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Changes in the Prefermentation Static Washing Regime of Kalamata Olives Affect the Fermentation Profile

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Summary

Traditional methods of naturally black olive production employ a series of static washings prior to fermentation. This work investigates the static washings and the effects they have on the subsequent spontaneous fermentation of Kalamata olives. Significant quantities of organic carbonaceous material, including phenolic compounds, were removed during the static washings. The rate of removal peaked after four static washings, and then declined. Bacteria (including lactic acid bacteria) and yeast were found to be present in high numbers throughout the static washings. An increase in the number of static washings resulted in the removal of inhibitory phenolic compounds. This led to a reduction in the lag phase and an increase in the specific growth rate for both the yeast and lactic acid bacteria during the subsequent spontaneous fermentations. However, an increased incidence of spoilage moulds was observed in the fermentations when the olives underwent thirteen static washings.

Key words: Kalamata olives, static washings, naturally black olives, table olives, yeast, lactic acid bacteria

Introduction

Table olives may be produced according to a variety of processing methods, of which naturally black (Greek style), green (Spanish style) and black ripe (Californian style) are of the greatest economic importance. Naturally black olives are harvested when mature and spontaneously fermented in brine by a mixed florae of yeasts and lactic acid bacteria. A number of traditional production methods employ a series of static washings prior to fermentation for up to 40 days (*1*). During static washings ripe olives are submerged in water, which is changed every two to three days to facilitate the partial debittering of the fruit. Few details have been reported on the changes that occur during these pre-brining static washings, although the proposed benefits of this technique are partial elimination of the natural bitterness (oleuropein) by diffusion into the water, cleaning of the fruit, and, if a light brine is used in place of water, an increase in the NaCl content of the olives (*2,3*).

The microorganisms involved in the fermentation of naturally black olives depend on a range of factors, including the indigenous microflora, brine concentration, pH, water activity, nutrient availability, fermentation temperature, presence of natural antimicrobials (oleuropein) and the olive cultivar (*4–6*). Yeasts, non-sporulating Gram-negative bacteria, and to a lesser extent lactic acid bacteria, are responsible for the spontaneous fermentation of naturally black olives (*7*). However, lower salt concentrations (less than 8 %) favour the growth of lactic acid bacteria over yeasts resulting in a lactic fermentation (*8*). Recent studies have involved the identification and characterisation of the predominant yeast species in table olive fermentations (*9,10*). Although the role

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of individual yeast species in olive fermentations has not yet been established, it has been suggested that they positively contribute to the organoleptic properties of the product, and aid in the supply of essential nutrients for lactic acid bacteria (*7*,*11*,*12*).

The proliferation of lactic acid bacteria has been recommended to prevent malodorous and softening spoilage caused by some spontaneously occurring microorganisms (*13*). However, the phenolic compound oleuropein and its derivative products inhibit the growth of lactic acid bacteria (*14*,*15*). Debittering (oleuropein removal) may allow for the increased growth of these bacteria during the fermentation; as indicated by the growth of *Lactobacillus* spp. in Taggiasca olives processed with static washings (*1*). The increased survivability of lactic acid bacteria attributed to the debittering process may also have considerable benefits towards the recently proposed use of table olives as a vehicle for probiotic cultures (*16*).

The aim of this study is to investigate the changes that occur during the static washing of Kalamata olives, and to determine the effect such changes have on the spontaneous fermentation.

Materials and Methods

Olives, prefermentation treatment and fermentation conditions

Mature large and extra large Kalamata olives (300 kg) were harvested in July 2004. Each lot of 100 kg of olives was placed into a 200-litre lidded plastic vessel, which was filled with tap water. Every two days the washwater was removed and replaced, comprising one static washing. The olives underwent two, seven, or thirteen static washings. After the appropriate number of static washings, 14 kg of olives were placed in 28-litre plastic fermentation vessels (N=7), thus three sets of seven fermentations were monitored, starting after two, seven, and thirteen static washings. The fermentors were filled to capacity with 10 % (by mass per volume) NaCl brine and kept at 20 °C, and a sampling port and airlock were fitted to each fermentor. The salt concentration was neither adjusted nor monitored. The remaining 48 kg of olives were washed three more times for a total of 16 static washings, to further monitor microbial and physicochemical changes.

