# Microbial and chemical changes during the spontaneous ensilage of grape pomace

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C.G. de PINA AND T.A. HOGG. 1999. Pilot scale fermentations with grape pomace from two different wineries were investigated during the 24 weeks of the ensiling period, along with laboratory scale experiments in which the environmental temperatures were held constant at 20, 25, 30 and 35 °C. During this period, yeast and lactic acid bacteria (LAB) counts were made, after which the identity of both groups of organisms was studied, as were the major microbial metabolites present. Major microbial and chemical alterations occurred during the first 3 weeks of ensilage, leaving a more stable product differing significantly from the initial substrate. The results obtained indicated that after initial growth, yeast and LAB populations undergo progressive inactivation at environmental temperatures above 20 °C, although LAB seem to adjust better to this specific, post-fermentation environment. Homofermentative species of *Lactobacillus* were the dominant LAB. The initial yeast flora of non-*Saccharomyces* species was replaced by a typical wine yeast flora, i.e. predominantly *Saccharomyces cerevisiae.* At the chemical level, major alterations were due to an alcoholic fermentation and a malolactic conversion within the first 3 weeks.

## INTRODUCTION

Grape pomace is the solid residue left after juice extraction in the wine-making process. It includes grape skins and seeds and, to a varying extent, stalks. In total, these components make up 10–20% of the total grape mass (Rice 1976; Famuyiwa and Ough 1982; Hang and Woodams 1986). In Portugal, this figure represents around 100 000–200 000 tons year$^{-1}$ (Anon. 1997). Possessing a moisture content of about 60%, it constitutes the most important solid waste product of the wine industry (Rice 1976; Famuyiwa and Ough 1982).

Until recently, the most widespread use of this by-product has been in the production of a spirit beverage; in Portugal, such products are termed 'Bagaceiras', and the French and Italian equivalents are known as 'Marc' and 'Grappa', respectively. In many wine-growing countries, certainly in Portugal, the production and commercialization of such beverages has suffered a considerable decrease in recent years. A combination of changing tastes and drinking habits, and strict environmental legislation for distilleries, has made all but the highest value products non-viable.

For these reasons, it is of interest that alternative uses for this substantial by-product are found, both for the production of commercially valuable products, and for the proper management of waste. By-products such as grape seed oil, anthocyanins, tannins, tartrates, fertilizer (Rice 1976), citric acid produced by solid state fermentation (Hang and Woodams 1986) and animal feed supplement (Famuyiwa and Ough 1982) have limited markets, and can only absorb a small proportion of the waste generated. In Portugal, as in other wine-growing countries, this grape pomace is widely used as a fertilizer/soil conditioner.

The initial microflora of pomace is significantly influenced by the wine environment from which the pomace derives. In the case of white wine pomace, an alcoholic (yeast) fermentation occurs (both in wine and pomace) after separation of the pomace. This will normally precede or be concurrent with other microbial activities such as those affected by lactic acid bacteria (LAB). In the case of red wine pomace, the alcoholic fermentation occurs before the separation of the pomace. After separation of liquid in the wine-making process, that pomace which is destined for distillation is normally pressed, ensilaged in horizontal silos or concrete 'earthrep' tanks and eventually distilled. During this solid fermentation process, a number of biochemical conversions occur which greatly affect the subsequent composition of the

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product and any by-products obtained. It is widely accepted that LAB play an important part in these alterations, but this has not been studied in detail. These bacteria convert sugars under anaerobic conditions into lactic acid; as a result, pH decreases and the silage is preserved (Weinberg *et al*. 1988).

The aim of this study was to investigate the diversity and identity of the yeast and LAB associated with the spontaneous fermentation of ensiling grape pomace, and correlate these with the profile of major microbial metabolites in the matrix. In this study, a pilot-scale fermentation was used with white pomace from two different wineries, and laboratory-scale experiments were carried out in which the environmental temperatures were varied between 20 and 35 °C.

## MATERIALS AND METHODS

### Grape material and fermentations

White grape pomace samples (*Vitis vinifera* var. *Alvarinho* and *Vitis vinifera* var. *Loureiro*) were collected in two different wineries from the Vinho Verde region of the north of Portugal during the 1994 and 1995 vintages. Pilot-scale experiments were performed in polyethylene tanks (250 kg) under essentially anaerobic conditions, the fermentations being effected at the prevailing ambient temperature. Laboratory-scale experiments were in hermetically sealed glass flasks (500 g), the incubation temperatures being varied between 20 and 35 °C.

