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Production of 4-ethylphenol in alperujo by *Lactobacillus pentosus*

Running title: Lactobacillus pentosus in alperujo

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Abstract

BACKGROUND: Alperujo is the paste generated from the two-phase extraction system of olive oil. This wet pomace must be stored for months and during this period, the formation of 4-ethyphenol provokes a strong off-odour. The aim of this work was to identify the microorganisms able to produce this volatile phenol.

RESULTS: Yeast and bacterial strains were isolated from stored alperujo and tested for their ability to metabolize *p*-coumaric acid and form 4-ethylphenol. Among them, *Lactobacillus pentosus* was the microorganism that both in synthetic medium and alperujo gave rise to 4-ethylphenol formation. This microorganism did not grow in alperujo acidified up to pH 2, thereby confirming the acidification as the best method to control odour emissions during alperujo storage.

CONCLUSION: Lactic acid bacteria, particularly *Lactobacillus pentosus*, can be responsible for the formation of the off-odour caused by 4-ethylphenol during the storage of alperujo, stink that can be prevented by acidifying the alperujo.

Keywords: Olive, alperujo, 4-ethylphenol, *Dekkera bruxellensis*, storage

INTRODUCTION

The two-phase centrifugation system is the technique used predominantly by most producer countries to obtain olive oil. With this technique, olive oil is separated from solid components, hydrosoluble substances and water that are present in the olive fruits. This olive pomace composes a paste or semisolid waste called alperujo, which represents the main residue of the olive oil extraction by weight.[1] In Spain, which is the most important olive-oil-producer-country, and many other Mediterranean countries, alperujo is generated in large quantities during the olive oil season and it is stored in large open air ponds for months until the residual oil is extracted by chemical or physical methods.[2,3] Chemical characterization of alperujos has been carried out in order to determine their suitability for agricultural use,[4,5] or as a source of interesting phenolic compounds.[6] Many changes occur in the alperujo paste during this storage period such as the enrichment of the oily phase in triterpenic acids,[7] acidity and fatty acid esters with short-chain alcohols.[8] Besides, a natural biodegradation of alperujo takes place,[9] which is provoked by the growth of different types of microorganisms, being yeasts the most widespread.[10,11] As a consequence of the microbial growth, offensive odours in the stored alperujo are generated, which create great concern for oil extracting plants, neighbouring communities and local governments. This off-odour is mainly caused by the formation of 4-ethylpheno, [12] which is a lipophilic substance that accumulates in the oily phase of stored alperujo.[3] This substance has been detected at low concentration in some olive products such as olive oil,[13] and table olives,[14] although most researches have been carried out to investigate and prevent its presence in wine, cider and other foodstuffs.[15,16]

Yeasts classified as *Brettanomyces/Dekkera* spp. have been associated with the formation of 4-ethylphenol in wine via the decarboxylation of *p*-coumaric acid to give 4-vinylphenol and further reduction of the latter substance.[16-18] *Brettanomyces bruxellensis* (anamorph of *Dekkera bruxellensis*) is the species more frequently related to this type of wine spoilage. To our knowledge, *B. bruxellensis* has never been detected in any olive products except in samples of Greek-style black olives.[19] Nonetheless, other yeasts, moulds, and different bacteria have been detected in alperujo and they could also be the responsible for 4-ethylphenol production in stored alperujo. For instance, lactic acid bacteria (LAB) have been occasionally found in alperujo samples,[11] and some species within this group of bacteria can produce 4-ethylphenol.[15,20]

It has been mentioned that alkalization,[21] and acidification,[11] of fresh alperujo may control odour emissions during its storage. However, if yeasts, particularly those of the *Brettanomyces/Dekkera* genus, are responsible for 4-ethylphenol formation in alperujo, some substances such as potassium sorbate, natamycin, copper and dimethyl dicarbonate,[16,22] could limit the growth of these microorganisms. We undertook a study to find out whether *B. bruxellensis* was the microorganism responsible for the formation of 4-ethylphenol in alperujo during its storage under open air conditions, and the effectiveness of several methods to control the appearance of the off-odour was also assessed. The feasibility that other microorganisms different to *B. bruxellensis* were involved in the production of the odour emissions was also investigated.

