identical in the sample from the tap and in the mean of the other locations. A smaller ratio denotes a higher concentration and, and a ration larger than 1 a lower concentration in the tap.
Tap: sampling valve

fermentation. Even if there are occasionally moulds that grow on samples taken from the sampling valve, this does not interfere with the quantifying process, because they cannot be mistaken for either yeasts or bacteria. This allows taking only one sample from a fermentation tank rather than sampling top, middle and bottom to assess the microbial population. As can be seen in Figure 1, sampling from the top of a tank can only be representative during active alcoholic and malolactic fermentation when the CO_2 evolution ensures good mixing.

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