### Sampling

The sampling period included the 'normal processing period' and the 'post-processing abuse period' of ensiling grape pomace. The 'normal processing period' corresponds to the maximum period (12 weeks) recommended for the distillation of grape pomace to beverage spirit. Fermentation/bulk storage can occur after the 12 weeks of ensilage; this is called the 'post-processing abuse period'. Samples were taken progressively throughout 'normal processing' and 'post-processing' and analysed microbiologically. Progressive samples of all experiments were frozen at −25 °C for subsequent extraction and chemical analysis.

### Microbiological analysis

*Yeast enumeration and identification.* Samples of 30 g of the grape pomace were collected randomly from the centre section of the pilot-scale pomace vessels. Samples were homogenized for 10 min in a Stomacher (Seward Medical, London, UK) with 270 ml 1/4 strength Ringer solution. Homogenates were decimally diluted from $10^{-1}$–$10^{-8}$ in the same diluent.

Diluted homogenates obtained at the various sampling times and at the different temperatures were plated using the Drop Count Technique (Miles and Misra 1938) on YM agar (Difco) previously acidified to pH 4·5 with lactic acid and incubated at 25 °C for 3 d. All counts are the results of duplicate platings. The various macroscopic colonies formed were counted, and representative colony forms were isolated and maintained on YM slopes at 4 °C prior to identification. Four to eight colonies were picked from each sampling (0, 3, 6, 9 and 12 weeks) from *Alvarinho* and *Loureiro* varieties until 12 weeks of the ensiling period. After this period, two colonies were picked from both varieties of grape pomace until 24 weeks of ensiling time (15, 18, 21 and 24 weeks). All colonies were selected based on a careful appreciation of colony colour and morphology. The morphological, physiological and biochemical characterization of these yeasts was investigated on media and under the incubation conditions described by Van der Walt and Yarrow (1984). Sugar assimilation tests were carried out using API ID 32C galleries (Biomérieux) with Yeast Nitrogen Base (Difco) as suspending diluent. The other tests included: fermentation of glucose and saccharose; assimilation of nitrogen compounds (cadaverine, L-lysine and nitrate); temperature tolerance (growth at 30, 37 and 42 °C); growth in the presence of 0·01 and 0·1% cycloheximide and in the presence of 1% acetic acid; and the presence of pseudohyphae and true mycelium. *Saccharomyces cerevisiae* 14 C-3507 (Gulbenkian Institute) was used as a control strain for sugar reactions. The commercially available computer program of Barnett *et al*. (1994) was used for identification.

*LAB enumeration and identification.* The same diluted pomace homogenates were inoculated using the Drop Count Technique (Miles and Misra 1938) on MRS agar (Lab M) with 0·25 g $l^{-1}$ cyclohexamide (Sigma) and incubated at 30 °C for 3 d under aerobic and anaerobic conditions (Difco Anaerobic System). The various macroscopic colonies formed were counted, and representative distinct colonies were picked and purified by streak plating on the same medium. Four to ten colonies were picked at each sampling (0, 3, 6, 9 and 12 weeks) from *Alvarinho* and *Loureiro* varieties until 12 weeks of the ensiling period. After this period, two to five colonies were picked from both varieties of grape pomace until 24 weeks of ensiling time (15, 18, 21 and 24 weeks). A pre-screening of the bacteria was performed on the basis of cell morphology, Gram stain reaction and catalase test. The Gram-positive and catalase-negative colonies were considered to be lactic acid bacteria. Representative strains of LAB were preserved at 4 °C as deep-stabs in MRS agar, prior to identification. The specific identification of LAB was made by numerical comparison of SDS solubilized whole-cell protein patterns of isolates by SDS-PAGE, with the patterns of type and reference strains of species commonly associated with wines (Table 1). The method was performed as described by

