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Microbial Stability and Quality of Seasoned Cracked Green Aloreña Table Olives Packed in Diverse Chloride Salt Mixtures

J. BAUTISTA-GALLEGO,[1,2]* F. N. ARROYO-LÓPEZ,[2] V. ROMERO-GIL,[2] F. RODRÍGUEZ-GÓMEZ,[2] P. GARCÍA-GARCÍA,[2] AND A. GARRIDO-FERNÁNDEZ[2]

[1]*Dipartimento di Valorizzazione e Protezione delle Risorse Agroforestali, Agricultural Microbiology and Food Technology Sector, Faculty of Agriculture, University of Turin, Via Leonardo da Vinci 44, 10095 Grugliasco, Torino, Italy; and* [2]*Department of Food Biotechnology, Instituto de la Grasa, Avda. Padre García Tejero 4, 41012 Seville, Spain*

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ABSTRACT

This work was conducted to determine the effect of the partial replacement of NaCl by KCl and $CaCl_2$ (expressed as percentages, wt/vol) on the microbial stability and physicochemical characteristics of seasoned cracked olives using a simplex centroid mixture design. Neither *Enterobacteriaceae* nor lactic acid bacteria were found during the 50 days that olive packages were monitored. Therefore, microbial instability was considered due to the growth of yeasts, which were the only detected microorganisms; *Saccharomyces cerevisiae* and *Pichia membranifaciens* were the most relevant species. Yeasts decreased during the first 21 to 30 days after packing, but their populations rose to 3.5 log CFU/ml by the end of the storage period, clearly causing product deterioration. The partial substitution of NaCl with the other chloride salts slightly altered the phase of microbial inhibition and regrowth. Most of the quality characteristics were not affected by the use of the alternative salt mixtures, but the pH values and Cl^- concentrations in brine decreased as the $CaCl_2$ concentration increased. Hence, seasoned cracked table olives can be produced using a lower proportion of NaCl without causing significant changes in the shelf life and product quality, although further detailed studies are necessary to guarantee the stability of products packed with specific salt mixtures.

Spain is the main producer of table olives, with an annual production of about 550,000 tonnes *(22)*. Seasoned olives are gaining acceptance among consumers because of their natural ingredients and association with local traditions. Aceituna Aloreña de Málaga is a cracked seasoned olive presentation that is characterized by its green surface color and may be prepared from fresh fruits or after storage in brine *(24)*. This preparation is the only type of table olive with Protected Denomination of Origin in Spain.

During table olive processing, fruits are in contact with brines containing sodium chloride, which progressively increases the sodium concentration in the flesh and decreases the concentration of other minerals or organic nutrients through subsequent dilutions *(17)*. The relationship between sodium intake and cardiovascular diseases is well established *(34)*. A recent survey revealed that sodium intake in the U.S. adult population was above the recommended levels *(9)*. The average salt intake by the Spanish population is about 9 g/day *(30)*. Efforts to reduce the amount of salt in foods have been initiated *(32)*, but this problem remains unsolved *(1)*. Although the impact of table olive sodium concentration on the overall consumer intake of this element is limited, a more equilibrated composition of minerals in this product could contribute to improving the consumer's diet and the table olive image.

Table olive salt concentration can be easily modified by processors by simply changing the composition of the packing brines. However, Sleator and Hill *(35)* emphasized the importance of a better understanding of the microbiological food safety issues associated with product reformulations because the new products may have different intrinsic physicochemical properties, which could lead to spoilage or support the growth of foodborne pathogens. Hence, microbial and physicochemical studies on the key parameters of new low-salt or salt-substitute table olive products are essential to prevent deterioration and safety risks. Aceituna Aloreña de Málaga, which has relatively limited shelf stability *(4)*, may be a good matrix for studying the effects of mineral brine composition changes on seasoned products already on the market. Yeasts have been identified as the main cause of spoilage during the shelf life of packed "seasoned" Manzanilla Aloreña table olives. These microorganisms cloud brines and cause accumulation of CO_2, leading to swollen containers and brine leakage *(3)*. Yeasts are very commonly found in diverse table olive processing plants, where they can act as both desirable and spoilage microorganisms *(6)*. Yeasts also can form polymicrobial communities with lactic acid bacteria (LAB) adhered to the Spanish-style green olive surface *(2)*.

* Author for correspondence. Tel: +34 954 692 516; Fax: +34 954 691 262; E-mail: joaquinbg@ig.csic.es.

Boziaris et al. *(10)* found that NaCl and KCl produced almost similar growth–no growth interfaces for *Listeria monocytogenes* Scott A at different pH values and nisin concentrations. A first attempt to replace common salt during the fermentation of cracked green olives produced promising results *(7)*. Initial mixtures of NaCl (12.8 g/100 ml) with $Ca(OH)_2$ (0.025 M) led to natural black olive products free of spoilage and with a high level of consumer acceptance *(26)*. The addition of $CaCl_2$ during the fermentation of Greek naturally black olives was studied by Tassou et al. *(36)*. Panagou et al. *(31)* tested five different combinations of NaCl, KCl, and $CaCl_2$ and concluded that only the combination containing 4% NaCl and 4% KCl produced an acceptable product. Di Silva *(12)* produced naturally green olives using brines containing KCl and $CaCl_2$. However, studies on NaCl substitution in seasoned cracked packed table olives have not been conducted.