MATERIALS AND METHODS

Microbial strains and maintenance

Pure cultures of *Dekkera bruxellensis* were obtained from the Spanish Type Culture Collection (CECT, Burjasot, Valencia, Spain). Strains CECT 1009, CECT 1451 (Type strain), and CECT 11045 were propagated on GYP medium containing (g L^{-1}) glucose (Panreac, Barcelona, Spain) 20, yeast extract (Oxoid, Basingstoke, Hampshire, England) 5, peptone (Oxoid) 5, and agar (Cultimed, Panreac) 15 when required. Different wild strains of lactic acid bacteria (LAB) and yeasts were isolated from successive samples of industrial olive alperujos. LAB were cultured in de Man, Rogosa and Sharpe (MRS) (Biokar, Beauvais, France) broth or with 15 g L^{-1} agar. In some instances, sodium azide (Sigma-Aldrich, St. Louis, MO, USA) was added to melted MRS agar before pouring the medium to get a final concentration of 0.2 g L^{-1}, in order to selectively allow the growth of LAB exclusively. Yeasts were routinely propagated in GYP medium. Nevertheless, a modification of the WL medium,[23] was also used to search for *D. bruxellensis* from alperujo samples. Modified WL medium consisted of (g L^{-1}) glucose 40, yeast extract 10, peptone 15, bromocresol green (Merck, Darmstadt, Germany) 22 mg L^{-1}, and agar 18 g L^{-1}. Final pH was adjusted at 5.5 and cycloheximide (Sigma-Aldrich) was added to the melted medium to get a final concentration of 50 mg L^{-1}. Identification of LAB isolates was based on carbohydrate fermentation pattern determined with the system API 50 CHL (bio-Mérieux), followed by discrimination between *Lactobacillus plantarum* and *Lactobacillus pentosus* species using the PCR method described by Torriani et al.[24] This method is necessary to differentiate between the species *L. pentosus*, *L. plantarum* and *L. paraplantarum* as nucleic acid sequencing is not accurate for these proximal species.

Inoculation of *D. bruxellensis* in alperujo samples

Alperujo samples from both industrial origin (Oleícola el Tejar SCA) and the experimental olive oil mill at the Instituto de la Grasa CSIC were inoculated with strains of *D. bruxellensis*. The procedure consisted of adding *p*-coumaric acid (Fluka, Buchs, CH) to the alperujo (final concentration of 100 mg kg^{-1}), and part of the batch was sterilized in autoclave (121 ºC, 15 min) whereas the other part was not heat treated, but different antifungal compounds were added instead. Natamycin (Panreac, Barcelona, Spain) 200 mg kg^{-1}, dimethyl dicarbonate (Sigma) 250 mg kg^{-1}, sodium benzoate (Panreac) 500 mg kg^{-1}, potassium sorbate (Panreac) 500 mg kg^{-1}, and copper sulphate (Panreac) 100 and 200 mg kg^{-1} were assayed and compared with controls without any antimycotic. For all treatments, duplicate samples were left without inoculation and duplicate lots were inoculated with a cocktail of cultures in GYP broth of the strains of *D. bruxellensis* CECT 1009, CECT 1451 and CECT 11045. Initial population after inoculation was 4.9 x 10^5 *D. bruxellensis*-CFU g^{-1}. Samples were incubated under anaerobiosis at 25 ºC for 5 months.