**Table 1** List of type and reference lactic acid bacteria strains used in this study

| Species | Strain* |
|---|---|
| *Lactobacillus acidophilus* | NCFB 1748$^T$ |
| *Lactobacillus brevis* | NCFB 1749$^T$ |
| *Lactobacillus brevis* subsp. *gravesensis* | LMG 7934$^{T0}$ |
| *Lactobacillus buchneri* | NCFB 110$^T$ |
| *Lactobacillus bulgaricus* | NCFB 1489$^T$ |
| *Lactobacillus casei* | NCFB 161$^T$ |
| *Lactobacillus collinoides* | NCFB 2805$^T$ |
| *Lactobacillus fermentum* | NCFB 1750$^T$ |
| *Lactobacillus fructivorans* | LMG 9202 |
| *Lactobacillus fructivorans* | NCFB 2167$^T$ |
| *Lactobacillus helveticus* | NCFB 2712$^T$ |
| *Lactobacillus hilgardii* | NCFB 264$^T$ |
| *Lactobacillus homohiochi* | NCFB 2402 |
| *Lactobacillus kefir* | NCFB 2737$^T$ |
| *Lactobacillus mali* | NCFB 2168$^T$ |
| *Lactobacillus plantarum* | NCFB 1752$^T$ |
| *Lactobacillus viridiscens* | NCIB 8965$^T$ |
| *Lactobacillus lactis* subsp. *lactis* | LMG 6890$^T$ |
| *Lactobacillus lactis* subsp. *cremosis* | LMG 6897$^T$ |
| *Leuconostoc oenos* | NCFB 1674$^T$ |
| *Pediococcus acidilactici* | NCFB 2767$^T$ |
| *Pediococcus damnosus* | NCFB 1832$^T$ |
| *Pediococcus dextrinicus* | NCFB 1561$^T$ |
| *Pediococcus pentosaceus* | NCFB 990$^T$ |

* Type strains are indicated by superscript T.
LMG : Culture Collection Laboratorium voor microbiologie, State University of Gent, Belgium ; NCFB : National Collection of Food Bacteria, Shinfield, UK ; NCIB : National Collection of Industrial Bacteria.

Laemmli (1970) modified by Kiredjian *et al*. (1986) under the conditions specified by Couto and Hogg (1994).

### Chemical analysis

*Sampling preparation.* Frozen grape pomace (30 g) was blended for 5 min with 270 ml hot (55 °C) distilled water (for sugars, organic acids and glycerol analysis) or cold distilled water (for ethanol analysis) in a Stomacher. The extract was successively filtered through folded filters (Macherey-Nagel, Düren, Germany) and membrane cartridges (pore 0·45 $\mu$m; Costar Corporation, Cambridge, MA, USA) with 320 mm and 25 mm diameter, respectively.

Sugars (glucose and fructose), organic acids (lactic, malic, tartaric, succinic and acetic), glycerol and ethanol were analysed by HPLC (Beckmann Gold 126, San Ramon, CA, USA) with a Polyspher OA KC, Merck column (300 × 7·8 mm), RI (Knauer) and u.v. 210 nm detection (Beckman Diode Array 168); the mobile phase was $H_2SO_4$ (0·00425 mol $l^{-1}$), the flow rate was 0·4 ml $min^{-1}$ and the injection rate was 20 $\mu$l.

## RESULTS

### The effect of temperature on the succession of microbial populations during the spontaneous fermentation of grape pomace

The overall changes in the total LAB and yeast counts during the normal processing and post-processing abuse periods in ensilaged grape pomace are shown in Fig. 1. This figure represents results for the pilot-scale experiments and laboratory-scale experiments at the various temperatures used. The results were obtained by averaging the viable counts from the two types of grape pomace studied, i.e from *Alvarinho* and *Loureiro* grapes. The changes in total yeast numbers at 20 °C and at pilot scale were very similar throughout the normal processing and abuse periods, as were those for total LAB, justifying to a certain extent the validity of the laboratory-scale experiments at 20 °C. The fresh grape pomace showed a yeast population higher than the LAB population of around 1 log cycle. After 3 weeks of ensilage, the total yeast numbers showed a dramatic decline, whereas the LAB population showed an increase at all temperatures tested. As described in the same figure, both groups of organisms underwent progressive inactivation at environment temperatures above 20 °C after the first 3 weeks of ensilage. This inactivation appeared to be temperature-dependent above 20 °C.

