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Off-Odor Compounds Produced in Cork by Isolated Bacteria and Fungi: A Gas Chromatography−Mass Spectrometry and Gas Chromatography−Olfactometry Study

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The risk of development of specific olfactory profiles in cork was evaluated after inoculation of cork granules and agglomerated and natural cork stoppers with isolated bacteria and fungi. The highest incidence of off-odor development was found in assays when fungi were inoculated. Cork granules with musty-earthy, musty-earthy-TCA, and vegetative deviations were inspected by gas chromatography−olfactometry (GC-O) and gas chromatography−mass spectrometry (GC-MS). Sixteen odor zones were clearly recognized in the GC-O analyses. Among these, octanal, 2-methoxy-3,5-dimethylpyrazine (MDMP), *Z*-2-nonenal, 2-methylisoborneol, 2,4,6-trichloroanisole (TCA), geosmin, and guaiacol were the most significant odorants and helped in the discrimination of sensory deviations. Only TCA and guaiacol were detected above their respective detection limits by HS-SPME-GC-MS. The fungi *Cryptococcus* sp. isolate F020, *Rhodotorula* sp. isolate F025, *Penicillium glabrum* isolate F001, and *Pennicillium variabile* F003A and the bacterium *Pseudomonas jessenii* isolate A1 were found to produce TCA to a greater extent. Additionally, 13 of 38 isolated microorganisms (2 bacteria and 11 fungi) proved able to produce unpleasant musty-earthy or vegetative odors that were not related to a significant TCA accumulation.

KEYWORDS: Musty-earthy taint; olfactometry; 2,4,6-trichloroanisole; 2-methoxy-3,5-dimethylpyrazine; *Penicillium*; *Pseudomonas*

INTRODUCTION

Cork has been the most popular material used for wine stoppers for many years and is regarded as the norm in the case of quality wines. It is widely accepted that cork stoppers have the desired level of elasticity and strength to ensure effective, safe closure for bottles of most wine types (*1*, *2*). However, cork is continuously being targeted as responsible for specific alterations found in wine. One of the most significant organoleptic defects in wines is ambiguously reported as "cork taint" and is related to a moldy/musty off-flavor. Although the list of possible compounds producing unpleasant aromas in cork is relatively large, the highest incidence of musty-earthy off-odors is due to 2,4,6-trichloroanisole (TCA), 2-methoxy-3,5-dimethylpyrazine (MDMP), and 2,4,6-tribromoanisole (TBA) (*2*−*4*). Other volatile organic compounds, such as 1-octen-3-ol, 1-octen-3-one (mushroom aroma), guaiacol (smoky, phenolic aroma), geosmin, and 2-methylisoborneol (earthy aroma), have also been reported as being responsible for unpleasant aromas (*5*).

The natural origin of cork and the long manufacturing process of stoppers contribute to the development of a true microbial ecosystem over the cork surface that has implicated biological activity in the formation of sensory deviations. Bacteria and fungi are among the main contributors to the formation and accumulation of taint compounds. Some of the reactions leading to the formation of unpleasant odors have recently been established and unequivocally proven to occur on the cork surface. For instance, the synthesis and accumulation of chloroanisoles, mainly TCA, by the biomethylation of 2,4,6-trichlorophenol (TCP) is a well-known process detected in fungi isolated from cork (*6*−*8*). Although differences exist in TCA formation between species, most of the isolates are able to accumulate this compound when growing on cork spiked with TCP as a substrate (*6*, *9*, *10*). Most of the reported work using isolated bacteria and fungi shows the production of TCA under ideal conditions, that is, using either complex culture media or TCP-amended cork matrices. However, little information exists about TCA accumulation under real conditions of storage and maintenance of cork slabs and stoppers. Similarly, the participation of bacteria in the production of guaiacol, geosmin, and pyrazines has been described experimentally in amended cork matrices and other substrates (*11*−*13*).

The study of the compounds contributing to the aroma of cork, usually present at trace levels, is a laborious task involving three different steps: extraction, concentration, and determination with gas chromatography. Gas chromatography−mass spectrometry (GC-MS) has been shown to be a powerful separation tool with

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very low detection limits and has been used for the above-mentioned purpose. However, some molecules can be odor-active at a concentration well below the detection limit of the technique, so additional methods are required. Gas chromatography−olfactometry (GC-O) has been demonstrated to be an excellent alternative, which allows those compounds that are aromatically important to be located and ranked according to their respective intensities (*14*, *15*). GC-O has also been used as a discriminant technique in experiments devoted to unequivocally assigning a specific chemical compound to a certain odor description (*14*, *16*). GC-O is mainly used in the flavor investigation of foods and beverages (coffee, meat, and fruits, among others) (*17*, *18*), but to the best of our knowledge only a few studies related to cork have been reported (*16*, *19*).

