

Enumeration and Identification of 4-Ethylphenol Producing Yeasts Recovered from the Wood of Wine Ageing Barriques after Different Sanitation Treatments

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Abstract

Aims: This work was aimed at the evaluation of several sanitation procedures on the reduction of total microbial flora and of *D. bruxellensis* recovered from the inner layers of the barrique's wood.

Methods: A group of used oak barrels tainted by 4-ethylphenol and contaminated with *D. bruxellensis* were differently sanitized and, afterwards, were dismantled to analyse samples of shaves taken from wood surfaces at different depths. Microbial counts were obtained by the Most Probable Number Technique using broths of general purpose medium and of *Dekkera/Brettanomyces* differential medium (DBDM).

Results: The least inefficient treatment included barrique steaming at low pressure. Uncontaminated samples were only detected under this treatment and in the upper level (0-2 mm) of the staves. With this treatment complete destruction of the contaminating flora was not achieved in any level of stave side surfaces and in grooves. The presence of *D. bruxellensis* was detected in depths up to 6-8 mm in the wood corresponding to the maximum level of wine penetration.

Significance: this work demonstrated that even after current sanitation procedures barriques used in wine maturation pose a severe risk to wine stability due to the presence of *D. bruxellensis*.

Keywords: wooden barrels, disinfection, volatile phenols, *Dekkera bruxellensis*, *Pichia guilliermondii*

1. Introduction

The utilisation of wood barrels for wine ageing is widespread and is aimed at the improvement of wine quality. However, it also increases the risk of microbial spoilage because of the difficulty to sanitise properly the wood or to keep adequate levels of sulphur dioxide in wines. Wooden barrels are particularly known as a preferential ecological niche for yeasts of the genera *Dekkera/Brettanomyces* which are the agents of phenolic-odour taints described as "band-aid", "stable" and "horse sweat", due to the production of 4-ethylphenol and 4-ethylguaiacol (Chatonnet et al., 1995, 1997). Therefore, utmost care should be taken in barrel cleaning and disinfection. Chemical agents, mainly those with chlorine, are not suitable for wood treatment because of off-flavour release and so most common empirical sanitation procedures include hot water or steam utilisation followed, or not, by addition of sulphur dioxide to the barrels prior to wine filling. Even with thorough sanitation barrels always remain as a critical point in microbiological control (Guzzon et al., 2011). Recent reports have described the use of other sanitising agents like ozone (Marko et al., 2005), high-power ultrasonics (Schmid et al., 2011, Porter et al., 2011) and microwaves (González-Arenzana et al., 2013). However, these new strategies require the investment in specific equipments. Therefore, our work was directed to compare the effectiveness of sanitation

procedures commonly used in wineries on the total microbial flora and on the yeasts able to produce high levels of 4-ethylphenol in the inner wood layers of ageing barrels.

2. Material and Methods

2.1 Barrel Treatments and Sample Collection

French oak barrels (250 l), with 4 year use, were selected to this study because of the detection of red wine with high levels of 4-ethylphenol and contaminated with *Dekkera bruxellensis*. Wine was from the 1996 vintage and analysed in 1999 giving 0.9949 g ml⁻¹ volumic mass, 12.55% (v/v) ethanol, 16 mg l⁻¹ free SO₂, 60 mg l⁻¹ total SO₂, 5.29 g l⁻¹ total acidity in tartaric acid, 0.72 g l⁻¹ volatile acidity in acetic acid, 2.8 g l⁻¹ reducing sugars and pH 3.86. Average 4-ethylphenol level was 1.67 mg l⁻¹ and *D. bruxellensis* counts ranged from 0.4 to 9.5x10³ CFU ml⁻¹, determined previously according to Rodrigues et al. (2001). After emptying, each barrel was subjected to commonly used cellar practices: (A) blank, without treatment; (B) pre-rinsing with cold water, rinsing with hot water (70°C) for 3 times and air-drying; (C) pre-rinsing with cold water, rinsing with hot water (70°C), filling with sulphur dioxide solution (200 mg l⁻¹) acidified at pH 3.0 and storage for one month; (D) pre-rinsing with cold water, filling with hot water (90°C) up to ¾ of the volume, for 15 min; (E) pre-rinsing with cold water, rinsing with hot water (70°C), steaming under pressure (0.5 kgf cm⁻²) for 10 min. Afterwards barrels were dismantled and from each one were selected different sampling points (Table 1). From each point were taken shaves with 2 mm thickness until the layer where no wine was observed, by means of a flame sterile chisel. The sampling areas were 50 cm² for the internal surface of the staves, 15 cm² for the side surface of the staves, 7.5 cm² for the external surface of the stave groove and 20 cm² in the river reed stalks. The staves were kept in sterile plastic bags until used.