### Succession of microbial populations during the spontaneous fermentation of grape pomace

*Specific identification of yeast.* Table 2 shows the yeast identifications obtained according to the test results in combination with the commercial computer program of Barnett *et al*. (1994). The total number of yeast strains identified was 25, belonging to the genera: *Torulaspora* (*T*.), *Debaryomyces* (*Deb*.), *Rhodotorula* (*R*.), *Saccharomyces* (*Sac*.), *Kloeckera* (*K*.), *Leucosporidium* (*L*.), *Zygosaccharomyces* (*Zyg*.) and *Dekkera* (*Dek*.). These genera have been reported to appear in the wine-making environment (Barnett *et al*. 1990). Four strains were identified as *Saccharomyces cerevisiae* which is widely accepted as the principal wine yeast and as that which normally conducts the alcoholic fermentation at least in the latter stages (Fleet *et al*. 1990). Two strains were identified as *Zygosaccharomyces bailii* (formerly known as *Saccharomyces bailii*). The presence of this species very often leads to secondary fermentation in wines, causing deterioration of wine flavour (Couto and Huis in't Veld 1995). The genus *Dekkera* included two species, which have also been reported as normal wine spoilage yeasts (Kalathenos *et al*. 1995).

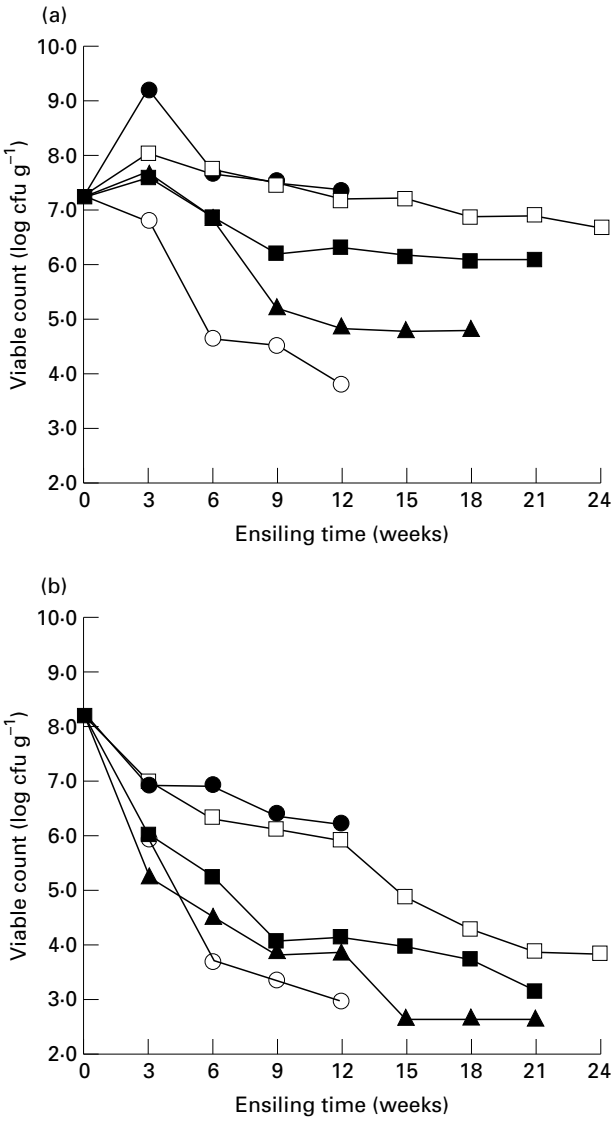


**Fig. 1** Changes in average counts of total viable lactic acid bacteria (a) and total viable yeasts (b) during the spontaneous fermentations on grape pomace ensiling period. Coefficient of variation $\leqslant 32\%$ (a), coefficient of variation $\leqslant 34\%$ (b). Pilot scale (●), 20 °C (□), 25 °C (■), 30 °C (▲) and 35 °C (○)

**Table 2** Identification of yeast strains isolated from grape pomace during the ensiling period

| Yeast species | Identification probability (%)* | Number of strains |
|---|---|---|
| *Torulaspora delbrueckii* | 93·8 | 2 |
| *Debaryomyces hansenii* | 98·2 | 7 |
| *Rhodotorula mucilaginosa* | 96·4 | 2 |
| *Saccharomyces cerevisiae* | 100 | 4 |
| *Kloeckera lindneri* | 97·1 | 2 |
| *Leucosporidium scottii* | 98·1 | 2 |
| *Zygossaccharomyces bailii* | 99·2 | 2 |
| *Dekkera custersiana* | 98·6 | 2 |
| *Dekkera bruxellensis* | 98·7 | 2 |
| *Saccharomyces cerevisiae*† | 100 | 1 |

* According to the Computer Program of Barnett *et al.* (1994).
† Type strain from Instituto Gulbenkian (14 C-3507).