Experiments to avoid formation of 4-ethylphenol during alperujo storage

Different treatments were applied to an industrial batch of alperujo with the aim of preventing the growth of *D. bruxellensis* and hinder the release of 4-ethylphenol. A total of 12 PVC containers were filled with 75 kg alperujo each and the following treatments were carried out: Controls left without any change; Acidified to about pH 2 with nitric acid; Alkalized to about pH 11 with calcium hydroxide; treated with Fungicide Chlorothalonil at 1500 mg kg^{-1} dose; treated with copper sulphate at a final concentration of 100 mg kg^{-1} (Cu-100); and treated with copper sulphate at a final concentration of 500 mg kg^{-1} (Cu-500). All treatments were done in duplicate and the drums were placed out in the open air to mimic the industrial circumstances. Samples

were withdrawn along time for microbiological and chemical analyses. The microbial population in alperujo throughout storage was monitored by spreading alperujo samples and their decimal dilutions onto different culture media. Alperujo samples were aseptically taken from 5–10 cm depths after removing the dry surface with sterile tools, directly spread with Drigalski spatula, and their dilutions (in sterile 1 g L^{-1} peptone water) were spread with a Spiral Plater (Don Whitley Sci. Ltd., Model Wasp 2, Shipley, UK). Lactic acid bacteria were counted on MRS agar (Biokar), and yeasts and molds on Oxytetracycline-Glucose-Yeast extract (OGYE) agar (Oxoid). Plates were incubated at 32 ºC for up to 72 h, and colony forming units were counted (Counter-mat, IUL Instruments, Barcelona, Spain).

Inoculation of isolated microorganisms in GYP culture broth enriched with *p*-coumaric acid

Metabolization of *p*-coumaric acid by the different microbial isolates was investigated after their cultivation in GYP broths with only 1.5 g L^{-1} glucose and enriched with 50 mg L^{-1} of *p*-coumaric acid (Fluka). In order to assure a heavy initial inoculum, each strain was cultured onto GYP or MRS agar plates for yeasts and LAB, respectively. Cells gathered from each plate were first homogenized in sterilized saline (9 g L^{-1} NaCl), then 0.1 mL of each suspension were inoculated into test tubes with 5 mL GYP + *p*-coumaric broth, and finally all the tubes were incubated at 30 ºC. Uninoculated tubes were used as controls, and duplicate tubes for each sample were analyzed by HPLC to determine their concentrations in *p*-coumaric acid, and in 4-ethylphenol and 4-vinylphenol as metabolic products.

Inoculation of isolated *Lactobacillus pentosus* in several alperujo batches

L. pentosus strains isolated from industrial alperujo were inoculated in several batches of alperujo supplied by the company Oleícola el Tejar SCA from two different olive mill factories located in southern Spain (Pedro Abad and Palenciana, Córdoba, Spain). The codes of the batches were 4, 5, 21, 26 and 34. Alperujo 4 and 5 were sterilized in autoclave (121 ºC, 15 min), inoculated with the *L. pentosus* strains, and incubated at 30 ºC for 4 months. A part of the batches were acidified up to pH 2 with phosphoric acid previously to sterilization and inoculation. Another part of the batches were not acidified nor inoculated to be considered as control. Growth of LAB was checked periodically, and the concentrations of *p*-coumaric acid, comselogoside and 4-ethylphenol were analyzed in the whole alperujo pastes and in their lipid phases at the end of the experiment.

Due to the presence of LAB inhibitors in olive products,[25] alperujo samples coded as 21, 26 and 34 were chosen because of their low concentration in these substances, sterilized in autoclave (121 ºC, 15 min), and inoculated with the *L. pentosus* strains. Subsequently, they were incubated at 30 ºC for 45 days. A part of the samples were not inoculated to be considered as controls. The concentration of *p*-coumaric acid, comselogoside and 4-ethylphenol was analyzed in the whole alperujo paste at the end of the experiment.