Table 1. Sampling points in the wooden barrels

Reference	Sampling points	Sampling depth
I	Internal surface of the stave located in opposite side of bung hole	0-2, 2-4, 4-6, 6-8 mm
II	Side surface of the stave located in opposite side of bung hole	0-2, 2-4 mm
III	Groove of the stave located in the opposite side of the bung hole	External, internal
IV	Internal surface of the stave of the bung hole	0-2, 2-4, 4-6, 6-8 mm
V	Side surface of the stave of the bung hole	0-2, 2-4 mm
VI	Groove of the stave of the bung hole	External, internal
VII	Internal surface of the bung hole	0-2, 2-4, 4-6 mm
VIII	Internal surface of the stave adjacent to the bung hole (I)	0-2, 2-4, 4-6, 6-8 mm
IX	Side surface of the stave adjacent to the bung hole (I)	0-2, 2-4 mm
X	Groove of the stave adjacent to the bung hole (I)	External, internal
XI	Internal surface of the stave adjacent to the bung hole (II)	0-2, 2-4, 4-6, 6-8 mm
XII	Side surface of the stave adjacent to the bung hole (II)	0-2, 2-4 mm
XIII	Groove of the stave adjacent to the bung hole (II)	External, internal
XIV	Internal surface of the side stave (I)	0-2, 2-4, 4-6, 6-8 mm
XV	Side surface of the side stave (I)	0-2, 2-4 mm
XVI	Groove of the side stave (I)	External, internal
XVII	Internal surface of the side stave (II)	0-2, 2-4, 4-6, 6-8 mm
XVIII	Side surface of the side stave (II)	0-2, 2-4 mm
XIX	Groove of the side stave (II)	External, internal
XX	Internal surface of the side stave of the head	0-2, 2-4, 4-6, 6-8 mm
XXI	Side surface of the side stave of the head	0-2, 2-4 mm
XXII	Groove of the side stave of the head	External, internal
XXIII	Internal surface of the central stave of the head	0-2, 2-4, 4-6, 6-8 mm
XXIV	Side surface of the central stave of the head	0-2, 2-4 mm
XXV	Groove of the central stave of the head	External, internal
XXVI	River reed stalks of the staves of the head	Shredded pieces

2.2 Microbial Counts

Samples of wood shaves were inoculated in 100 ml of 30 g l⁻¹ Tryptic Soy broth (Merck, Darmstadt, Germany), 20 g l⁻¹ Yeast extract (Merck) and 1 ml/l of Tween 80 (Merck), with orbital shaking (125 rpm) for 24 h. These solutions were serially diluted (10⁻¹ to 10⁻⁵) in Ringer solution (Oxoid) and inoculated in series of 3 tubes for each dilution according to Most Probable Number (MPN) technique (Vaz-Oliveira et al., 1995).

Total microbial counts were obtained using the medium GYP (10 g l⁻¹ glucose, 5 g l⁻¹ yeast extract, 10 g l⁻¹ peptone and 20 g l⁻¹ agar, pH 6.0) incubated for 5 days at 25°C. Positive results were determined by visual observation of medium turbidity.

Yeasts of the genera *Dekkera/Brettanomyces* sp. were quantified using the medium DBDM (Rodrigues et al., 2001). Positive results were recorded after turbidity observation and phenolic smell detection as described by Rodrigues et al. (2001). Samples from the most diluted positive tubes were streaked onto DBDM plates. Growth characteristics in DBDM medium were evaluated on the basis of medium acidification, colony morphology and detection of phenolic taint by smelling, after incubation at 25°C for up to 14 days (Rodrigues et al., 2001). Typical colonies were further purified to confirm the presence of *D. bruxellensis* by PNA hybridization and to evaluate 4-ethylphenol production, as described below. The strains were maintained in GYP medium (20 g l⁻¹ glucose (Merck), 5 g l⁻¹ yeast extract (Difco Laboratories, Detroit, USA), 10 g l⁻¹ peptone (Difco) and 20 g l⁻¹ agar, pH 6.0) added of 5 g l⁻¹ of calcium carbonate (Merck), at 4°C.