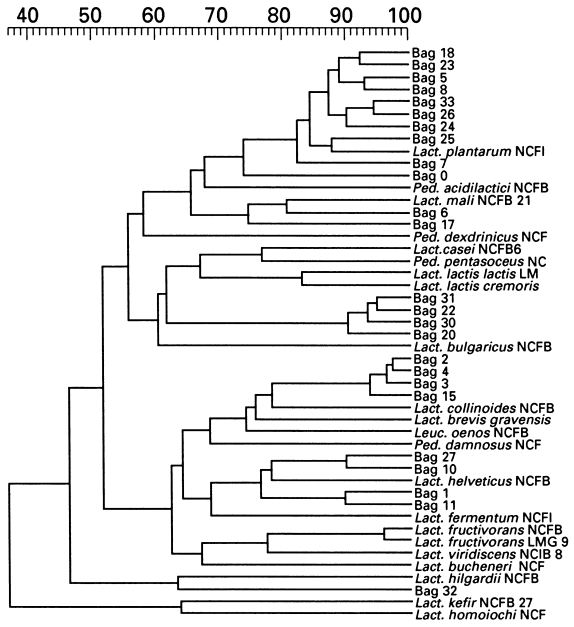


**Fig. 2** Dendrogram showing the clustering analysis based on the electrophoretic protein patterns of the grape pomace isolates and the relevant reference strains included in this study (vertical numbers). The horizontal numbers represent the percentage similarities as determined by the Pearson product-moment correlation coefficient and UPGMA clustering

*Specific identification of LAB.* All colonies isolated from grape pomace were generically identified as lactobacilli (Gram-positive, catalase-negative rods – data not shown). Following numerical analysis of the total protein profiles of 32 representative isolates, five clusters were found, as shown in the dendrogram in Fig. 2. Type and reference strains which did not cluster with any of the wild-type strains were omitted. Each of the clusters grouped at a level of at least 75% homology. Cluster I includes the majority of the grape pomace isolates and the type strain of *Lactobacillus plantarum* at a level of 82% homology. Cluster II is composed of the type strain of *Lactobacillus mali* and two isolates at a level of 75% homology. Cluster III contains four isolates which did not cluster with any reference strain. Cluster IV includes the type strain of *Lactobacillus collinoides* and four isolates at a

level of 78% homology. Cluster V includes the type strain of *Lactobacillus helveticus* with four isolates at a level of 77% homology.

*Succession of yeast and LAB populations.* Table 3 represents the microbial succession of yeast and LAB species occurring during the spontaneous fermentation of grape pomace until 24 weeks of the ensiling period. The predominant yeast species encountered in the fresh pomace samples was consistently *Torulaspora delbrueckii*, which is a wild species usually appearing in grapes, or in earlier stages of fermentation, such as *Alvarinho* musts (Lema *et al.* 1996). *Saccharomyces cerevisiae* is the dominant yeast after 3 weeks of ensilage and this species still dominates until 9 weeks of ensilage. These results are in agreement with studies previously reported by Mauricio *et al.* (1991), which showed that the growth of *T. delbrueckii* during spontaneous fermentation of musts only takes place in the early stages, while *S. cerevisiae* survives to the end of the fermentation. After 9 weeks of ensilage, the dominant yeasts were from the genera *Debaryomyces* and *Dekkera*, although after 12 weeks, *S. cerevisiae* was still present, becoming undetectable only in the few weeks following. During the post-processing abuse period, spoilage wine yeasts such as members of the genus *Dekkera* became dominant. With respect to LAB, the homofermentative lactobacilli species were dominant in this particular environment. *Lactobacillus plantarum* is a common species in the wine environment and appeared in this study throughout the ensilage period (from the beginning until the end of sampling). *Lactobacillus collinoides* and *Lact. mali* appeared in the earlier stages until 9 and 12 weeks, respectively, while *Lact. helveticus* appeared until 18 ensilage weeks.