Chemical analyses

Analyses of the acidity, wax content, sterols, and fatty acid composition of alperujo oil samples were carried out according to the official methods described by the European Community Regulation EEC/2568/91.[26] Organic acids (lactic, acetic and butyric acids) and ethanol were analyzed in the aqueous phase of the alperujo samples by HPLC as described elsewhere.[27]

Phenolic compounds were extracted from the alperujo paste with dimethyl sulfoxide (DMSO).[28] Around 0.1 g of the paste were put into contact with 0.5 mL of DMSO, vortexed for 1 min and sonicated for 5 min. This process was repeated twice and the mixture was centrifuged at 6000 x g for 5 min. Then, 0.25 mL of the supernatant were diluted with 0.5 mL of DMSO and 0.25 mL of 0.2 mmol L^{-1} syringic acid (internal standard). Finally, the mixture was filtered through a 0.22 µm pore size nylon filter and 20 µL were injected into the chromatograph. The chromatographic system consisted of a Waters 717 plus autosampler, a Waters 600E pump, and a Waters heater module (Waters Inc. Mildford, MA). A Spherisorb ODS-2 (5 µm, 25 cm x 4.6 mm i. d., Waters Inc.) column was used. Separation was achieved using an elution gradient with an initial composition of 90 % water (pH adjusted to 2.7 with phosphoric acid) and 10 % methanol. The concentration of the later solvent was increased to 30 % over 10 min and maintained for 20 min. Subsequently, the methanol percentage was raised to 40 % over 10 min, maintained for 5 min, and then increased to 50 %. Finally, the methanol percentage was increased to 60, 70, and 100 % in 5 min periods. Initial conditions were reached in 10 min. A flow of 1 mL min^{-1} and a temperature of 35 ºC were used in all of the assays. Phenolic compounds were monitored using a Waters 996 diode array detector (280 and 340 nm) and a Jasco fluorescence detector (emission at 280 nm and excitation at 320 nm) connected in series.

The oily phase was separated from the alperujo paste by centrifugation at 6000 x g for 15 min. Subsequently, the supernatant oil was taken by using a Pasteur pipette. Phenolic compounds were extracted from the oil with *N,N*-dimethylformamide, following the procedure described elsewhere.[7] These substances were analyzed by HPLC as described above.

Statistics

The experiments included duplicates and all the measurements carried out were done in duplicate. Basic statistics were calculated using the Microsoft Excel spreadsheet. Mean values and standard deviation (SD) are given in tables and figures.

RESULTS AND DISCUSSION

Inoculation of *D. bruxellensis* in alperujo samples

All the pure cultures of *Dekkera bruxellensis* CECT 1009, CECT 1451 and CECT 11045 cultivated in GYP medium enriched with *p*-coumaric acid gave rise to the formation of 4-ethylphenol and the consumption of the former substance (data not shown). Then, a cocktail of these strains was inoculated in two fresh alperujo spiked with several antimycotics such as sodium benzoate, potassium sorbate, natamycin, copper sulphate and dimethyl dicarbonate. After months of incubation of the paste at 30 ºC, it was not detected off-odour in any of the experiments. Olfactory detection of 4-ethylphenol in alperujo is a sensible method. In fact, odour threshold in wines is 450 µg L^{-1}.[29] The presence of the inoculated microorganisms was not checked in the alperujo but it must be said that growth of *D. bruxellensis* in real matrixes as wine is not easy, and the dilution with synthetic medium has been used.[17] Besides, researchers have not found on many occasions a good correlation between levels of 4-ethylphenol in wines and growth of these yeasts.[30,31]