2.3 Production of Volatile Phenols

A loopful of fresh culture (24-48 h) was suspended in Ringer solution and used to inoculate the YNB medium (Difco) (6.7 g l⁻¹) supplemented with glucose (20 g l⁻¹) and *p*-coumaric acid (100 mg l⁻¹) (Sigma Chemical Co., St. Louis, USA), adjusted to pH 5.4 and filter sterilized. Volatile phenols were measured according to a protocol described by Rodrigues et al. (2001). Briefly, the volatile phenols were extracted by ether-hexan from a 50 ml sample with pH adjusted to 8 with NaOH. The volatile phenols were separated by collecting the organic phase of the mixture. The quantitation was achieved by gas chromatography using a DB-Wax capillary column (J & W Scientific, Folsom, California, USA).

2.4 PNA FISH Hybridization

Colonies were smeared onto microscope slides (Erie Scientific, Portsmouth, NH), heat fixed and analyzed by fluorescence in situ hybridization (FISH) using peptide nucleic acid PNA probes targeting 26S rRNA of *D. bruxellensis* (CGGTCTCCAGCGATT) as described by Stender et al. (2001). Microscopic examinations were performed using a fluorescence microscope (Dialux 20, Leica Microsystems, Cambridge, United Kingdom) and *D. bruxellensis* was identified as bright fluorescent yeast cells.

2.5 PCR Amplification and 5.8S-ITS Restriction Analysis

Isolates obtained from samples of wood, which were not identified as *D. bruxellensis* by PNA probe, were identified by restriction analysis of 5.8S-ITS region according to Esteve-Zarzoso et al. (1999). PCR amplification was carried out in an Eppendorf Thermal Cycler using DyNAzyme™ II DNA Polymerase (Finnzymes OY, Espoo, Finland). PCR products were digested with *Cfo*I, *Hae*III and *Hinf*I (Roche Molecular Biochemicals, Mannheim, Germany), and fragments separated on 3% agarose gel.

3. Results

3.1 Effect of Sanitation Treatments

The enumeration of total microbial flora after different sanitation treatments of the barrels is shown in Table 2. In order to be statistically compared results higher than 7.15 log₁₀ MPN counts assumed the value 7.15, and to the absence of counts in 10⁻¹ dilution was given the value 0. In this way, statistical analysis (ANOVA, at 0.05 level) of the mean of the log₁₀ MPN counts obtained with the treatment A (blank) and the mean obtained with the other treatments, showed that: i) B treatment yielded higher counts than A; ii) the differences between A and C or D treatments were not statistically different; iii) E treated barriques yielded significantly lower counts than the blank A. In addition, most of the lowest counts (56 samples in a total of 68) were obtained with E treatment (Table 2) and this was the single treatment to yield null counts in the initial suspension (10⁻¹ dilution) of the oak shaves. These sampling points without measurable contamination were all in the 0-2 mm layer of the internal surface of the staves. However, even with E treatment there were spots of inefficient sanitation. These were the grooves between body staves and head stave, particularly in the external surface where E treatment produced the lowest counts only in 2 of the 8 sampling points. In this case, C treatment was the most efficient by showing the

lowest counts in 4 out of the 8 sampling points. The side surface of the staves was also a point of inefficient sanitation, for which not even the most efficient treatment E was able to reduce microbial counts substantially.