**Table 3** Microbial succession of yeast and lactic acid bacteria species during the spontaneous fermentation of grape pomace

| Silage period (T = weeks) | Yeast species | LAB species (lactobacilli) |
|---|---|---|
| T0 (fresh grape pomace) | *Torulaspora delbrueckii** | *Lactobacillus plantarum* |
| | *Debaryomyces hansenii* | *Lact. collinoides* |
| | *Rhodotorula mucilaginosa* | *Lact. helveticus* |
| T3 | *Saccharomyces cerevisiae** | *Lact. plantarum* |
| | *D. hansenii* | *Lact. mali* |
| | *Kloeckera lindneri* | *Lact. helveticus* |
| | *Leucosporidium scottii* | |
| T6 | *S. cerevisiae** | *Lact. plantarum* |
| | *Kloeckera lindneri* | *Lact. helveticus* |
| | *Zygossaccharomyces bailii* | |
| | *Rhodotorula mucilaginosa* | |
| T9 | *S. cerevisiae** | *Lact. plantarum* |
| | *D. hansenii* | *Lact. collinoides* |
| | *Z. bailii* | |
| T12 | *Dekkera custersiana** | *Lact. plantarum* |
| | *S. cerevisiae* | *Lact. mali* |
| T15 | *D. hansenii* | *Lact. plantarum* |
| | *D. custersiana* | *Lact. helveticus* |
| T18 | *D. hansenii* | *Lact. plantarum* |
| | *D. custersiana* | *Lact. helveticus* |
| T21 | *D. hansenii* | *Lact. plantarum* |
| T24 | *D. bruxellensis* | *Lact. plantarum* |

* Dominant yeast.

## Substrates and products within pomace fermentation

The major chemical changes attributed to microbial metabolism occurred in the first 3 weeks of ensilage (Figs 3, 4 and 5). The fresh grape pomace contained about 11 g of sugar and after 3 weeks of ensilage, this fell to 0·5 g 100 g$^{-1}$ of grape pomace while the ethanol concentration increased (Fig. 3), which indicated an initial alcoholic fermentation. During this initial period, malic acid was depleted concurrently with an increase in lactic acid (Fig. 4), indicating a malolactic conversion, although it is probable that lactic acid was also produced via other routes. Figure 5 represents the alterations of tartaric, acetic, citric and succinic acids, and glycerol, in grape pomace during the ensiling period. An increase in glycerol is noted throughout the ensilage period and was one of the end products of yeast sugar metabolism (Boulton *et al.* 1996). Tartaric acid decreases abruptly during the first 3 weeks of ensilage, probably due to LAB activity (Sponholz 1993) or to precipitation as bitartrate crystals (Peynaud 1981). Acetic acid appeared after 3 weeks of the ensiling period and showed a

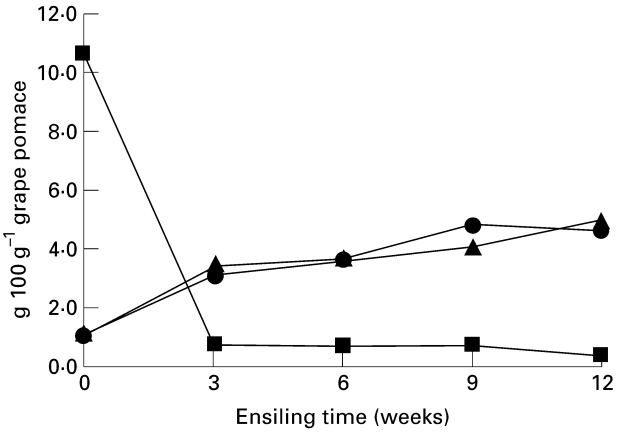


**Fig. 3** Changes in the average values of the sugar (glucose+fructose) and ethanol content in grape pomace during the ensiling period on a pilot scale and a lab scale at 20 °C. Sugar (■), ethanol on pilot scale (▲), ethanol on laboratory scale (●)

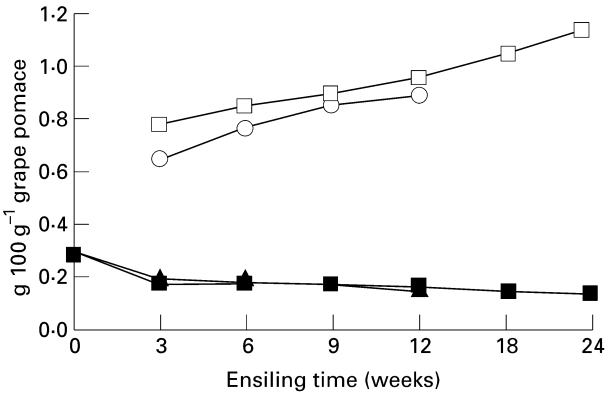


**Fig. 4** Changes in the average values of the malic and lactic acid content in grape pomace during the ensiling period on a pilot scale and a laboratory scale at 20 °C. Malic acid on pilot scale (▲), malic acid on laboratory scale (■), lactic acid on pilot scale (○), lactic acid on laboratory scale (□)

slight increase until the end of sampling. Citric and succinic acid levels did not alter during the entire 24 weeks of the experiment.