Experiments to avoid formation of 4-ethylphenol during alperujo storage

New experiments were carried out at pilot plant scale with fresh alperujo obtained from a local processor. Twelve PVC containers with 75 kilograms of alperujo were stored under air conditions for months and subjected to different treatments to restrain yeast growth. Acidification of the paste up to pH 2,[11] alkalization up to pH 11,[21] and the use of several antimycotics such as copper sulphate and Chlorothalonil was assessed to control odour emissions. Figure 1 shows the population of yeasts with the different alperujo treatments at three different points along the storage period. At day 1, Control containers displayed a yeast population slightly above 10^4 CFU g^{-1}, whereas close to 10^2 was present in Alkalized, Chlorothalonil, and 100 mg kg^{-1} Copper sulphate treatments. Yeasts were not detected this first day in Acidified (Ac), and Copper sulphate 500 mg kg^{-1} treatments. At day 100, yeast population had increased with all treatments except in the alkalized containers, which presented a similar value to the initial, and the acidified containers, which again did not contain yeasts. Finally, the results obtained at day 180 confirmed that acidification was the only successful method to prevent yeast growth throughout the storage period. Apart from yeasts, other microorganisms tested were lactic acid bacteria. This group of microorganisms were detected in the alkalized treatments (>10^7 CFU g^{-1}), whereas they were not detected in any of the other containers (data not shown). These results agreed with the level of lactic acid found in the aqueous phase of alkalized alperujo and absence of this substance in the rest of treatments (Table 1). Moreover, a higher concentration of acetic acid was found in the alkalized alperujo than in the rest of treatments, and a significant amount of butyric acid (4800 mg L^{-1}) was formed in one of the alkalized alperujo duplicates. Olive mill wastes are very complex ecosystems from a microbiological point of view.[32] Although yeasts seems to account for around 90 per cent of the total microbiota,[10] there are differences among batches and among olive varieties.[33] In our case, it is presumable that

alkalinization favoured the growth of lactic acid bacteria and, in one of the containers, allowed the formation of butyric acid, which is a typical final product of clostridial metabolism. Although Clostridia were not analysed, both *Lactobacillaceae* and *Clostridiaceae* are among the taxa that have been identified in olive mill wastes.[32]

Few changes were observed in the pH of the alperujo paste of treatments with no acid or alkali added, ranging from 4.5 to 5 units. The pH of the acidified paste slightly increased from 2 to 2.5 at the end of the storage period, and a marked decrease in the initial pH of the alkalized paste from 11 to 5 units occurred during storage, which can be explained by the higher formation of acids in the alkalized containers. Additionally, the acidity and sterols content of the oily phase of alkalized alperujo were significant higher than in the rest of treatments (data not shown). All these data indicate that, in our case, alkalization of the alperujo did not guarantee the stability of the paste along time, but it may suffer fermentation that can provoke formation of off-odour such as butyric acid in some instances. In contrast, acidification of the paste prevented the growth of yeasts (Figure 1) and formation of the off-odour 4-ethylphenol was not detected (Table 1). However, 4-ethlyphenol was only found in alkalized alperujo (Table 1), and it was not detected in any other container despite the fact that a significant yeast population was recorded in all the control, treated with fungicide and treated with copper containers (Figure 1), and thereby the absence of this compound cannot be related to the population of yeasts detected in the pastes. The fact that this volatile phenol was only found (Table 1) in alkalized alperujo, where lactic acid bacteria grew, revealed a possible role of these microorganisms in the formation of 4-ethylphenol . Olive products such as table olives, olive oil and alperujo contain substances with phenolic nature that

show anti-lactic acid bacteria activity,[25] and lactic acid bacteria are not always present in all batches of alperujo.[11]

Inoculation of isolated microorganisms in GYP culture broth enriched with *p*-coumaric acid