In the present work the biological origin of off-odors has been determined for a collection of bacteria and fungi isolated from either tainted or control cork samples. Tests have been conducted on three different cork matrices consisting of cork granules, agglomerated cork stoppers, and natural cork stoppers, in undisturbed conditions. In addition, we have made use of GC-O analyses to evaluate the contribution of different volatile compounds to off-odor development in inoculated cork granules with different olfactory profiles. Odorous compounds have been quantified using solid phase microextraction (SPME) and GC-MS.

MATERIALS AND METHODS

Reagents and Standards. Octanal, *E*-2-nonenal, 2-acetylpyrazine, 2,4,6-trichloroanisole (TCA), 2-methoxyphenol (guaiacol), *p*-cresol, ethyl cinnamate, 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (Furaneol), and 4, 5-dimethyl-3-hydroxy-2(5*H*)-furanone (sotolon) were supplied by Sigma-Aldrich (Madrid, Spain). *Z*-2-Nonenal was obtained from its isomer *E*-2-nonenal, which contains 5−10% of *Z*-2-nonenal as a major impurity. Geosmin and 2-methylisoborneol were supplied by Supelco (Bellefonte, PA). NaCl and 2,4,6-trichloroanisole-d_5 (used as an internal standard, IS) were purchased from Riedel-de-Haën (Seelze, Germany). LiChrolut EN resins and polypropylene cartridges were from Merck (Darmstadt, Germany). Dichloromethane and methanol of LiChrosolv quality were from Merck; absolute ethanol, pentane, and ammonium sulfate, all of ARG quality, were from Panreac (Barcelona, Spain). Pure water was obtained from a Milli-Q purification system (Millipore, Billerica, MA). Separated stock solutions were prepared in HPLC-grade methanol (Carlo Erba Instruments, Milan, Italy) at a concentration level of 1 mg L^{-1}. A stock solution of 1.074 mg L^{-1} of 2-methoxy-3,5-dimethylpyrazine in ethanol was kindly supplied by Dr. Sefton (University of Adelaide, Australia). All stock solutions were stored in a refrigerated environment at 4 °C and kept in darkness. Working solutions in the range of 1−100 ng L^{-1} were made daily by diluting the standard solutions with double-deionized water and adding the internal standard (TCA-d_5) at a concentration of 25 ng L^{-1}.

The Ringer isotonic solution was provided by Scharlau (Barcelona, Spain). Microbiological media Luria−Bertani (LB), Rose Bengal agar (RBA), and potato dextrose agar (PDA) were provided by Conda (Madrid, Spain), Liofilchem (Teramo, Italy), and Difco BD (Le Pont de Claix, France), respectively. All media were supplied with antibiotic supplements. Chelex 100 molecular biology grade resin was provided by Bio-Rad Laboratories (Hercules, CA). All reagents for PCR amplification and DNA purification, including the Taq DNA polymerase, were from Qiagen (Hilden, Germany). Finally, DNA sequencing was performed with the BigDye Terminator v3.0 ready reaction cycle sequencing kit obtained from Applied Biosystems (Foster, CA).

Cork Samples. For the isolation of microorganisms, we used agglomerated or natural cork stoppers, which either showed an obvious colonization of molds on their surface or had previously been classified as harboring strong off-odors. These were provided by TESA (Catalonia, Spain).

For the inoculation and off-odor development experiments, cork granules (approximate diameter, 3−4 mm; apparent density, 70−130 kg m^{-3}), natural cork stoppers for still wine (o.d. = 24 mm, 49 mm thick), and agglomerated stoppers for Spanish sparkling wine D.O. Cava (o.d. = 30 mm, 47 mm thick) were kindly supplied by TESA, J. Vigas SA, and Geyru SA (Catalonia, Spain).

Isolation of Microorganisms. Microorganisms were isolated using conventional isotonic solution washes and a dilution series. Single corks were washed twice in 50 mL of an isotonic solution under agitation for 30 min. The two washing solutions were pooled and serially diluted. LB plates were supplemented with nystatin (final concentration = 60 μg mL^{-1}) and used for bacterial isolation. Chloramphenicol-supplemented (100 μg mL^{-1}) RBA and PDA were used for the isolation of fungi and yeasts. Colonies of different shapes and colors were selected and repeatedly stroked on fresh medium agar plates until pure cultures were obtained. A minimum of five replating steps were performed. Additionally, six bacterial isolates and one yeast isolate were provided by the cork manufacturing companies. All strains were grown at 25 °C.

Identification of Strains. Identification of strains was done by sequencing the 16S rRNA gene in the case of bacteria and the intergenic spacer regions, ITS1 and ITS2, and the 5.8S rRNA gene (ITS1-5.8S-ITS2) in the case of fungi. Nucleic acids were extracted from colonies picked directly from agar plates using the Chelex 100 resin. Briefly, 1 mL of fungal or bacterial suspension was centrifuged for 5 min at 12000 rcf at 4 °C. Cell pellets were suspended in 200 μL of 6% Chelex 100 resin, thoroughly mixed, and incubated at 56 °C for 20 min. Extracts were subsequently mixed, boiled for 8 min, and finally cooled on ice. Cell debris was pelleted by centrifugation at 14000 rpm at 4 °C for 5 min, and 50 μL aliquots of the supernatant were transferred into a new microtube and stored at −20 °C until use. Nucleic acid concentrations were quantified spectrophotometrically using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE).