Experimental design and response surface methodology are powerful tools for investigating the effect of several variables at the same time *(29)* and were used in studies conducted to minimize the salt concentration in natural cucumber fermentations *(20)*, substitute NaCl with KCl in the fermentation of olive juice *(38)*, and determine the effect of diverse chloride salt mixtures during the storage of cracked green olives *(7)*. In the present study, we used this methodology with a simplex centroid mixture design to investigate the effect of replacing NaCl with KCl and $CaCl_2$ on the stability (changes in the microbial population) and quality (changes in the physicochemical characteristics of fruits) during the shelf life of packed seasoned cracked olives. Multivariate techniques also were applied to elucidate the relationships among variables and grouping treatments.

MATERIALS AND METHODS

Olives and experimental design. Fruits of the Aloreña cultivar (240 fruits/260 kg), that had been mechanically cracked and stored in an initial 11% NaCl (wt/vol) brine for 6 months were used in the present study. The experiment consisted of replacing 50% of the salt normally used in the packing brine (5%, equivalent to 5 g/100 ml) with selected proportions of KCl and $CaCl_2$. The concentrations were expressed in percentages (wt/vol) to facilitate the direct application of results to the industry, which uses these units. The simplex centroid mixture design was generated with the software Design Expert release 6.0.1 (Stat-Ease, Minneapolis, MN) and consisted of 12 treatments in which packing brines were mixtures of the three salts in the proportions specified in Table 1. The current conditions used by the industry correspond to runs 1, 2, and 15 (added to the initial experimental design). Apart from salts, other components of the packing brine were potassium sorbate at 1,700 g/1,000 liters, citric acid at 2,630 g/1,000 liters, ascorbic acid at 640 g/1,000 liters, and lactic acid at 1,500 g/1,000 liters.

Fruits (400 g) and seasoning material (16 g of a mixture of diced garlic, pepper strips, and small pieces of fennel and thyme) were placed in plastic 750-ml containers. The containers were then filled with 310 ml of brine, closed, and kept at room temperature (20 ± 3°C) for a period similar to that of the shelf life of this product in the market (~50 days). Periodically, samples of the brines from two replicate containers were withdrawn with a sterile syringe via a septum previously fixed to each container cover, and a microbial analysis was performed. At the end of the shelf life, the containers were opened and the color and firmness of the fruits and physicochemical characteristics of the brines were determined.

TABLE 1. *Simplex centroid mixture design used to study the effect of diverse mixtures of NaCl, KCl, and $CaCl_2$ in the packing brine of seasoned cracked Aceituna Aloreña de Málaga during shelf life*[a]

Run no.[b]	NaCl (g/100 ml)	KCl (g/100 ml)	$CaCl_2$ (g/100 ml)
1 (a)	5.000	0.000	0.000
2 (a)	5.000	0.000	0.000
3 (b)	2.500	0.000	2.500
4 (b)	2.500	0.000	2.500
5	2.917	1.667	0.417
6 (c)	2.500	1.250	1.250
7 (d)	2.500	2.500	0.000
8	4.167	0.417	0.417
9	3.750	0.125	0.000
10	3.750	0.000	0.125
11	3.333	0.833	0.833
12 (d)	2.500	2.500	0.000
13	2.917	0.417	1.667
14 (c)	2.500	1.250	1.250
15 (a)	5.000	0.000	0.000

[a] The maximum allowed amount (wt/vol) of total salt (NaCl + KCl + $CaCl_2$) was 5 g/100 ml (5%). The concentration ranges of the salts were 2.5 to 5.0 g/100 ml for NaCl, 0 to 2.5 g/100 ml for KCl, and 0 to 2.5 g/100 ml for $CaCl_2$.

[b] Run numbers followed by the same letters are replicates.

Microbiological analyses. Brine samples and their decimal dilutions were plated onto the appropriate media using a spiral system (Don Whitley Scientific, Shipley, UK). Subsequently, the plates were counted with an image analysis system (CounterMat v. 3.10, IUL, Barcelona, Spain), and the results were expressed as log CFU per milliliter. *Enterobacteriaceae* were counted on crystal violet neutral red bile glucose agar (Merck, Darmstadt, Germany), LAB were counted on de Man Rogosa Sharpe agar (Oxoid, Basingstoke, UK) with sodium azide at 0.02 g/100 ml (Sigma, St. Louis, MO), and yeasts were counted on YM (yeast malt peptone glucose) agar (Difco, BD, Sparks, MD) supplemented with oxytetracycline and gentamicin sulfate as selective agents. The plates were incubated at 30°C for 24 h (*Enterobacteriaceae*) or 48 h (LAB and yeasts).

Microbial populations were assessed by determining the minimum and maximum counts and estimating the area under the corresponding growth-decline curves. The areas were calculated by integration with OriginPro 7.5 software (OriginLab Corporation, Northampton, MA). The Weibull model was also fit to the decline phase of microorganisms *(19)*:

$$\ln(N_t) = \ln(N_0) - (t/\delta)^{\beta} \quad (1)$$

where N_t and N_0 are the microbial counts at time t and time 0, respectively, δ is the time for the first decimal reduction, and β is the shape parameter of the inhibition curve.