Chemical analysis

The titratable acidity of the washwater and the brine was determined by titrating 10 mL of brine against 0.1 M NaOH with thymol blue indicator. Total organic carbon (TOC) was measured using the Degtjareff acidic dichromate oxidation method (*17*). For this method, sodium dichromate (10 mL, 0.5 M) was mixed with 10 mL of a washwater sample, concentrated H_2SO_4 (20 mL) was added, and the resulting solution was heated to 110 °C for 30 min. The heated solution was made up to 100 mL with distilled water and cooled to room temperature before measuring the absorbance at 600 nm (Varian Cary 50, Australia). Total phenolics in the washwater were quantified by the Folin-Ciocalteu assay (*18*). Filtered washwater (1 mL) diluted 1:10 was added to 5 mL of diluted (1:10) 2 M Folin-Ciocalteu reagent (Sigma-Aldrich, Australia). This mixture was allowed to stand for 5 min, after which 4 mL of 7.5 % (by mass per volume) Na_2CO_3 were added. Absorbance was measured at 740 nm (Varian Cary 50, Australia) after 2 h. Total phenolics were expressed as gallic acid equivalents. All chemical analyses were carried out in triplicate.

pH measurements

The pH measurements of the washwater were taken with a TPS-LC 80A (TPS, Australia).

Microbiological analysis

Brine samples were drawn from the fermentor *via* the sampling ports, serially diluted in 0.1 % peptone water, and 0.1 mL of aliquot was plated onto the three media described below. Yeasts were enumerated on YPG medium containing (in g/L): yeast extract 5, peptone 5, glucose 20, agar 15, supplemented with ampicillin 0.1 and chloramphenicol 0.1, and incubated at 25 °C for 5 days. Lactic acid bacteria were enumerated on acidified MRS agar (Oxoid, Australia) (*4*), supplemented with cycloheximide (0.2 g/L) to inhibit yeast, and incubated at 30 °C for 5 days. Mesophilic bacteria were enumerated on nutrient agar (Oxoid, Australia) incubated at 30 °C for 3 days. All microbial analyses were carried out in duplicate.

Statistical analysis

The specific growth rates and lag phases of the fermentations were examined by ANOVA using SPSS 11. Differences between treatment mean values were determined using Bonferroni's post-hoc test at $p<0.05$.

Results

The titratable acidity decreased steadily for the first ten static washings (Fig. 1), but there was no apparent change in pH. Total organic carbon levels in the wash-

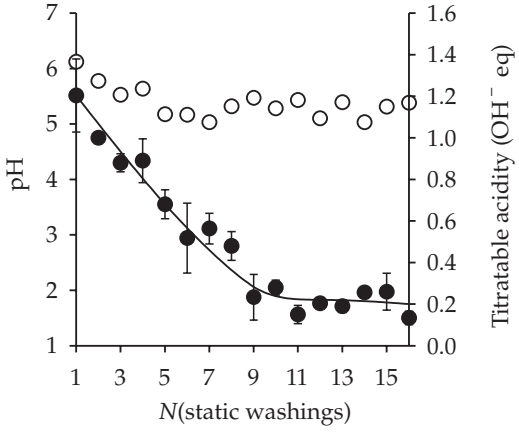

Fig. 1. Changes in the pH and titratable acidity of washwater during subsequent static washings prior to brining of Kalamata olives
○ pH (data points are mean values of duplicates), ● titratable acidity as hydroxy ion equivalents (data points are mean value ±standard deviation of triplicate samples)

water were measured as an indicator of the rate of olive-associated material leaching into the water (Fig. 2). The rate of loss of carbon compounds to the washwater increased over the first four washings, after which the amount of material lost during each washing declined (Fig. 2). A continuous decrease in the rate of loss of phenolic compounds into the washwater was observed over the 16 static washings.

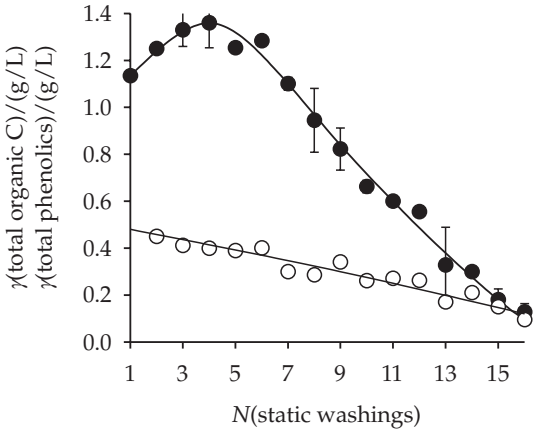

Fig. 2. Changes in the total phenolics and total organic carbon of the washwater during subsequent static washings prior to brining of Kalamata olives
○ total phenolics (as gallic acid equivalents) and ● total organic carbon. Data points are mean value±standard deviation of triplicate samples

Changes in the microbial populations during the washings are shown in Fig. 3. Over the first six static washings, the yeast levels increased as the lactic acid bacteria population declined. During the subsequent washings this initial trend was reversed until the yeast and lactic acid bacteria populations were restored to their initial levels. There was little change in the cell counts of the mesophilic bacteria over the course of the static washings.