PCR amplification was performed with primers 27f and 1492r (*20*) for bacterial strains and with ITS1F and ITS4 (*21*, *22*) for fungi. Amplification reactions were contained in a total volume of 50 μL: 1× PCR buffer, 0.8 mM deoxynucleoside triphosphate, 0.3 μM of each primer, 3 mM $MgCl_2$, and 2.5 U of Taq DNA polymerase. Extracted DNA (10−100 ng) was added as template for the PCR reactions. The sizes of the PCR products were checked in TAE agarose gels (1%) and ethidium bromide staining. Positive products of the correct size were purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany), prior to sequencing. All fragments were sequenced using the BigDye Terminator v3.0 kit in an ABI Prism 310 sequencer. Primer 27f was used to sequence the 16S rRNA gene of bacteria (*20*). The fungal ITS1-5.8S-ITS2 fragment was sequenced using primer ITS1F. Sequences were aligned using Clustal W software (European Bioinformatics Institute http://www.ebi.ac.uk). Similarity searches were performed using BLASTn software (http://www.ncbi.nlm.nih.gov/BLAST/) and the results used for the identification of isolates (*23*).

Incubation of Microorganisms in Cork Matrices and Sensory Evaluation. Bacterial and fungal suspensions were prepared from LB and RB agar plates and adjusted to approximately 10^8 cells mL^{-1} and 5 × 10^4 spores mL^{-1}, respectively. Agglomerated and natural cork stoppers were soaked in the corresponding microbial suspension for 3 s to ensure an even inoculum distribution. Single corks were used in every experiment and distributed individually in sterile screw-capped glass bottles. Incubations using cork granules were performed using autoclave-sterilized 5 g aliquots distributed in 100 mL glass bottles. Cork granules were inoculated with 1 mL of the bacterial or fungal suspensions and mixed thoroughly to homogenize. All sample types, both stoppers and cork granules, were incubated at 25 °C for 30 days. Three replicate tests were performed for every isolate and cork matrix.

After the incubation period, the cork samples were macerated for 24 h in double-distilled water following the method proposed by AENOR regulation UNE 56928 (*24*). Sensory evaluation was performed by trained judges at the providing companies. Three specific 1 h training sessions were carried out before the panel evaluated the macerates. In the first training session, the judges generated descriptive terms for the sensory deviations present in the different cork samples. In the second and third sessions, different macerates were presented and the terms were discussed by the panel. Through these discussions, eight terms were selected and used for further sensory analysis: musty-earthy, musty-earthy-TCA, vegetative, green olive, sulfur, chemical, spicy, and smoky. After the training periods, the macerates were evaluated.

The specific incidence of isolated microorganisms in off-odor development was calculated as the proportion of positive experiments in relation

to the total number of incubations, regardless of the cork matrix used, for every single isolate. Additionally, the risk of off-odor development due to the inoculation of isolated microorganisms in the cork matrices used was calculated as the proportion of tainted cork incubations in relation to the total number of incubations regardless of the inoculated microorganism. The ratios were independently estimated for each aromatic descriptor.

GC-O. *Extract Preparation.* Volatile compounds present in aqueous cork macerates were collected using a purge-and-trap system, which was designed in our laboratory. The headspace strategy followed was proposed by Campo et al. (*25*). The trap consisted of a standard polypropylene SPE tube (0.8 cm i.d., 3 mL internal volume) packed with 400 mg of LiChrolut EN resin. The bed was washed with 20 mL of dichloromethane and dried by circulating air (negative pressure = 0.6 bar, 10 min). The tube was placed on top of a bubbler flask containing 80 mL of aqueous cork macerate, which was continuously stirred with a magnetic stir bar and kept at a constant temperature of 37 °C by water bath immersion. A controlled stream of nitrogen (100 mL min^{-1}) was passed through the sample for 200 min. Volatile constituents released into the headspace were trapped in the cartridge containing the sorbent and were further eluted with 3.2 mL of dichloromethane with 1% methanol. The extract was kept at −30 °C for 2 h to eliminate any water content by freezing and further decantation. The extract was then concentrated under a stream of pure N_2 for a final volume of 200 μL.

GC-O Analysis. The concentrated cork extracts were used in the GC-O study. Sniffings were carried out in a Trace gas chromatograph from ThermoQuest equipped with flame ionization detection (FID) (ThermoQuest, Madrid, Spain) and a sniffing port (ODO-1 from SGE GmbH, Griesheim, Germany) connected by a flow splitter to the column exit. The column was a DB-WAX from J&W Scientific, Folsom, CA (30 m × 0.32 mm ID, 0.5 μm film thickness). A constant pressure of 52 kPa was maintained throughout the analysis. The carrier was H_2. One microliter was injected in splitless mode for 1 min of splitless time. The injector and detector were both kept at 250 °C. The temperature program was 40 °C for 2 min, rising by 12 °C min^{-1} to 105 °C, followed by 6 °C min^{-1} to 220 °C, and finally kept at 220 °C for 20 min. To prevent condensation of high-boiling compounds on the sniffing port, it was heated sequentially with a laboratory-made rheostat.