For yeast identification, a total of 150 isolates (10 for each treatment) were selected randomly at the end of the shelf life. These cultures were purified by subsequent restreaking on YM agar and observed under a phase contrast microscope (Olympus Optical Co., Tokyo, Japan) to distinguish cell morphology. The colonies were then molecularly identified by restriction fragment length polymorphism (RFLP) analysis of the rDNA 5.8S internal

transcribed spacer (ITS) region according to the procedure described by Esteve-Zarzoso et al. *(15)*. The restriction patterns obtained were compared with those in the yeast-id.com database developed by the University of Valencia and the Institute of Agrochemistry and Food Technology (IATA) of the Spanish Council for Scientific Research (CSIC; Valencia, Spain).

Physicochemical analyses. The analyses of brines for pH, chloride concentration, and titratable and combined acidity were carried out using the standard methods developed for table olives *(17)*.

Firmness was measured objectively using a Kramer shear compression cell coupled to a universal testing machine (Instron, Canton, MA). The cross-head speed was 200 mm/min. The firmness of the olives was expressed as the mean of 20 measurements, each of which was performed on one cracked and pitted fruit. Shear compression force was expressed as kilo-Newtons per 100 grams of pitted olives.

Surface color analyses were performed on olives using a Color-View spectrophotometer (model 9000, BYK-Gardner, Columbia, MD) equipped with computer software to calculate the CIE coordinates: L* (lightness), a* (negative values indicate green and positive values indicate red), and b* (negative values indicate blue and positive values indicate yellow) with an illuminant to 10°C. Interference by stray light was minimized by covering samples with a box that had a matt black interior. Each measurement recorded was the mean of the values for 20 olives. The green color evolution of vegetables has also been expressed as the ratio –a*/b* (a kind of internal standardization), the hue angle, and the chroma (c*) *(27, 37)*. Hue is the angular component of the polar representation, and chroma is the radial component. Hue values were estimated from the equation

$$h_{ab} = \arctan\frac{b^*}{a^*} \quad (2)$$

and c* values were obtained from the equation

$$c^* = \sqrt{a^{*2} + b^{*2}} \quad (3)$$

The color index (Ic) was determined according to Sánchez et al. *(33)*:

$$Ic = \frac{-2R_{560} + R_{590} + 4R_{635}}{3} \quad (4)$$

where the R values are reflectance at 560, 590, and 635 nm, respectively.

Effect of mixture composition on the microbial and physicochemical characteristics. In this work, response surface methodology based on mixture designs *(29)* was applied to model each studied variable (response) as a function of the initial mixtures of NaCl, KCl, and $CaCl_2$ in the packing brines, according to the following equation, expressed in the canonical (Sheffé) form:

$$R = \sum_{i=1}^{3} \beta_i x_i + \sum \sum_{i<j=2}^{3} \beta_{ij} x_i x_j + \sum \sum \sum_{i<j<k=2}^{3} \beta_{ijk} x_i x_j x_k + \varepsilon \quad (5)$$

where x_1, x_2, and x_3 stand for NaCl, $CaCl_2$, and KCl, respectively, R are the responses to be modeled (variable under study or their transformed values), and the β values are the coefficients to be estimated (the applied model is able to estimate up to special cubic effects). The procedure for obtaining models and their interpretations can be found elsewhere *(8)*.

Multivariate analysis. Data from all variables were auto-scaled *(28)* before multivariate analysis to prevent bias due to differences in scales. The procedure standardizes a variable m according to

$$y_{mj} = \frac{(x_{mj} - \bar{x}_m)}{s_m} \quad (6)$$

where y_{mj} is the value j for the variable m after scaling, x_{mj} is the value j of the variable m before scaling, $\bar{x}_m$ is the mean of the variable m, and s_m is the standard deviation for the variable m. The result is a variable with zero mean and a unit standard deviation. The multivariate analysis was carried out using standardized data.

Standardized data were subjected to hierarchical clustering analysis based on Euclidean distances. The results allowed for the detection of dissimilarities and/or similarities among runs, based on microbiological, physicochemical, and color characteristics.

Principal components analysis, a mathematical procedure that transforms the overall original variables into smaller ''constructs'' or principal components (PCs) *(23)*, was carried out using a varimax rotation. For the selection of the number of PCs, the Kaiser criterion 25 was followed, and only factors with eigenvalues greater than 1 unit variance were retained *(13)*. Then the loadings of the original variables or case scores were projected onto the PC1 and PC2 plane.

Statistical data analysis. Statistica software package version 7.0 (Statsoft, Tulsa, OK) and Design Expert v. 6.06 (Stat-Ease) were used for GLM and multivariate data processing.

RESULTS AND DISCUSSION

Microbial stability. Neither *Enterobacteriaceae* nor LAB were found during the 50 days that the packaging were monitored, but yeasts were initially present in all analyzed treatments (Table 2). In agreement with previous studies on packed seasoned cracked Aceituna Aloreña de Málaga *(3, 4)*, the average yeast count just after packing was 4.27 log CFU/ml. As result, stabilization of these table olives requires the use of a preservative to control yeast growth, which could be affected by the use of different chloride salt mixtures. The presence of potassium sorbate in the packing brines caused a general progressive decrease in the initial yeast population in all treatments (mean of ~1.50 log CFU/ml), which reached its minimum between 305 h (~13 days) in runs 7, 8, and 9 and 689 h (~29 days) in runs 1, 3, and 15. However, most of the minimum populations were observed between 521 h (~22 days) and 689 h (~29 days). When used as responses, the minimum yeast population per treatment and the required times to reach these values were not related to the initial mixture concentrations. Therefore, the yeast decline phase did not depend on the initial salt mixtures in the packing brine.