Taking into mind that 4-ethylphenol was not present in any of the treatments assayed except in alkalized alperujo, yeasts and lactic acid bacteria were isolated from several industrial batches of not fresh, but stored alperujo with strong off-odour, and the capability of the isolates of producing volatile phenols in GYP broth with *p*-coumaric acid was studied (Table 2). Some of the isolates (P8g, P8h, P8j and P8x) slightly reduced the concentration of the spiked *p*-coumaric acid but they did not produce 4-ethylphenol or 4-vinylphenol; a high number of the isolates consumed the precursor but did not produce any of the volatile phenols; and only two isolates (P6k and P6L1) consumed the *p*-coumaric acid and gave rise to 4-ethylphenol. It should be remembered that 50 mg L^{-1} of *p*-coumaric acid were added to the GYP broth (see section 2.4.), which means 0.30 mmol L^{-1}. The low value of this compound in the control tubes (25.2 mg L^{-1}) can be explained due to the low hydrosolubility of this phenol, although that difficulty did not hindered its metabolization . Both 4-ethylphenol (0.13 mmol L^{-1}) and 4-vinylphenol (0.04 mmol L^{-1}) were detected in the synthetic medium inoculated with the isolate P6L1, and only 4-ethylphenol (0.24 mmol L^{-1}) was detected in broth inoculated with the isolate P6k. These microorganisms were identified as lactic acid bacteria, and according to their carbohydrate fermentation pattern determined with the system API 50 CHL and their PCR band pattern,[24] both isolates were allocated to the species *Lactobacillus pentosus*. This species is the prevalent microorganism in brines of

Spanish-style green olives,[34] and the strain LP99 isolated from an olive brine also produced 4-ethylphenol (0.15 mmol L^{-1}) in the synthetic medium (Table 2). It has been reported that several species of the genus *Lactobacillus* are able to produce 4-ethylphenol such as *L. collinoides*,[15] *L. plantarum*,[20] and *L. brevis*,[35] but to our knowledge, it has never been associated with *L. pentosus* either in food or particularly in olive products. It should be highlighted that the attempts to isolate *D. bruxellensis* from the smelling industrial samples of alperujo did not succeed, even by using the medium specifically designed with that aim.[23] Moreover, this yeast species has not been found in the studies by Giannoutsou et al.[36] nor it is present in the comprehensive review by Ntougias et al.[32]

Inoculation of isolated *Lactobacillus pentosus* in several alperujo batches

The strain *L. pentosus* P6k, chosen for its higher 4-ethylphenol production, was inoculated in sterilized samples of industrial Alperujo 4 and 5 and incubated at 25 ºC for 4 months. Figure 2 shows that the population of LAB decreased during the first week but it increased thereafter and reached more than 10^8 CFU g^{-1} at day 20. It must be noticed that LAB were not detected in the uninoculated control paste or the acidified paste up to pH 2 and inoculated. Inoculation of *L. pentosus* P6k in Alperujo 4 did not lead to a clear effect on the formation of 4-ethylphenol (Figure 3), the concentration of this substance being higher in the control paste than in the acidified alperujo although similar to the inoculated treatment. In contrast, both the control and the acidified paste inoculated with *L. pentosus* P6k in Alperujo 5 were absent of 4-ethylphenol after 4 months of incubation whereas a significant concentration of this substance was detected in the inoculated paste. As it has been commented above,[7] 4-ethylphenol is a lipophylic

compound that concentrates in the oily phase of alperujo, thereby it was analyzed in the oil pomace of Alperujo 4 and 5 (Figure 4). Consequently, a much higher concentration of the volatile phenol was found in the oily phase (Figure 4) than in the alperujo paste (Figure 3). It was confirmed the presence of 4-ethylphenol in the oil of inoculated Alperujo 5 but not in control or acidified treatments of this paste. Moreover, a higher concentration of 4-ethylphenol was also observed in the oil of inoculated Alperujo 4 than in the control or acidified treatments.

Due to differences between Alperujo 4 and 5, more than 10 batches of alperujo from several olive oil factories were obtained and the concentration of antimicrobials in them was tested. Those with low concentration in antimicrobial compounds such as the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol,[25] were chosen for a new inoculation experiment with *L. pentosus* P6k. In the three olive pastes of inoculated Alperujo 21, 26 and 34 the concentration of 4-ethylphenol was higher than in the non-inoculated control (Figure 5). In contrast, the concentration of the precursors comselogoside and *p*-coumaric acid was lower in the inoculated than in the control. All these data confirmed that formation of the off-odour 4-ethylphenol in alperujo during its storage can be due to the growth of LAB, in particular *Lactobacillus pentosus*.