A panel of six judges carried out the sniffings of each extract. Sniffing time was approximately 30 min, and each judge performed one session per day. The panelists were asked to assess the overall intensity of each odor on a seven-point category scale (0 = not detected; 1 = weak, hardly recognizable odor; 2 = clear, but not intense odor, 3 = intense odor). Half values were allowed. Each judge evaluated the macerate extracts once, and the six intensity scores obtained for each odorant in each macerate sample were averaged to give the mean intensity score for that odorant in that sample.

Odorants were identified by comparing aroma, chromatographic retention indices in the DB-WAX column and the DB-5 column, and MS spectra with those of pure reference compounds.

Headspace Solid-Phase Microextraction Gas Chromatography Analysis (HS-SPME-GC-MS). A DVB/CAR/PDMS (50/30 μm) fiber (Supelco) was used with a 5 mL sample containing 1.2 g of NaCl. Extraction was performed under stirring conditions for 30 min at 50 °C (*26*). GC analysis was performed with a Trace GC 2000 series gas chromatograph coupled to a PolarisQ ion trap mass spectrometer detector (Thermo, Milan, Italy), operating in the electron impact (EI) mode at 70 eV. A SGE BPX-5 capillary column (Scientific Instrument Services, Ringoes, NJ) (30 m × 0.25 mm i.d., 0.25 μm film thickness) was used. The carrier gas was helium at 1 mL min^{-1}. A split/splitless injector was used in the splitless injection mode (5 min) with an injector temperature of 250 °C. The oven temperature program was 5 min at 40 °C, increasing by 8 °C min^{-1} to 100 °C, then by 5 °C min^{-1} to 200 °C, then by 15 °C min^{-1} to 270 °C, and finally held for 5 min at 270 °C. The ion source was set at 225 °C, and the transfer line was held at 270 °C. Full-scan acquisition was used in two ranges of masses (60−170 and 100−350) to characterize the compounds, which were clearly identified by comparison with reference spectra (Wiley7 database) and with pure standards. The selected mass fragments for identification and quantification (in bold) were **95**, 108 for 2-methylisoborneol (MIB); **112**, 125 for geosmin; **210**, **212**, 195, 197 for TCA; 120, **138**, 137, 120 for 2-methoxy-3,5-dimethylpyrazine (MDMP); **124** for guaiacol; and **215**, **217** for TCA-d_5. The chromatographic data were analyzed with Xcalibur 1.4 software.

The concentration of analytes in cork samples was calculated by interpolating in the SPME calibration graph obtained by the internal standard method (TCA-d_5). Unless otherwise stated, every determination was made in duplicate.

RESULTS AND DISCUSSION

Isolation and Identification of Microorganisms. A total of 31 microorganisms comprising 5 yeasts, 13 filamentous fungi, and 13 bacteria were isolated from tainted cork samples (**Table 1**). One additional yeast isolate and six bacteria were obtained from routine microbiological controls and generously provided by one of the manufacturing companies. Sequencing of both the 16S rRNA gene and the ITS1-5.8S-ITS2 gene fragment yielded good-quality sequences of approximately 770 and 550 nucleotides that were used for identification purposes. In all cases except two (isolates F003A and F020), Blastn similarity values were > 99.5% and matched previously cultured microorganisms. Isolated yeasts were identified as *Aureobasidium pullulans*, *Cryptococcus* sp., *Rhodotorula mucilaginosa*, and two additional unidentified *Rhodotorula* species. All genera have been previously isolated from cork samples (*12*). Filamentous fungi were identified as *Penicillium glabrum* (six isolates), *Penicillium glandicola*, *Penicillium roquefortii* (*2*), *Penicillium variabile*, and *Trichoderma viride* (*3*) according to sequence homologies of the ITS1-5.8S-ITS2 region. All of the fungi except *P. roquefortii* have been previously reported as common species in cork slabs and are perhaps present from the onset of the cork manufacturing process (*6*, *9*, *12*, *27*).

Among the bacteria, *Pseudomonas* were the most frequently isolated (10 isolates). Other γ-proteobacteria, such as *Enterobacter* sp. and *Stenotrophomonas maltophilia*, were also identified on the basis of partial 16S rRNA gene sequence similarities. The rest of the isolates were identified as *Phyllobacterium myrsinacearum* (α-proteobacteria) and *Curtobacterium flacumfaciens* (Actinobacteria). Interestingly, four of the six bacterial isolates provided by the cork manufacturing companies belonged to the genus *Bacillus*, which was not found in tainted corks. *Pseudomonas* isolates differed from those previously reported in cork using culture-independent techniques in accordance with their 16S rRNA gene sequence signatures (*28*). Bacterial contamination of cork is a rather complex process, and species may vary according to many environmental factors. Dias-Machado et al. (*29*) reported the isolation of *Bacillus* and *Stenotrophomonas* species in wastewater samples obtained from a manufacturing company.