The inhibitory phase of yeasts was also quantitatively studied by fitting experimental data to the Weibull model, which permitted the mathematical calculation of the corresponding inhibition parameters according to runs. In general, the model fit well (an example is shown in Fig. 1), explained a high proportion of the variance (except in run 13), and always had significant probability values (Table 3). Runs 7, 8, and 9, in which the brines included KCl, had the shortest time for a 90% reduction of the initial population; apparently the combination of NaCl and KCl may be responsible for this increase in the killing rate. In contrast, the longest periods of decline were observed in runs 1, 3,

TABLE 2. *Yeast populations in the brines of Aceituna Aloreña de Málaga packed in solutions with diverse proportions of NaCl, KCl, and $CaCl_2$*

	Mean yeast population (log CFU/ml)[a]														
Time (h)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8	Run 9	Run 10	Run 11	Run 12	Run 13	Run 14	Run 15	Mean (SD)
0	4.21	4.50	4.23	4.48	4.33	4.52	4.18	4.47	4.05	4.32	4.09	4.26	4.05	4.19	4.15	4.27 (0.16)
17	3.92	4.09	4.05	4.13	3.86	3.93	3.66	4.18	3.92	3.92	3.98	4.00	3.74	4.08	4.04	3.97 (0.14)
41	3.59	3.80	3.63	3.56	3.28	3.64	3.13	3.86	3.40	3.40	3.53	3.58	2.95	3.35	3.58	3.49 (0.23)
65	3.36	3.56	3.26	4.08	3.20	3.37	3.21	3.79	3.29	3.33	3.32	3.48	2.90	3.26	3.22	3.38 (0.27)
161	3.26	3.27	2.96	3.58	3.15	3.19	2.53	3.38	2.93	3.19	2.56	3.05	3.31	3.08	3.15	3.11 (0.27)
233	2.85	3.10	2.72	3.74	3.01	2.92	2.26	3.46	2.94	2.91	2.94	3.13	3.16	3.03	3.25	3.03 (0.32)
305	2.76	2.68	2.66	3.28	3.11	2.81	**1.60**	**3.27**	**2.64**	2.51	2.79	2.79	2.00	2.98	3.09	2.73 (0.43)
401	2.72	2.53	2.82	3.31	2.86	2.72	2.00	3.76	2.82	2.81	2.70	2.66	2.89	2.97	2.88	2.83 (0.36)
521	2.26	**2.08**	2.68	**3.09**	**2.73**	**2.64**	1.90	4.33	3.06	**2.20**	**2.56**	**2.58**	**1.90**	**2.85**	2.68	**2.64** (0.58)
689	**2.20**	2.76	**2.30**	3.35	3.20	4.18	3.03	4.04	4.20	2.26	2.95	3.65	3.87	3.92	**2.30**	3.22 (0.71)
857	3.36	3.84	3.15	3.41	3.35	3.91	3.96	3.85	3.76	3.39	2.90	3.89	4.08	3.78	3.79	3.63 (0.33)
1,025	3.24	3.43	3.84	3.60	3.21	3.74	3.51	3.56	3.49	3.33	3.32	3.63	3.67	3.65	3.58	3.52 (0.18)
1,193	2.98	2.87	3.67	3.79	3.10	3.31	3.18	3.71	3.63	3.25	4.13	3.44	3.33	3.85	4.08	3.49 (0.37)
AUC[b]	3,461	3,649	3,628	4,161	3,731	4,061	3,409	4,515	4,048	3,488	3,609	3,953	3,846	4,081	3821	3,831 (297)

[a] Standard deviations were intentionally omitted to facilitate readability and were always below 0.5 log CFU/ml for yeast counts and 260 for AUC values. Minimum populations are highlighted in bold.
[b] AUC, area under the curve for yeast counts versus time for each different run.

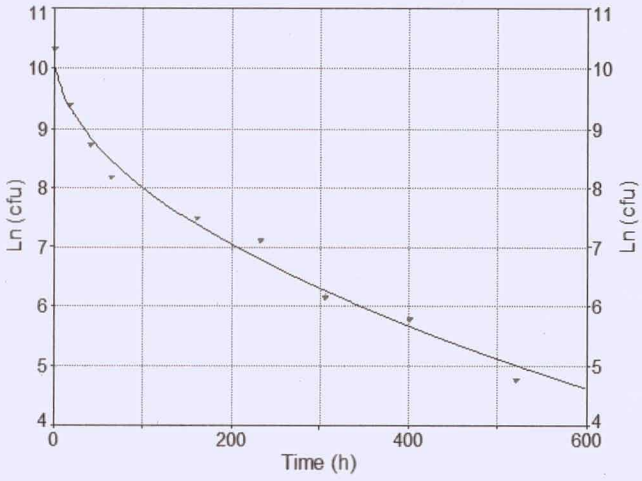

FIGURE 1. *Example of Weibull model fit to changes in yeast populations in olive brines during the decline phase in run 3.*

and 15 (without KCl in the brine), which had fairly high δ values, indicating that the reduction rate was low and maintained for a long time. In these runs, the presence of NaCl may have caused a slight decrease in the killing rate. Because the brine composition in these runs was similar to that used by the olive industry, the commercial products may have longer shelf lives (the phase of regrowth began later) than will olives packed with salt mixtures, particularly those containing KCl. In run 11 (with both KCl and $CaCl_2$), the yeast population remained controlled below 3 log CFU/ml from 161 to 857 h and then grew rapidly to reach the highest final population among runs. In contrast, in runs 8 and 9 (both containing KCl) the yeast control was more limited and the regrowth phase after the minimum began earlier. In both cases, the presence of KCl may have had a promoting effect on yeast growth. In the rest of the runs (all except run 2 were packed with salt mixtures), yeasts initiated regrowth after 521 h, an intermediate period between the two groups previously described. Thus, in