Table 2. Enumeration of yeasts ($\log_{10}$ MPN cm^{-2} of wood shave) recovered from different levels of the wood of barrels after sanitation (treatments A to E)

Origin	Sampling depth (mm)	A	B	C	D	E
I	0-2	2.95	4.95	2.45	2.25	0*
	2-4	2.15	5.34	2.15	3.70	0.70
	4-6	1.48	1.90	1.90	0.70	1.26
	6-8	1.26	1.70	1.70	1.95	1.26
II	0-2	3.22	4.22	3.86	3.70	2.47
	2-4	3.80	3.47	3.80	3.22	3.22
III	External	4.52	5.52	5.00	4.78	4.73
	Internal	5.78	5.78	4.52	4.52	3.52
IV	0-2	2.28	2.28	3.78	3.18	0
	2-4	Nd	3.95	3.51	3.70	1.70
	4-6	2.70	2.70	2.70	3.17	2.70
	6-8	2.48	2.48	2.70	2.70	2.18
V	0-2	3.00	5.87	4.48	4.48	1.78
	2-4	4.87	4.48	4.87	4.70	3.22
VI	External	>7.15	>7.15	5.78	5.78	4.78
	Internal	3.97	>7.15	4.08	5.00	3.52
VII	0-2	3.52	3.52	4.78	3.00	0
	2-4	5.52	3.52	4.00	4.78	2.78
	4-6	4.52	5.78	4.52	4.73	3.73
VIII	0-2	>7.15	>7.15	>7.15	3.18	0.70
	2-4	0.90	3.95	3.08	3.90	2.95
	4-6	1.95	3.95	3.18	3.95	3.70
	6-8	3.48	3.48	3.48	3.70	2.70
IX	0-2	3.70	3.48	5.48	3.48	2.48
	2-4	4.87	>7.15	5.48	3.70	3.22
X	External	3.52	5.78	4.52	5.78	5.78
	Internal	4.43	4.43	2.78	4.00	3.52
XI	0-2	4.95	4.95	4.95	2.70	0
	2-4	3.70	3.70	5.18	3.18	2.18
	4-6	1.26	1.26	>7.15	3.34	3.18
	6-8	0.70	0.70	4.70	3.18	2.90
XII	0-2	2.80	4.80	4.12	3.70	1.78
	2-4	4.70	4.48	4.48	3.87	3.70
XIII	External	5.30	5.30	6.17	5.30	4.30
	Internal	5.30	>7.15	3.73	4.78	4.00

XIV	0-2	2.28	5.28	2.28	1.95	0
	2-4	4.95	3.95	4.70	2.70	0.70
	4-6	3.34	3.18	3.78	3.18	2.90
	6-8	2.90	2.90	3.18	2.90	2.18
XV	0-2	2.22	3.22	4.30	3.48	1.78
	2-4	3.43	2.48	3.70	3.70	3.22
XVI	External	4.78	5.78	4.78	5.17	5.78
	Internal	5.78	4.52	5.30	4.00	3.30
XVII	0-2	4.70	4.95	4.18	2.95	0
	2-4	3.60	3.95	3.26	1.95	1.28
	4-6	2.78	2.70	2.78	3.18	2.70
	6-8	3.34	3.34	5.28	3.18	2.18
XVIII	0-2	5.48	5.48	5.87	4.22	3.22
	2-4	4.80	4.80	5.87	4.80	4.42
XIX	External	5.10	5.78	5.00	6.00	5.78
	Internal	4.67	>7.15	>7.15	5.52	4.52
XX	0-2	2.70	2.70	3.60	2.95	1.95
	2-4	3.85	3.85	3.85	3.85	1.95
	4-6	2.70	2.70	2.70	3.18	3.18
	6-8	2.90	2.90	4.70	3.70	2.70
XXI	0-2	>7.15	>7.15	5.48	4.70	3.70
	2-4	5.22	5.22	3.78	4.48	4.22
XXII	External	5.78	6.18	5.30	6.18	5.78
	Internal	5.78	>7.15	>7.15	5.78	4.52
XXIII	0-2	>7.15	>7.15	4.28	3.18	0
	2-4	3.95	3.95	3.70	3.70	2.70
	4-6	3.60	4.70	3.95	4.18	3.34
	6-8	3.95	3.95	3.51	3.95	2.70
XXIV	0-2	4.22	>7.15	4.22	5.87	3.22
	2-4	5.87	5.87	4.80	5.48	3.48
XXV	External	>7.15	>7.15	3.10	>7.15	>7.15
	Internal	4.52	5.78	>7.15	4.00	4.00
XXVI	Pieces	>7.15	5.10	5.10	4.57	4.35
Mean [†] ± standard deviation		4.03±1.61	4.58±1.65	4.32±1.29	3.93±2.85	2.85±1.59

*The lowest counts in each row of table are written in bold.