Evaluation of Taint Incidence in Inoculated Cork. We have investigated the ability of the isolated microorganisms to develop compounds that may be responsible for unpleasant odors over three different cork matrices. The incubated corks were first inspected by sensory analyses. Most of the isolates were able to introduce sensory deviations in cork to a certain degree (**Table 1**). The highest incidence of off-odor development was found in fungal assays of the inoculated samples, which reached values above 30% in 9 of 19 isolates. *A. pullulans* F030 and *P. glabrum* F007 were the only isolates for which no unpleasant odors were detected regardless of the cork matrix used. In contrast, other isolates such as F003A (tentatively identified as *P. variabile*), F003B (*P. glabrum*), and F019 (*A. pullulans*) exhibited the highest capacity of taint development and accounted for incidences of 40% or higher. The results obtained suggest that high intraspecies heterogeneity may exist in the ability of taint development for a defined fungal species. This indicates that a strain level analysis is needed to define an isolated organism as a potential taint producer.

Bacteria showed a relatively lower incidence in the development of taint compounds and rarely exceeded 20% of the inoculated cork replicates. Isolates N5 (*P. fluorescens*), A1 (*P. jessenii*), and N9_1 (*S. maltophilia*) were the only ones showing higher taint

Table 1. Taint Incidence after Inoculation of Isolated Microorganisms on Cork[a]

isolate	closest cultivated strain	similarity (%)	length (bp)	taint incidence[b]
	Yeasts			
F019	*Aureobasidium pullulans* ATCC16629 (AF121283)	100.0	523	40.0
F030	*Aureobasidium pullulans* CO-4 (EU547495)	99.7	598	0.0
F020	*Cryptococcus* sp. F6 (AY518273)	98.9	534	18.2
F014	*Rhodotorula mucilaginosa* Saar1 (DQ386306)	100.0	628	22.2
F013	*Rhodotorula* sp. SY-92 (AB026006)	99.8	592	22.2
F025	*Rhodotorula* sp. SY-74 (AB025984)	99.5	607	36.4
	Filamentous Fungi			
F001	*Penicillium glabrum* 296P (EU128643)	100.0	554	27.3
F003B	*Penicillium glabrum* 296P (EU128643)	100.0	554	54.6
F005	*Penicillium glabrum* 296P (EU128643)	100.0	554	33.3
F006	*Penicillium glabrum* 296P (EU128643)	100.0	554	33.3
F007	*Penicillium glabrum* 296P (EU128643)	100.0	554	0.0
F018	*Penicillium glabrum* 296P (EU128643)	100.0	553	9.1
F017	*Penicillium glandicola* NRRL 985 (DQ339573)	99.8	560	40.0
F002	*Penicillium roquefortii* FRR 849 (AY373929)	100.0	562	18.2
F004	*Penicillium roquefortii* FRR 849 (AY373929)	100.0	559	41.7
F003A	*Penicillium variabile* FRR 1290 (AY373936)	98.6	576	50.0
F011	*Trichoderma viride* GJS 89-142 (DQ109532)	100.0	580	45.5
F012	*Trichoderma viride* GJS 89-142 (DQ109532)	100.0	582	22.2
F016	*Trichoderma viride* GJS 89-142 (DQ109532)	100.0	582	28.6
	Bacteria			
A3	*Bacillus megaterium* (FJ174655)	99.9	776	11.1
A4	*Bacillus megaterium* (FJ174655)	99.9	776	8.3
A6	*Bacillus* sp. MM1 (FJ228145)	100.0	781	0.0
A7	*Bacillus* sp. MM1 (FJ228145)	100.0	781	0.0
N1_2	*Curtobacterium flacumfaciens* DSM 20129 (AM410688)	99.9	752	16.7
N4	*Enterobacter* sp. B5 (EU240200)	99.6	767	0.0
N6	*Phyllobacterium myrsinacearum* STM948 (AY785315)	100.0	714	11.1
N5	*Pseudomonas fluorescens* TM5-2 (AB203715)	100.0	761	33.3
N10	*Pseudomonas fluorescens* TM5-2 (AB203715)	100.0	761	0.0
N1	*Pseudomonas fluorescens* TM5-2 (AB203715)	100.0	761	0.0
A1	*Pseudomonas jessenii* (AF501361)	100.0	759	25.0
N13_1	*Pseudomonas putida* RS-4 (DQ112332)	100.0	773	11.1
N7	*Pseudomonas putida* WH3 (FJ262368)	100.0	761	11.1
N8	*Pseudomonas putida* WH3 (FJ262368)	100.0	761	8.3
N9_2	*Pseudomonas putida* WH3 (FJ262368)	100.0	761	0.0
N14	*Pseudomonas* sp. FA20 (AY131224)	100.0	768	8.3
N3	*Pseudomonas* sp. FA20 (AY131224)	100.0	768	11.1
N9_1	*Stenotrophomonas maltophilia* DTQ-MD18 (EF061454)	100.0	767	22.2
A2	*Stenotrophomonas maltophilia* DTQ-MD18 (EF061454)	100.0	766	0.0

[a] Isolates in boldface type correspond to cultures provided by the manufacturing industry. Similarity values are based on the results of a Blastn search of the fungi ITS1-5.8S-ITS2 region and the prokaryotic 16S rRNA gene sequences. GenBank accession numbers of the closest sequence appear in parentheses. Lengths of the compared sequences are also indicated. [b] Percentage of experiments in which off-odors were detected after the incubation period for every strain.

incidences and accounted for 33.3, 25.0, and 22.2% of the inoculated cork samples, respectively.