TABLE 3. *Parameters and other statistics resulting from the fit of the Weibull model to changes in the yeast decay phase*[a]

Run no.	δ	β	R^2	Adjusted R^2	$P > F$
1	20 (5)	0.44 (0.04)	0.978	0.972	<0.00001
2	18 (4)	0.50 (0.03)	0.987	0.984	<0.00001
3	12 (7)	0.36 (0.06)	0.925	0.904	<0.00001
4	22 (16)	0.35 (0.10)	0.830	0.773	0.00063
5	2 (2)	0.23 (0.06)	0.930	0.906	0.00003
6	4 (1)	0.30 (0.02)	0.988	0.985	<0.00001
7	5 (3)	0.38 (0.06)	0.933	0.911	0.00002
8	21 (7)	0.39 (0.06)	0.965	0.947	0.00008
9	25 (10)	0.47 (0.09)	0.943	0.915	0.00027
10	9 (5)	0.38 (0.06)	0.939	0.919	0.00002
11	27 (9)	0.44 (0.66)	0.965	0.951	0.00001
12	17 (5)	0.41 (0.04)	0.972	0.963	<0.00001
13	10 (15)	0.36 (0.16)	0.663	0.551	0.00752
14	10 (8)	0.30 (0.08)	0.871	0.828	0.00024
15	30 (13)	0.43 (0.07)	0.917	0.894	0.00001

[a] δ, time for the first decimal reduction; β, shape of the inhibition curve.

TABLE 4. *Effect of the partial substitution of KCl and $CaCl_2$ for NaCl on the frequency of the two yeast species identified in packing brines*[a]

Run no.	Frequency (%)	
	S. cerevisiae	*P. membranifaciens*
1	80	20
2	100	0
3	5	95
4	50	50
5	40	60
6	20	80
7	100	0
8	50	50
9	10	90
10	50	50
11	60	40
12	0	100
13	5	95
14	10	90
15	30	70
Avg	40.67	59.33

[a] Restriction fragments for *S. cerevisiae* were 390, 310, and 140 bp (*Cfo*I), 330, 250, 180, and 140 bp (*Hae*III), and 360, 360, and 110 bp (*Hin*fI). Those for *P. membranifaciens* were 175, 110, 90, and 75 bp (*Cfo*I), 325, 90, and 50 bp (*Hae*III), and 275 and 210 bp (*Hin*fI).

general the usual packing conditions (NaCl only) result in a shelf life of 689 h (~29 days), but the use of salt mixtures may reduce the shelf life. However, the time for the first decimal reduction was not linked to the initial salt conditions in the covered solutions; therefore, statistically the use of the various salt mixtures in packing did not affect the preservation of seasoned cracked Aceituna Aloreña de Málaga.

The yeast populations at the end of the study ranged from 2.87 log CFU/ml (run 2, standard NaCl-only packing conditions) to 4.13 log CFU/ml (run 11) with an average population of 3.49 ($\pm$0.37) log CFU/ml. This final yeast population and the areas under the growth-decline curves also were not correlated with the various chloride salt mixtures.

Hence, in agreement with Sleator and Hill *(35),* the replacement of 2.5 g/100 ml NaCl with other salts may reduce the period after which the minimum population and the first decimal reduction time were reached. Changes were not particularly related to initial salt mixtures but introduced an unpredictable instability in the packed product. As result, to properly fix the shelf life of eventual new specific presentations with various salt mixtures in the packing brines, further detailed studies are required.

At the end of the shelf life of the products, only two yeast species were isolated and identified by RFLP analysis of the 5.8S ITS region, *Saccharomyces cerevisiae* and *Pichia membranifaciens,* with average frequencies of 40.67 and 59.33%, respectively (Table 4). When frequencies from both species were modeled as a function of the initial salt mixtures in the packing brine, a linear equation was suggested. This equation was nonsignificant ($P = 0.1174$) but explained about 88.26% of the total variance, which suggests a possible trend. The two-dimensional contour lines of the fit (data not shown) indicated that the *S. cerevisiae* frequency decreased from a 67% expected value to 27% as the amount of NaCl was reduced from 5 to 2.5 g/100 ml (and amount of KCl and $CaCl_2$ increased up to 2.5 g/100 ml). In contrast, a trend for higher frequencies of *P. membranifaciens* in brine with KCl and $CaCl_2$ versus brine with only NaCl was observed. Further research is needed to confirm the differential resistance of yeasts to the effects of various chloride salts in real table olive products.

These two yeast species are considered normal microbiota of fermenting table olives *(5, 6),* and both species have been recently found in natural black Conservolea olives packed in polyethylene pouches *(14).* However, *S. cerevisiae* has been linked to gas pocket formation in natural black olives *(17)* and to olive tissue degradation in directly brined olives *(18)*; thus, this yeast is considered a spoilage microorganism in packaging.