[†]The means of the log counts were estimated assuming the value 7.15 for counts higher than 7.15, and the value 0 for the absence of counts in 10⁻¹ dilution.

3.2 Quantification and Typing of 4-Ethylphenol Producing Yeasts

The enumeration of yeasts with positive results in the DBDM medium is shown in Tables 3 and 4. It was possible to evidence the presence of 4-ethylphenol producing yeasts in the wood up to the layers of 6-8 mm depth. In most sampling points these yeasts only represent a small proportion of the total contaminating flora.

The tests with *D. bruxellensis* specific PNA probe showed that almost all strains with positive growth response in DBDM hybridised with this probe (Table 3). These strains should then be assigned to the species *D. bruxellensis*.

Table 3. Enumeration of strains isolated at different levels in the wood of barriques and with positive hybridization with the PNA probe specific for *D. bruxellensis*

Strain	Treatment	Origin	Isolation depth (mm)	MPN counts* (DBDM/GYP)	Relative predominance (%)
400	A	I	0-2	50/900	5.6
402	A	I	4-6	5/18	27.8
405	A	III	External	6000/33333	18.0
411	A	VII	2-4	120/33333	0.4
416	A	IX	2-4	500/90000	0.6
418	A	XII	0-2	167/300000	0.06
419	A	XX	4-6	50/500	10.0
423	A	XXV	Internal	1267/33333	3.8
531	A	VII	4-6	333/33333	1.0
532	A	X	External	1267/3333	38.0
533	A	XIX	External	3333/126667	2.6
534	A	XXIII	4-6	<1/4000	<0.02
535	C	VIII	0-2	500/>140000000	<0.004
536	C	XII	0-2	1667/13333	12.5
537	C	XVI	Internal	14800/200000	7.4
538	C	XX	6-8	50/50000	0.1
539	B	I	2-4	90/220000	0.04
540	B	II	0-2	167/733333	0.02
541	B	VII	4-6	600/600000	0.1
542	B	VIII	4-6	90/9000	0.1
543	D	IV	6-8	50/500	10.0
544	D	VII	4-6	333/53333	0.6
545	E	V	2-4	17/1667	1.0
546	E	XIII	External	120/20000	0.6
547	E	XVI	External	120/600000	0.02
548	E	XXV	External	600/>140000000	<0.0004
549	E	VII	4-6	120/5333	2.3

* Ratio between the MPN counts in DBDM and GYP broths.

The strains with positive growth response in DBDM and not hybridising with the specific probe are listed in Table 4. The strains growing in DBDM but without releasing phenolic smell (negative response) are listed in Table 5.

The 4-ethylphenol production in synthetic medium was evaluated for all strains recovered from the DBDM broth. All *D. bruxellensis* strains produced high amounts of 4-ethylphenol (results not shown). Among the 3 strains not hybridising with the PNA specific probe the amount of 4-ethylphenol produced in synthetic medium was high only for the strain 430b (Table 4). In figure 1 are shown representative results of growth and 4-ethylphenol production by one strain of *D. bruxellensis* and by strain 430 b, showing similar maximum levels of 4-ethylphenol attained and much higher growth rates for *P. guilliermondii*.

As expected all strains in table 5 yielded rather low conversion rates of *p*-coumaric acid into 4-ethylphenol, producing less than 1.2 mg l⁻¹ of 4-ethylphenol.

Table 4. Enumeration of strains isolated at different levels in the wood of barriques with negative hybridization with the PNA probe specific for *D. bruxellensis* and growing in DBDM medium releasing phenolic smell

Strain	Treatment	Origin	Isolation depth (mm)	MPN counts (DBDM/GYP)	Relative predominance (%)	4-EP*
417	A	XVI	External	1267/60000	2.1	1.2
420	A	XXII	External	600/600000	0.1	0.7
430b	A	I	2-4	1/140	0.7	51.0

* Maximum production of 4-ethylphenol (mg l⁻¹) in synthetic medium added of 100 mg l⁻¹ of *p*-coumaric acid (mean of 2 independent experiments).