The origin of isolates was found to be the determining factor in the taint development capacity of the isolated strains. Most of the isolates provided by the cork manufacturing companies failed to develop any of the deviations analyzed in any of the cork matrices used. The only exceptions were isolates A1 (*P. jessenii*) and A3 and A4, both identified as *B. megaterium*, which accounted for taint incidences of 25.0, 11.1, and 8.3%, respectively.

When the results of the incubation experiments with nonamended cork were compared in relation to the cork matrix used, interesting differences were observed (**Figure 1**). Globally, the descriptors musty-earthy-TCA, musty-earthy, and vegetative had the highest incidence. Cork granules were the most sensitive substrate for the development of unpleasant odors with the following descriptors: musty-earthy-TCA, vegetative, chemical, spicy, and smoky. Musty-earthy off-odors were most frequently encountered in cork granules and agglomerated cork stoppers. The higher taint incidences in cork granules can be attributed to their high surface-to-volume ratio, which will enhance the growth and metabolic activity of microorganisms. Moreover, cork granules are derived from a complex mixture of cork from different origins and of different qualities, which may enhance the presence of metabolizable compounds that will eventually lead to off-odor development.

Natural cork was found to be a safer material. Incidence of off-odor development when natural cork stoppers were used was restricted to musty-earthy, green olive, and sulfur descriptors and appeared always at levels below 5% of the inoculated corks. A plausible explanation for the differences observed when natural or agglomerated cork stoppers are used is the potential effect of the glue used in the latter. It is worth mentioning that the appraisal of the different deviations was not exclusive to a defined strain or isolate, and some of them exhibited the capacity to promote off-odors assigned to different types (results not shown).

Olfactometric and Chemical Inspection of Sensory Deviations in Inoculated Cork. The aqueous macerates of corks with different sensory deviations were inspected using GC-O and GC-MS analyses. The analyses were restricted to samples bearing musty-earthy, musty-earthy-TCA, and vegetative deviations because these showed the highest off-odor incidence (%) in cork granules. One control sample consisting of the noninoculated cork granules incubated in the same experimental conditions was

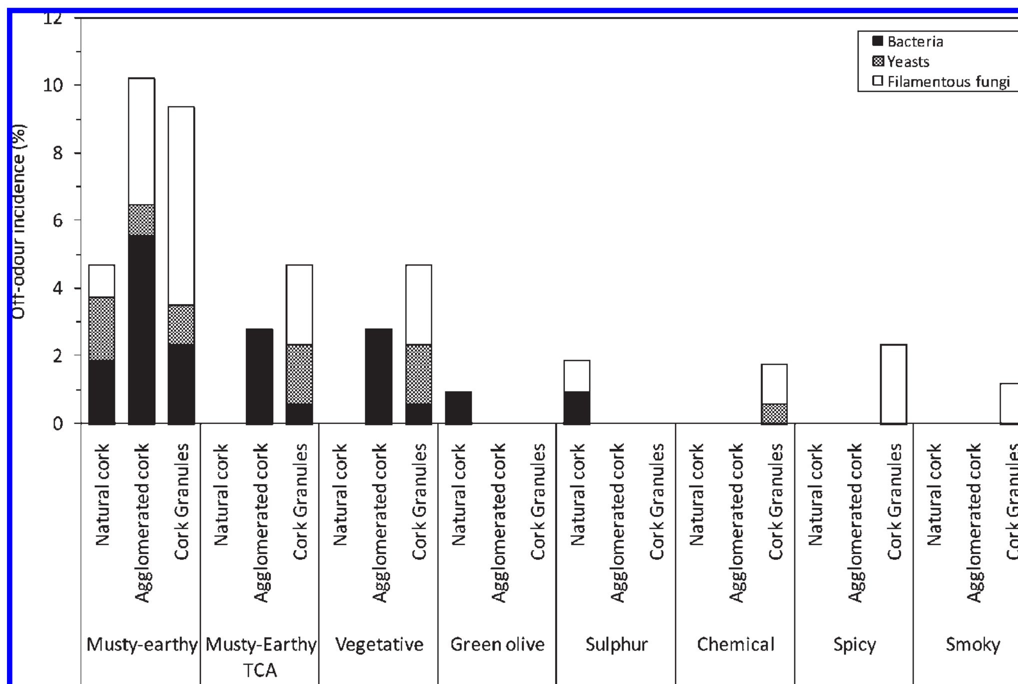

Figure 1. Incidence (%) of different off-flavors in cork matrices after 30 days of incubation at 25 °C with different fungal, bacterial, and yeast strains. Three replicates of every cork matrix were used for each strain isolated.