## DISCUSSION

White grape pomace, due to its microbial composition and sugar content, is an unstable product which progressively produces a variety of different chemical and microbial profiles during the spontaneous fermentation ensilage. In this study, the major microbial and chemical alterations occurred during the first 3 weeks of fermentation, leading to a more stable product which differed significantly from the initial substrate.

The growth and metabolism of yeast and LAB is dependent on various factors, one of which is certainly ambient temperature. The data presented above suggest that the apparent death of yeasts and LAB were strongly dependent on environment temperatures above 20 °C. These results were probably due to the synergistic effects of temperature and ethanol (D'Amore and Stewart 1987; Gao and Fleet 1988) which led to a loss of viability of the cells.

Yeast populations were found to be numerically dominant over bacteria in fresh pomace, although after 3 weeks of the ensiling period, the LAB population numerically dominated the yeast population at all of the various environment temperatures tested, suggesting that LAB can better adjust to this highly specific environment. For silage fermentations generically, once anaerobic conditions are fully established, the total yeast numbers decline, and a dramatic increase in the populations of LAB occurs until they rapidly dominate the other microflora. However, during the first week of ensilage, air is still present in the containers and this enables aerobic microbial activity to continue (Weinberg *et al.* 1988).

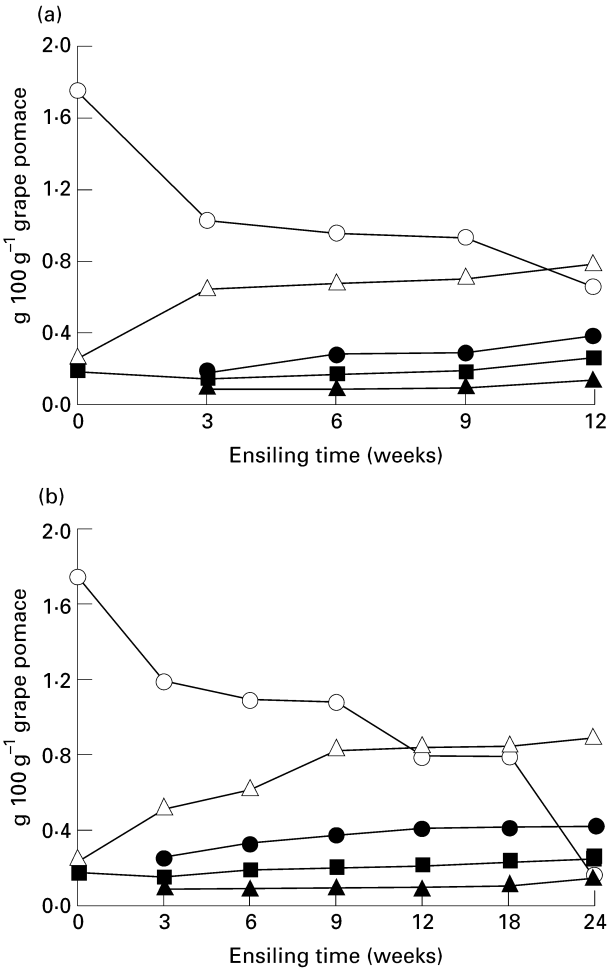


**Fig. 5** Changes in the average values of the tartaric acid, acetic acid, citric acid, succinic acid and glycerol content on a pilot scale (a) and on a laboratory scale at 20 °C (b). Tartaric acid (○), acetic acid (●), citric acid (■), succinic acid (▲) and glycerol (△)

The homofermentative lactobacilli were the dominant LAB encountered. The succession of homofermentative to heterofermentative species, such as that reported for wine fermentations (Lafon-Lafourcade *et al.* 1983) and some silage scenarios (Woolford 1984), was not apparent in this study. As silage fermentation is generically affected by many factors, numerous ensiling environments result and allow different LAB species or strains to become involved in the microbial succession (Lin *et al.* 1992). *Lactobacillus plantarum* seems to be the LAB that can best adjust to the specific environment studied here.