Table 5. Enumeration of strains isolated at different levels in the wood of barriques with negative hybridization with the PNA probe specific for *D. bruxellensis* and growing in DBDM medium without releasing phenolic smell

Strain	Colony colour	Treatment	Origin	Isolation depth (mm)	MPN counts (DBDM/GYP)	Relative predominance (%)	4-EP*
403	Pink	A	I	6-8	5/18	27.8	-†
404	Pink-orange	A	II	0-2	1667/1667	100.0	0.2
406	Yellow grey	B	IV	0-2	5/190	2.6	1.2
407	Yellow grey	A	IV	2-4	5/-	-	0.9
408	Yellow white	A	IV	4-6	90/500	1.8	0.9
409	Yellow grey	A	V	0-2	633/1000	63.3	0.7
410	Olive green	A	VI	Internal	-/9333	-	0.6
412	Olive green	A	VIII	4-6	50/90	55.6	0.3
413	Yellow white	A	IX	2-4	300/733333	0.04	0.6
414	Yellow grey	A	XI	6-8	18/50	36.0	1.0
415	Olive green	A	XIII	Internal	600/200000	0.03	0.1
421	Yellow white	A	XIII	6-8	8/9000	0.09	0.7
422	Yellow	A	XXIV	2-4	167/733333	0.02	0.1

* Maximum production of 4-ethylphenol (mg l⁻¹) in synthetic medium added of 100 mg l⁻¹ of *p*-coumaric acid.

† Not determined.

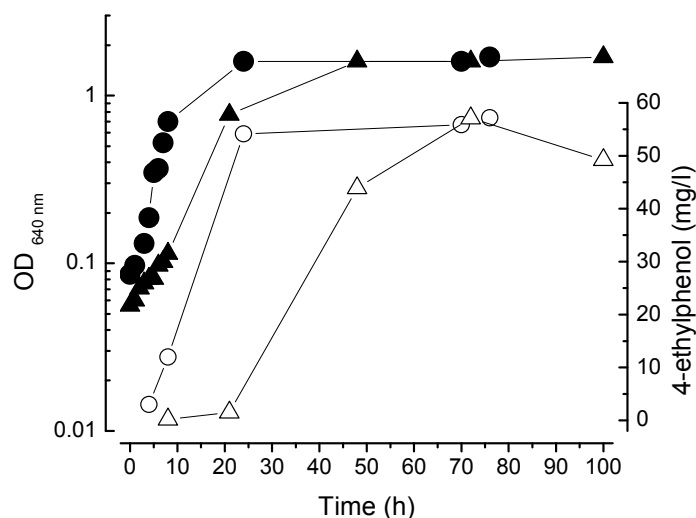


Figure 1. Growth (filled symbols) and production of 4-ethylphenol (open symbols) by strains 430b (circles) and *D. bruxellensis* strain 535 (triangles). Maximum growth rates (h^{-1}) were 0.37 for strain 430b and 0.11 for strain 535

3.3 Strain Identification by RFLPs of 5.8S-ITS

The yeast species able to grow on DBDM medium and not hybridising with the PNA probe were identified by RFLP of 5.8S-ITS region. The identifications are listed in table 6 showing that the most common species was *P. guilliermondii*. Other strains were assigned to *Candida parapsilopsis*, *Rhodotorula mucilaginosa* and *Torulaspora globosa*. Both *P. guilliermondii* and *Candida parapsilopsis* released phenolic smell in DBDM medium but only strain 430b of *P. guilliermondii* yielded high amounts of 4-ethylphenol in synthetic medium (see Tables 4 and 5).

Table 6. Strain identification by RFLPs of the 5.8-ITS region of isolates from different levels in the wood of barriques