Effect of salt mixtures on the physicochemical characteristics of brines and the quality of fruits. The pH value is an essential parameter for olive safety and stability *(17)* because it affects the growth of pathogens and the inhibitory efficacy of sorbate *(4).* The International Olive Oil Council *(21)* established a maximum pH of 4.3 for natural olives in brine. In this experiment, the pH among treatments ranged from 3.59 (run 14) to 4.02, with an overall change of 0.40 units (Fig. 2). The pH values were significantly related to the compositions of the initial packing brines. The sequential sum of squares (data not shown) suggested a linear equation. Fit parameters ($P = 0.0373$; lack of fit, $P = 0.419$; precision, 5.669) revealed that the model was suitable to navigate through the experimental region. The equation of the linear model in actual variables (salt proportions) was

$$\text{pH} = 0.78965 \times \text{NaCl} + 0.74297 \times \text{KCl} + 0.68857 \times \text{CaCl}_2 \quad (7)$$

According to the two-dimensional contour lines (Fig. 3), the highest pH value is expected for brines with only NaCl, and the lowest level is expected in the $CaCl_2$ vertex (at 2.5 g of $CaCl_2$ per 100 ml plus at 2.5 g of NaCl per 100 ml). Thus, the pH values decrease as the composition of the mixture moves from the base connecting the NaCl (5 g/100 ml) and KCl (2.5 g of NaCl per 100 ml plus 2.5 g of KCl per 100 ml) vertexes to the opposite vertex (2.5 g of NaCl per 100 ml plus 2.5 g of $CaCl_2$ per 100 ml). However, the contour lines are not parallel to that line; its inclination implies that a fixed pH change (e.g., moving from pH 3.82 to 3.78) requires the substitution of $CaCl_2$ for a lower percentage of NaCl than that of KCl. The substitution of KCl for NaCl has a small effect on pH changes; e.g., the pH of brine with NaCl alone is about 4.02, but half of the NaCl percentage must be replaced to reduce the pH to about 3.83 in the KCl vertex (2.5 g of NaCl per 100 ml plus 2.5 g of KCl per 100 ml). Thus, substitution of $CaCl_2$ for NaCl produced a greater decrease in pH than when KCl was substituted for NaCl, although KCl can also contribute to a slight decrease in the pH. The contour lines are almost perpendicular to the base connecting the NaCl

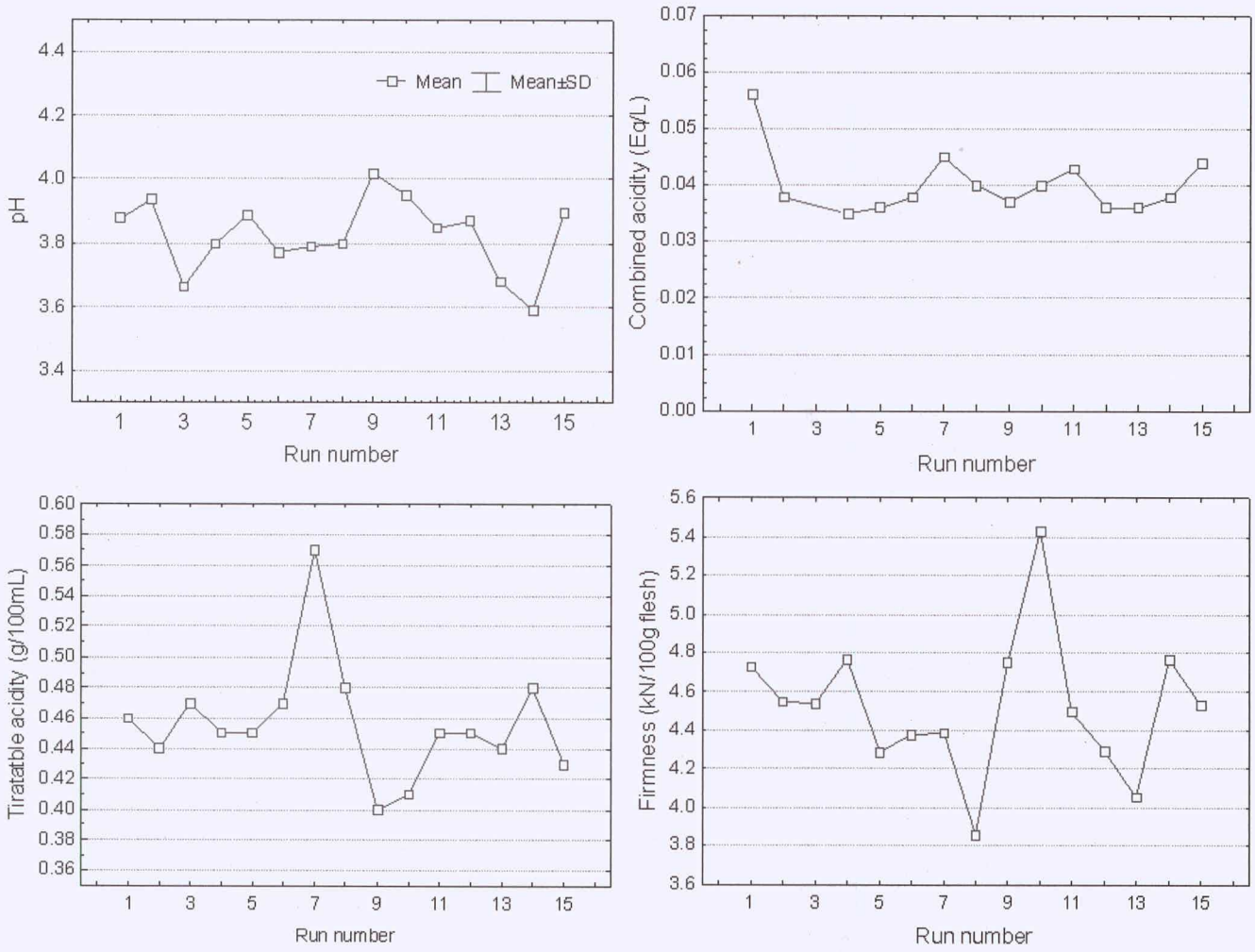

FIGURE 2. *Final physicochemical characteristics of olives at the end of the study, according to runs.*