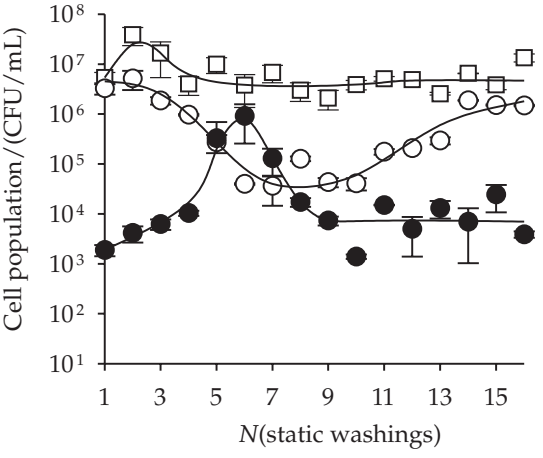

Fig. 3. Changes in the microflora during subsequent static washings prior to brining of Kalamata olives
● yeast, ○ lactic acid bacteria, □ mesophilic bacteria. Data points are mean value±standard deviation of triplicate samples

Beginning the brine fermentations after seven washes instead of two increased the specific growth rate of the fermentative microflora (Figs. 4a and 4b), however, regardless of the number of washings, both sets showed an instant drop in cell numbers, followed by a lag phase of 18–20 days. The fermentations of the olives washed thirteen times showed reduced inhibition of microorganisms, having only a very short lag phase (Fig. 4c). However, these fermentations showed an increased incidence of surface growth, which later supported the growth of mould (data not shown).

The number of static washings showed no discernable effect on pH, as the pH of all the fermentations ranged between 4.57 and 4.89. The evolution of the titratable acidity is shown in Fig. 5. All three sets of fermentations showed a general increase in titratable acid-

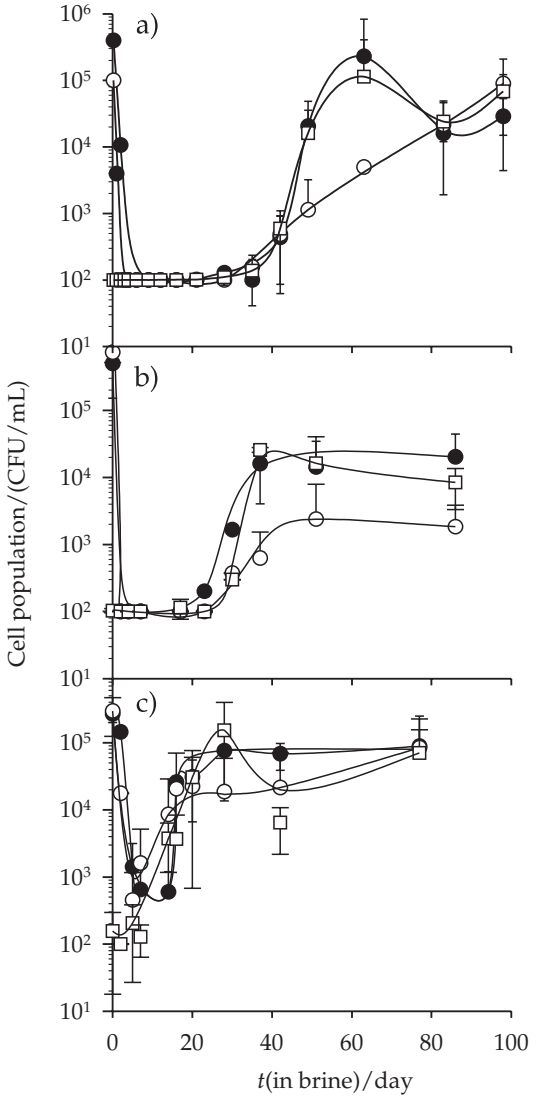

Fig. 4. Changes in the microflora during brining of Kalamata olives following different pre-brining washing regimes: a) olives placed in brine following 2 static washings; b) olives placed in brine following 7 static washings; c) olives placed in brine following 13 static washings
● yeast, ○ lactic acid bacteria, □ mesophilic bacteria. Data points are mean value±standard deviation of triplicate samples

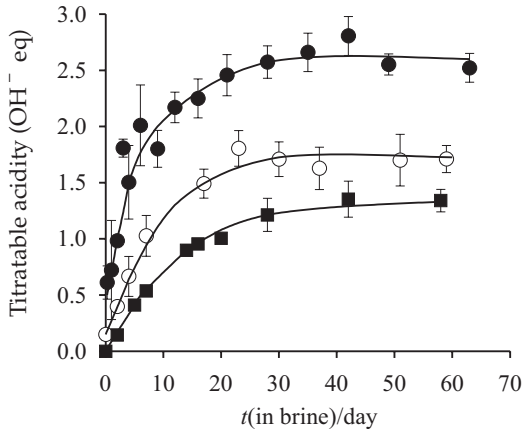

Fig. 5. Titratable acidity of Kalamata olive brines following different pre-brining washing regimes
● 2 static washings, ○ 7 static washings, ■ 13 static washings. Data points are mean value±standard deviation of triplicate samples

ity, which was more pronounced in those fermentations that underwent the least number of static washings. Note that it is likely that various phenolic compounds are being titrated against, in addition to organic acids such as lactic acid.