Table 2. Odorants Found in Inoculated Cork Granules: Gas Chromatographic Retention Data, Olfactory Description, Chemical Identity, and Mean Olfactometric Intensities (0−3 Scale, Six Judges)[a]

DB5 RI[b]	DBWAX RI[c]	odor descriptor	identity	control	vegetative	musty-earthy	musty-earthy-TCA
	1285	musty	unknown				**
1006	1300	fat, soap, lemon	octanal		**		
	1397	spicy	unknown		**		
1040	1450	musty, moldy	2-methoxy-3,5-dimethylpyrazine	*	***	***	*
1147	1520	chlorine	*Z*-2-nonenal	**	**		*
1210	1622	earthy	2-methylisoborneol			*	
1022	1646	roasty	2-acetylpyrazine	**			
1330	1832	musty	2,4,6-trichloroanisole	*	**	*	***
1497	1858	mossy, earthy	geosmin			**	
1089	1875	phenolic, chemical	2-methoxyphenol (guaiacol)	**	*	*	*
	1920	grass	unknown				**
	1968	musty	unknown				**
1064	2054	cotton candy	2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (Furaneol)	*	**	***	
1075	2100	bitumen, animal	*p*-cresol	*		**	
1469	2167	flowery	ethyl cinnamate	*			
1107	2217	curry, burnt	4,5-dimethyl-3-hydroxy-2(5*H*)-furanone (sotolon)	*	*		

[a] *, mean olfactometric intensity between 1 and 1.5; **, mean intensity between 1.5 and 2.5; ***, mean intensity between 2.5 and 3. [b] Retention index estimated in DB5 column. [c] Retention index estimated in DBWAX column.

included for analysis. Aroma profiles obtained with GC-O are shown in **Table 2**.

A total of 16 odor zones that scored a mean intensity of 2 in at least one sample were found. Most of them were identified, but four could not be assigned to a chemical compound. The aromatic profile for the control sample was found to be very simple. Nine odorants were detected in the control sample, three of which (*Z*-2-nonenal, 2-acetylpyrazine, and guaiacol) scored an average intensity of 2. In the sample described sensorially as vegetative, the presence of octanal (which was not found in any other macerate) and 2-methoxy-3,5-dimethylpyrazine should be highlighted. Moreover, 2,4,6-trichloroanisole and Furaneol have been detected in this sample with higher intensities than in the control one. These odorants, together with the other compounds detected, could contribute to the overall aromatic note.

The macerate sample with a "musty-earthy" note was characterized, again, by a high intensity of 2-methoxy-3,5-dimethylpyrazine, in addition to the presence of 2-methylisoborneol and geosmin. These two last odorants, both characterized by a potent "earthy, mossy" note, were found only in this cork sample, which had a moldy aroma. In addition, although they were perceived at a low intensity, a synergic effect was shown to exist between them. This means that when both odorants occur together, their aromatic potential is reinforced. The thresholds of these compounds are 10 ng L^{-1} for geosmin and 12 ng L^{-1} for 2-methylisoborneol in water solutions (*30*). La Guerche et al. also estimated these thresholds in a synthetic wine matrix (12% ethanol, 5 g L^{-1} tartaric acid, pH 3.5) (*30*). They found higher thresholds, attaining a value of 40 ng L^{-1}, for both odorants. However, a significant sensorial difference is appreciated when only 5 ng L^{-1}

Table 3. Concentrations of TCA and Guaiacol in Inoculated Cork Granules[a]

isolate	TCA (ng g^{-1} of cork)	guaiacol (ng g^{-1} of cork)
	Yeasts	
F019	0.6 ± 0.1	40.0 ± 49.9
F020	12.7 ± 16.7	56.9 ± 40
F025	5.3 ± 4.9	92.6 ± 15.5
	Filamentous Fungi	
F001	15.2 ± 14.0	127.9 ± 34.8
F003B	0.8 ± 0.3	98.6 ± 21.2
F005	0.5 ± 0.0	89.9 ± 1.1
F006	0.3 ± 0.0	93.3 ± 18.6
F018	0.4 ± 0.2	106.2 ± 10.2
F017	0.7 ± 0.2	nd
F002	0.6 ± 0.2	23.1 ± 5.2
F004	1.2 ± 0.7	82.0 ± 16.1
F003A	34.6 ± 24.5	51.1 ± 14.4
F011	0.3 ± 0.1	69.6 ± 13.9
	Bacteria	
A4	0.5 ± 0.0	95.6 ± 23.9
N1_2	1.4 ± 0.6	64.1 ± 18.7
N14	0.4 ± 0.0	104.6 ± 20.6
A1	26.1 ± 33.4	89.3 ± 26.2
N8	0.9 ± 0.5	114.9 ± 21.7
N5	0.5 ± 0.3	58.7 ± 9.0
noninoculated control	0.8 ± 0.2	127.0 ± 32.4

[a] Values are means and error standards of three replicates.

of each odorant is mixed in synthetic wine. Furaneol and *p*-cresol also have been detected with higher intensities in this sample than in the control one.