CONCLUSIONS

Although different microorganisms can generate 4-ethylphenol in other food products, the results discussed in the present work indicated that lactic acid bacteria, particularly *Lactobacillus pentosus*, can be responsible for the formation of the off-odour caused by 4-ethylphenol during the storage of alperujo. Because the growth of lactic acid bacteria is inhibited at low pH, acidification of the paste up to pH 2 can prevent appearance of

odour emissions during storage of alperujo. In contrast, alkalization of the paste does not seem a reliable preservation method because alperujo, in some instances, can ferment and develop off-odour compounds such as 4-ethylphenol and butyric acid.

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Figure captions

Figure 1. Yeast population in alperujo paste with different treatments during storage for 6 months. Bars indicate the standard deviation of duplicates.

Figure 2. Lactic acid bacteria population in Alperujo 4 and 5 inoculated with *L. pentosus* P6k and incubated at 25ºC for 4 months. Bars indicate the standard deviation of duplicates.

Figure 3. Concentration of *p*-coumaric acid, comselogoside and 4-ethylphenol in Alperujo 4 and 5 inoculated with *L. pentosus* P6k and incubated at 25ºC for 4 months. A batch of alperujo was also acidified up to pH 2, inoculated and incubated as described above. Bars indicate the standard deviation of duplicates.

Figure 4. Concentration of 4-ethylphenol in the oily phase of Alperujo 4 and 5 inoculated with *L. pentosus* P6k and incubated at 25ºC for 4 months. A batch of each alperujo was also acidified up to pH 2, inoculated and incubated as described above. Bars indicate the standard deviation of duplicates.

Figure 5. Concentration of *p*-coumaric acid, comselogoside and 4-ethylphenol in Alperujo 21, 26 and 34 inoculated with *L. pentosus* P6k and incubated at 25ºC for 45 days. Bars indicate the standard deviation of duplicates.

Table 1. Concentration (mg L^{-1}) of metabolic products in the aqueous phase of alperujo stored under different conditions for 6 months.

Treatment	Lactic acid	Acetic acid	Ethanol	4-Ethylphenol
Control	ND[a]	400	2900	ND
Acidified	ND	650	7050	ND
Alkalized 1	12400	3400	400	43
Alkalized 2[b]	7400	2700	1200	33
Chlorothalonil	ND	400	700	ND
Cu-100	ND	350	600	ND
Cu-500	ND	200	540	ND

[a]ND: not detected. [b]A concentration of 4800 mg L^{-1} of butyric acid was found in the aqueous phase of this alperujo

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Table 2. Grouping of the different microbial strains isolated from several batches of stored alperujo according to their activity in GYP broths spiked with 50 mg L^{-1} of *p*-coumaric acid, in relation to the final concentration ranges (mg L^{-1}) of *p*-coumaric acid, 4-ethyl phenol and 4-vinylphenol after 7-day incubation of the inoculated tubes.

Strains	*p*-Coumaric acid	4-Ethylphenol	4-Vinylphenol
Uninoculated control	25.2 (0.0)[a]	0.0	0.1 (0.0)
P8g; P8h; P8j; P8x	19.1 – 23.2	0.0	0.0 – 0.4
P6aB; P6aG; P6b; P6c; P6d; P6m; P6n; P8i; P8y; P10r; P10s; P10t	0.0 – 6.0	0.0	0.2 – 0.3
P6k	0.0	28.9 (1.2)	0.0
P6L1	0.0	15.4 (5.4)	4.5 (1.6)
LP99[b]	0.0	19.0 (0.9)	1.1 (0.3)

[a]Standard deviation of duplicate in parenthesis. [b]LP99 is a *Lactobacillus pentosus* strain isolated from a table olive brine.