Discussion

Total organic carbon was used as an indicator of the diffusion of olive-associated organic material into the washwater, such as phenolics, organic acids, amino acids, and sugars. The steady decrease in total organic carbon detected at the end of subsequent washings indicates that the amount of organic material released from the olives per washing cycle is diminishing. This phenomenon is reflected in the fact that the amount of phenolics detected in the washwater also decreased with every subsequent washing cycle. A decrease in phenolic content during the washings is in contrast with earlier reports that indicated only negligible change in phenols during static washings (*19*). However, their work involved only two static washings and different olive cultivars. The observed drop in titratable acidity despite the constant pH may be due to phenolic compounds being measured in addition to organic acids.

During the initial washings, yeasts dominated over the lactic acid bacteria. This trend continued until the fifth wash cycle, after which the yeast population reached its maximum and the lactic acid bacteria population reached the minimum ($9{\cdot}10^5$ and $4{\cdot}10^3$ CFU/mL, respectively). The suppression of lactic acid bacteria may be attributed to the presence of antimicrobial compounds such as oleuropein leaching out from the olives at a greater rate into the washwater. Oleuropein and its hydrolysis products have been shown to induce leakage of cellular material from lactic acid bacteria due to degradation of the cell wall (*20*). Most yeasts are unaffected by oleuropein, and it is thought to be the primary reason why black olive fermentations are dominated by yeasts (*20,21*). After six washes the lactic acid bacteria showed a period of enhanced growth, whilst the yeast population failed to reach the levels of the previous washings. At the end of the static washings, both the lactic acid bacteria and yeast populations had returned to their initial counts of $1.5{\cdot}10^6$ and $1.0{\cdot}10^4$ CFU/mL, respectively. This restitution of microbial growth indicates that the majority of antimicrobial bittering compounds have been eliminated from the olives at the completion of 13 washings. These findings appear consistent with artisan practices, where the producer 'taste tests' the olives for a reduction in bitterness prior to commencing fermentation.

Although sampling of the washwater will likely underestimate the actual microbial population (due to cells adhering to the olive surface) (*4,5*), the method employed allows an insight into the microflora associated with the static washings. It must also be noted that as the washwater was discarded at the end of each washing, the microbial population was continually renewed.

Following the static washings, the olives were placed in brine to undergo a spontaneous fermentation. The profiles of the microflora during the various brining arrangements are shown in Fig. 4. Table 1 details the specific growth rates and lag phases for the yeast and lactic acid bacteria. The implementation of only two washings causes a near complete reduction in viability and an extensive lag phase before the brine-adapted microflora initiate the growth. The use of seven static washings also caused a near complete reduction in viability and did not appear to reduce the lag phase, however, it allowed for a significantly increased specific growth rate ($p=0.008$) for the yeast population. Thirteen static washings removed a substantial amount of growth inhibitors (phenolics), allowing for a rapid progression of growth, with a significantly shortened lag phase ($p<0.001$). However, an initial drop in viability and short lag phase were still present due to the high salt content of the brine.

Table 1. Mean specific growth rates and lag phases for yeast and lactic acid bacteria for the olive fermentations started after two, seven, and thirteen static washings

N(static washes)	Specific growth rate/day		Lag phase/day	
	Yeast	LAB	Yeast	LAB
2	0.3471	0.2200	38.00	49.00
7	0.7093	0.0845	30.00	38.00
13	0.5636	0.2622	7.00	7.67

Values are means of seven replicates

Fermentations with fewer static washings showed increased titratable acidity (Fig. 5). This most likely reflects the higher level of phenolic compounds in these brines, rather than increased acidity due to the production of organic acids by lactic acid bacteria.

Although increased growth of beneficial microorganisms was observed with thirteen static washings, these fermentations suffered from a higher rate of surface mould contamination. This observation may be attributed to the excessive removal of phenolic compounds, as it had been proposed that the presence of the phenolic compounds in olive flesh may inhibit potential fungal contaminants (22). Excessive removal of phenolic compounds could also reduce the sensory appeal of the final product, as a degree of residual bitterness is commonly associated with black table olives.

Conclusions

During the static washings of naturally black Kalamata olives, the continual growth of lactic acid bacteria and yeast was observed. Organic material, including phenolic compounds, was removed during the static washings, allowing for an increased specific growth rate for both the yeast and lactic acid bacteria during the fermentation. Seven static washings appeared most beneficial, allowing for sufficient growth of a fermentative microflora, without allowing for mould spoilage.

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