Strain	AP* (bp)	Restriction enzymes			Species
		<i>CfoI</i>	<i>HaeIII</i>	<i>HinfI</i>	
417	550	310+240	420+130	280+270	<i>Candida parapsilopsis</i>
420	625	300+265+60	400+115+90	320+300	<i>Pichia guilliermondii</i>
430b	625	300+265+60	400+115+90	320+300	<i>Pichia guilliermondii</i>
403	620	300+220+100	410+210	350+220+50	<i>Rhodotorula mucilaginosa</i>
404	620	300+220+100	410+210	350+220+50	<i>Rhodotorula mucilaginosa</i>
406	550	310+240	420+130	280+270	<i>Candida parapsilopsis</i>
407	625	300+265+60	400+115+90	320+300	<i>Pichia guilliermondii</i>
408	625	300+265+60	400+115+90	320+300	<i>Pichia guilliermondii</i>
409	625	300+265+60	400+115+90	320+300	<i>Pichia guilliermondii</i>
410	625	300+265+60	400+115+90	320+300	<i>Pichia guilliermondii</i>
412	625	300+265+60	400+115+90	320+300	<i>Pichia guilliermondii</i>
413	625	300+265+60	400+115+90	320+300	<i>Pichia guilliermondii</i>
414	625	300+265+60	400+115+90	320+300	<i>Pichia guilliermondii</i>
415	650	300+300+50	420+150+80	325+325	<i>Torulaspora globosa</i>
421	625	300+265+60	400+115+90	320+300	<i>Pichia guilliermondii</i>
422	625	300+265+60	400+115+90	320+300	<i>Pichia guilliermondii</i>

* Amplification product.

4. Discussion

The comparison of the microbial counts among the different barrels must take into account that each treatments was done in independent barrels and that each barrel may have harboured different initial microbial loads. In addition, for a single barrel, microbial distribution in the wood is not uniform. Having in mind these considerations, the results obtained with the different sanitation procedures suggest that none was able to reduce significantly the contaminating microflora. However, the less inefficient treatment was that using steam under low pressure. The loss of efficiency was observed with increasing the depth of sampling in the wood and in points not reached by steam (external surfaces of grooves and side surfaces of staves). In this case only dismantling and further steaming could have reduced the contamination levels. It is surprising that the most common procedure in Portuguese cellars – B treatment – showed results statistically similar to those of barrel without treatment. This observation can be explained by higher initial counts in the B treated barrel.

Most of *D. bruxellensis* strains were isolated in the untreated barrel. However, this species was isolated after any of the sanitation procedures carried out. The presence of *D. bruxellensis* was evidenced up to the 6-8 mm depth layer, corresponding to the maximum depth of wine penetration. In most sampling points this species represented a small proportion of the contaminating flora, as already observed in bulk wines (Rodrigues et al., 2001). Concerning the most efficient E treatment, the presence of *D. bruxellensis* was evidenced in the external surface of grooves, side surface of staves (2-4 mm) and in the bunghole (4-6 mm). Therefore, these points should be regarded as critical during sanitation. Contaminated barrels should be treated after dismantling or discarded to minimise or avoid, respectively, the risk of wine contamination. Assuming an average internal area of 2.1 m² for a 250 l barrel (Schahinger & Rankine, 1992) and an average of 100 cells cm⁻² of *D. bruxellensis* per each of the 4 layers of staves, one reaches the initial inoculum level of 33 cells/ml of a hypothetical sterile wine introduced into a sanitised barrel. The fact that 6 cells ml⁻¹ of *D. bruxellensis* were reported as being able to produce 1 mg l⁻¹ of 4-ethylphenol during a period of 4.5 month barrel maturation (Chatonnet et al., 1993) leads to the conclusion that the most effective treatment does not eliminate the risk of contamination. According to González-Arenzana et al. (2013) also reported the isolation of *Brettanomyces* spp. from 8 mm depth after microwave treatment. Thus, steam treatment should be regarded as an additional procedure in the prevention of *D. bruxellensis* activity and not as the key to its elimination.

The species isolated from the wood other than *D. bruxellensis*, were *Pichia guilliermondii*, *Candida parapsilopsis*, *Rhodotorula mucilaginosa* and *Torulaspora globosa*. From this group only one strain of *P. guilliermondii* showed the ability to produce high levels of 4-ethylphenol as already observed by us (Dias et al., 2003). It remains to be seen if this ability also occurs in wines. If so, strains of this species should be regarded as well as spoilage agents. Otherwise, all mentioned species should be regarded as innocent contaminants concerning spoilage by 4-ethylphenol production.

5. Conclusion

From this study arises the conclusion that the recovery of used barrels is a difficult task to achieve. This operation must include the removal of all wood layers soaked with wine. Other treatments with ozone, microwave or ultrasonics may have similar efficiency. However, it is likely that the limiting factor of any treatment is the prevention of contact between the sanitising agent and the microbial cells which occurs when microorganisms are imbedded in deep wood layers.

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