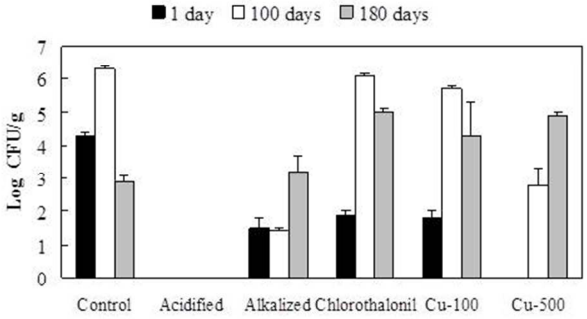

Figure 1
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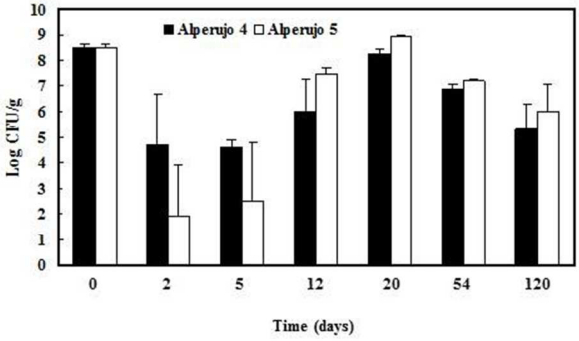

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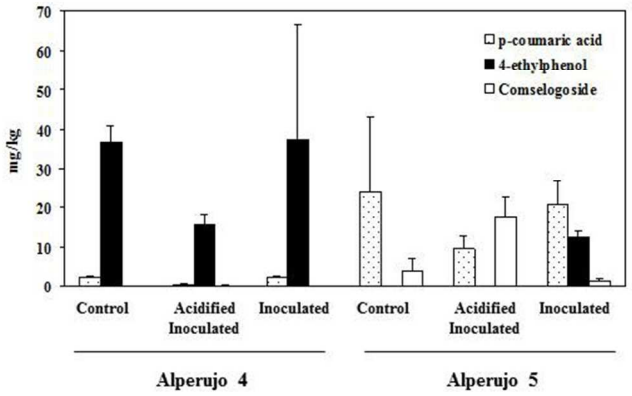

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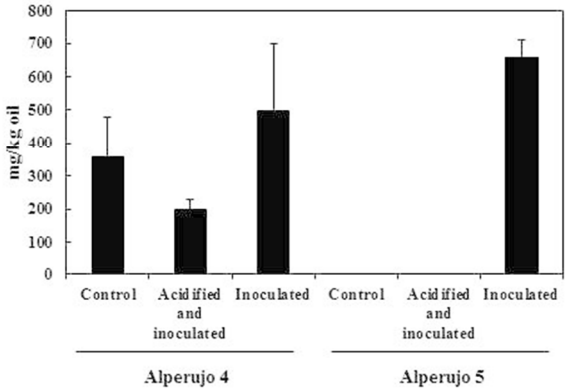

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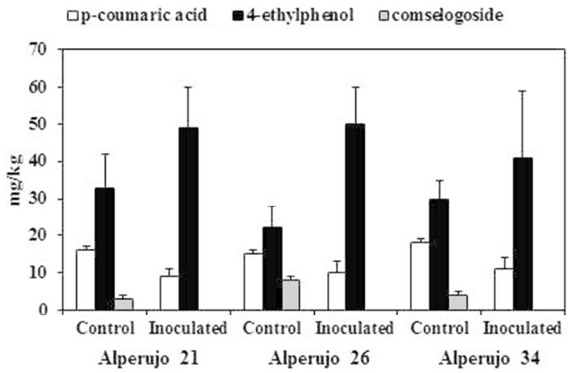

Figure 5
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