and $CaCl_2$ vertexes, indicating that for a specific NaCl-$CaCl_2$ relationship, the presence of KCl does not have any appreciable effect. As a result, the presence in the packing brines of increasing proportions of $CaCl_2$ (0.0 to 2.5 g/100 ml) may contribute to decreasing the pH and improving the stability and safety of the product. This hypothesis is supported by the results obtained with the same salt in the fermentation of green Gordal table olives *(8)* or in the storage of cracked Aceituna Aloreña de Málaga *(7)*.

The titratable acidity is responsible for the pH decrease in table olives (in addition to the presence of $CaCl_2$ when this salt is included in the brine mixtures) and therefore contributes to the product stability and safety. When the product is stabilized by preservatives, the minimum concentration established in the table olive standards for this parameter in natural brined olives is 0.3 g of lactic acid per 100 ml. In this work, the concentrations of lactic acid in the various treatments ranged from 0.40 to 0.57 g/100 ml of brine (Fig. 2) and were always above the required limit. Because of the absence of LAB growth in all treatments, titratable acidity was due to the acid added to the packing brine and was not related to the initial salt mixtures. Thus, the use of salt mixtures in packing did not affect titratable acidity.

Combined acidity is due to the presence of organic salts *(17)*, and the importance of these salts in table olives is related to their buffering capacity, which in turn controls the pH changes with acidity. In this study, combined acidity ranged from 35 to 56 meq/liter (Fig. 2), and the acidity values were not related to the initial concentration of salts in the packing brines. Thus, the use of salt mixtures in the packing brine did not affect combined acidity.

The Cl^- concentration was also analyzed in the present study because all the components in the mixture were chloride salts. The sequential sum of squares suggested a quadratic model (data not shown) whose fit parameters ($P = 0.0076$; lack of fit, $P = 0.2983$; precision, 7.533) revealed that it was suitable to navigate through the experimental region. The equation of the linear model in actual variables (salt proportions) was

$$Cl^-(g/100\ ml) = 0.819 \times [NaCl] + 1.118 \times [KCl] + 0.670 \times [CaCl_2] - 0.169 \times [NaCl] \times [KCl] \quad (8)$$

The triangular plot (Fig. 3) shows that the highest concentration in the brines corresponded to samples prepared with only NaCl. The proportions of salts in the initial brine situated in the base of the diagram (the experimental region where the proportions of KCl and $CaCl_2$ are higher) defined the region with the lowest Cl^- concentration. Apparently, the Ca diffusion into the flesh was also accompanied by the corresponding anion (Cl^-).

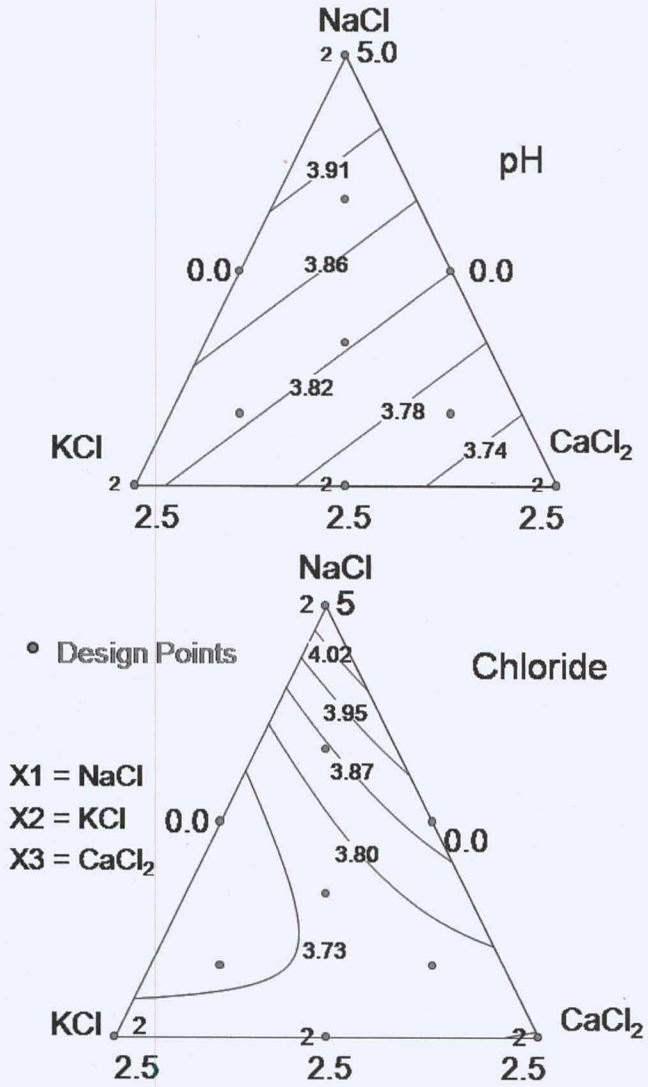

FIGURE 3. *Two-dimensional contour lines for final pH (upper panel) and chloride concentration (lower panel) as a function of the initial NaCl, KCl, and $CaCl_2$ concentrations in the olive packing brines.*

The results indicate that the contribution of table olives to Cl^- intake by consumers is approximately 2.2 to 2.5 g/100 g of olive flesh (considering an equilibrium between brine and moisture in flesh), which is remarkably higher than the daily recommend intake (800 mg/day) *(16)*. Currently, consumption of Ca is not a matter of concern, but its abundance in table olives is emphasized for the first time in the present study. As a result, the use of chloride salt mixtures in packing brines may lower the intake of sodium by consumers but may increase the intake of chlorides.