The predominant species of yeast encountered in the fresh pomace samples was *Torulaspora delbrueckii*. According to several studies reviewed by Fleet and Heard (1993), the apiculate yeasts of the genus *Hanseniaspora* are normally the predominant species on the surface of the grape, accounting for

about 50–75% of the total yeast population. However, these and other authors accept that the grape microbial flora is dependent upon many factors, such as rainfall, humidity and other climatic influences, use of fungicides and the grape variety (Fleet 1992; Boulton *et al*. 1996), and yeasts other than apiculate species can dominate. The yeast strains identified as *Torulaspora delbrueckii, Debaryomyces hansenii, Rhodotorula mucilaginosa* and *Kloeckera lindneri* are normally associated with the natural microflora of grapes (Herraiz *et al*. 1990; Mauricio *et al*. 1991; Salvadores and Cardell 1993; Briones *et al*. 1995; Cianni and Picciotti 1995; Salvadores *et al*. 1995; Lema *et al*. 1996) and are frequently referred to as wild or indigenous yeasts which exhibit limited growth during the first few days of fermentation, giving way to the dominant growth of the more ethanol-tolerant strains of *S. cerevisiae* (Fleet 1990). With longer ensilage periods, the indigenous yeast population decreased and was replaced by yeast species usually associated with alcoholic fermentation such as the *S. cerevisiae*. An exception to this tendency was the persistence of *D. hansenii*, which suggests a greater tolerance to this specific environment. Also, spoilage wine yeasts such as species of *Zygossaccaromyces* or *Dekkera* appeared within the total *Saccharomyces* species population. These micro-organisms are relatively common in winery installations and can produce unpleasant odours which cause deterioration of wine flavour (Chatonnet *et al*. 1995; Couto and Huis in't Veld 1995).

The relationship between the physiological properties of the epiphytic microflora in the silo and their natural succession is not very clear. According to some references reviewed by Woolford (1984), the change in the conditions within the silo from aerobic to anaerobic, coupled with a lactic acid fermentation, were the main plausible explanations for the microflora changes observed in forage silage. Although grape pomace ensilage has parallels with forage silage, especially as a solid state fermentation, the intrinsic characteristics of this solid viticultural residue make an important contribution to the natural succession of yeasts and LAB observed in this study. Factors such as specific epiphytic flora, free hexose sugar concentration and initial low pH (around 4·5) are sufficiently characteristic in grape pomace to provide an alcoholic sugar fermentation over the lactic acid fermentation normally occurring in forage silage. In this particular environment, ethanol is certainly a major factor in the natural yeast succession during the first weeks of grape pomace ensilage. It is generally accepted that *S. cerevisiae* (a strongly fermentative yeast) is present in very small numbers, if at all, on intact grapes, but is ubiquitous in the winery environment and hence will certainly be present in the initial stages of ensilage. It is worth noting that in the study presented at T0 (the fresh samples), *S. cerevisiae* was not the predominant species of yeast whereas after 3 weeks it was completely dominant, suppressing and outgrowing the indigenous yeast flora. Further studies are needed to clarify the relationship between the environmental changes imposed by ensilage and the natural microflora succession.

The initial metabolic alterations which occurred in grape pomace were probably affected by resident yeasts, most importantly, *S. cerevisiae*, and by LAB homofermentative species. From our results, an alcoholic fermentation by yeasts due mainly to the presence of *S. cerevisiae*, and a malolactic conversion by LAB, occurred in the first 3 weeks of the ensiling period. However, the results suggest that other metabolic conversions took place, such as homofermentative or heterofermentative fermentations. A dramatic depletion in tartaric acid probably due to LAB activity, and an increase in glycerol due to yeast activity, is also noted during the entire ensilage period. The detection of acetic and succinic acid in grape pomace after 3 weeks of ensiling was probably due to microbial activity. The presence of acetic acid was probably due to LAB activity through the heterofermentative route, while the succinic acid was probably formed during the alcoholic fermentation by yeast.

In this study, the results suggested that the LAB were better adjusted to this specific environment. Only those micro-organisms able to adjust to the ensiling environment could survive, grow, or maintain their populations (Lin *et al*. 1992). The rate and efficiency of acid production in the first stages of fermentation by the epiphytic LAB are important factors in efficient silage making. Conservation of forage crops by ensiling is based on natural fermentation in which the epiphytic LAB convert sugars under anaerobic conditions into lactic acid (Weinberg *et al*. 1988). In the case of grape pomace silage, ethanol as well as lactic and acetic acids, can influence the succession of microbial flora and the stability of the product.

## ACKNOWLEDGEMENTS

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