Finally, three notable odorants were found in the sample with the musty-earthy-TCA aromatic note. In addition to TCA, which scored a mean intensity of 3, two other compounds that have been described as musty (polar RI = 1285 and 1968) were found. Unfortunately, none of these compounds could be identified.

The characterization of macerates from inoculated cork granules was complemented by chemical analysis following the optimized HS-SPME-GC-MS method developed by Prat et al. (*26*). The compounds previously described in GC-O, including TCA, MDMP, guaiacol, MIB, and geosmin, were inspected. Limits of detection of the proposed chromatographic method were 1 ng L^{-1} for TCA, 2 ng L^{-1} for geosmin, and 5 ng L^{-1} for MIB and MDMP; for guaiacol the limit of detection was higher, at 0.5 μg L^{-1}. As shown in **Table 3**, only TCA and guaiacol could be quantified in the samples. The concentration of guaiacol was not significantly different ($p > 0.05$) between control and inoculated cork for most of strains. Only strains F002 and F017 presented a lower level of this compound when compared with the control experiments. In all cases the concentration that was determined was below its reported perception threshold (13–20 μg L^{-1}) (*31*); however, the possibility of its contribution to the aroma due to a synergistic effect cannot be discounted (*5, 32*).

Production of Specific Taint Compounds by Isolated Fungi and Bacteria. TCA production was above the value found in noninoculated cork in the yeasts *Cryptococcus* sp. F020 and *Rhodotorula* sp. F025, the filamentous fungi *P. glabrum* F001 and *P. variabile* F003A, and the bacterium *P. jessenii* A1 (**Table 3**). To the best of our knowledge, TCA accumulation in nonamended cork matrices is described for the first time with *Cryptococcus* sp. and *Rhodotorula* sp. isolates, which may be of particular interest because these are microorganisms commonly found in cork (*12*). Isolates F001 and F003A were found to be the strongest TCA producers (maximum concentration of 34.6 ± 24.5 ng g^{-1} of cork) among the filamentous fungi studied. In a previous work, we showed that some ITS1 sequences (accession no. FJ217293–FJ217296) were specifically found in samples exhibiting a strong TCA taint (*28*). A detailed comparison of those sequences with the ITS1-5.8S-ITS2 fragment obtained for isolate F003A shows an exact homology, evidence of the potential activity of this strain in TCA production.

Isolates identified as *P. glabrum* diverged greatly in both taint incidence and TCA production. In some cases (isolates F003B, F005, and F006) the taint incidence was >30% of the inoculated cork. Musty-earthy and vegetative notes were common for these strains. Key odorants for these notes were octanal, MDMP, and *Z*-2-nonenal according to GC-O analyses, but could not be quantified by the GC-MS method used.

The TCA generated by *P. jessenii* isolate A1 was similar (26.1 ± 33.4 ng g^{-1} of cork) to that obtained for fungi. Surprisingly, isolate A1 was obtained from colonies growing on plates used for regular microbiology analysis performed in the manufacturing companies with no selection of tainted cork samples. It is interesting to note that whereas *P. jessenii* has been identified in cork for the first time, unfortunately, no data are available to indicate its abundance in the cork manufacturing process and to evaluate the risk of taint development.

A detailed comparison of the results obtained for taint incidence and TCA accumulation in cork shows that there is a lack of coincidence for most strains. This result indicates that other odor-active compounds are being generated and accumulated in the cork. The inoculation of *P. fluorescens* N5 did result in a high taint incidence (33.3%) with a significant proportion of musty-earthy notes, which were characterized by the presence of MDMP, 2-methylisoborneol, and geosmin, according to GC-O analyses. Although the production of these compounds by isolate N5 has not been confirmed, it is likely to occur because other *Pseudomonas* strains have been implicated in the synthesis of MDMP and geosmin (*33*).

In conclusion, the use of GC-O has shown that it is a powerful tool with which to characterize the aromatic composition of cork samples bearing different and, so far, poorly studied taints such as non TCA-derived musty-earthy and vegetative. We have shown that 16% of the isolated microorganisms were able to produce TCA in nonamended cork granules but that a significantly higher proportion did contribute to taint development. Besides, the intrinsic diversity of microorganisms thriving over the surface of corks may suggest the existence of strain-specific differences in the TCA production capacity of several isolates. The GC-MS survey of suspected taint compounds resulted in a poor chemical characterization of incubated cork granules. Most of the compounds analyzed, except TCA and guaiacol, were found below the detection limit. Olfactometry of selected samples was used to characterize the odorants that may be responsible for the taints analyzed and allowed us to discuss the contribution of certain isolates to the formation of products such as MDMP and 2-methylisoborneol on cork. Nevertheless, further research using the isolates described is needed to fully characterize their production in complex growing media.

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