Firmness is an essential table olive quality that may vary according to olive style and presentation *(21)*. Firmness (Fig. 2) ranged from 3.86 to 5.43 kN/100 g for runs 8 and 10, respectively, both with Ca added, although no correlation between firmness and element concentration was apparent, i.e., the initial $CaCl_2$ concentration in the packing brine of run 8 was higher (0.417 g/100 ml) than that in run 10 (0.125 g/100 ml). When firmness was considered as a response of the mixture design, no relationship between its value and the initial concentrations of the various salts in the packing brines was found. Thus, the use of salt mixtures in the packing brine of traditional Aceituna Aloreña de Málaga did not affect instrumental texture. This lack of a Ca effect was unexpected because the presence of Ca has been traditionally related to texture improvement *(17)*, which could be related to the cracking of the fruits. An increase in firmness due to presence of Ca has already been mentioned in olives treated without lye *(11)*.

Color plays an important role in seasoned olives and particularly in Aceituna Aloreña de Málaga because the green color is an essential characteristic of the product. However, color preservation is usually a difficult task *(4)*. In this experiment, luminance (L*) was fairly high, ranging from 51.11 to 58.41 with an average within treatments of 54.67 ($\pm$1.89) (Table S1 in supplementary material available at https://docs.google.com/file/d/0BwMgslO6iMbOWWE5MEM3ZkFWUzA/edit). Values of a* were positive (reddish) and ranged from 3.17 to 4.44 ($\pm$1.00). Values of b* were also positive (yellow) and high, indicating that the original green color had turned to a more yellowish tone similar to that of fermented green Spanish olives. The high hue values (h_{ab}) mean that the olive colors form an angle fairly close to 90°, which is also characterized by high luminance (lightness) (Table S1). Changes in c* and –a*/b among treatments (Table S1) were also relatively low, with standard deviations of 2.85 and 0.04, respectively. The color index (developed for lye-treated green olives) ranged from 37.15 (run 8) to 42.90 (run 4) for treatments that contained both NaCl and $CaCl_2$ (but not those that contained KCl, run 8). The overall average color index was 39.05, with a standard deviation of 1.83, which is relatively low (Table S1). In general, the changes observed in all color parameters indicated a clear degradation of the green color regardless of treatments, which may have been due to the presence of about 0.50 g/100 ml of titratable acidity and the subsequent low pH *(17)*. The lack of effect of the initial salt mixtures on the color degradation was confirmed by using all these color parameters as responses of the experimental design; none of them were related to the concentrations of the diverse salts in the packing brines. Hence, instrumental color measurements indicate the difficulties associated with preserving the green appearance of this type of olive *(4)*, but the use of salt mixtures in the packing brine did not have an effect on color.

These results indicate that 50% of the NaCl in the packing brine can be replaced with KCl and $CaCl_2$ without significantly altering most of the traditional physicochemical characteristics (except pH and Cl^- concentration) and quality (firmness and color) of Aceituna Aloreña de Málaga. However, the nutritional value of this product will be improved because of the increase in potassium and calcium, which are important nutritional elements. However, because of the high proportion of Cl^- in table olives (disclosed for the first time in the present study), chlorides should perhaps be replaced by other mineral salts (e.g., lactates) in reformulated products.

Multivariate analysis. Multivariate analysis is a convenient technique for considering all variables simultaneously. Clustering analysis (Fig. 4) of runs (based on all

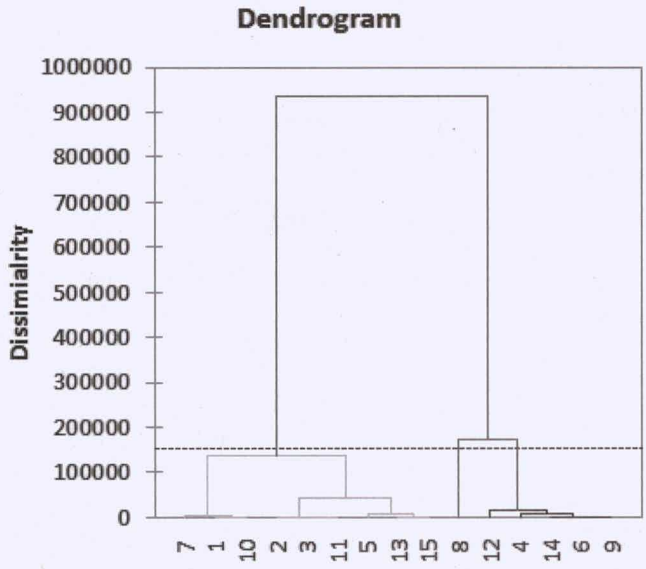

FIGURE 4. *Clustering analysis based on standardized data from microbiological and physicochemical parameters of brine and firmness and color of olives.*

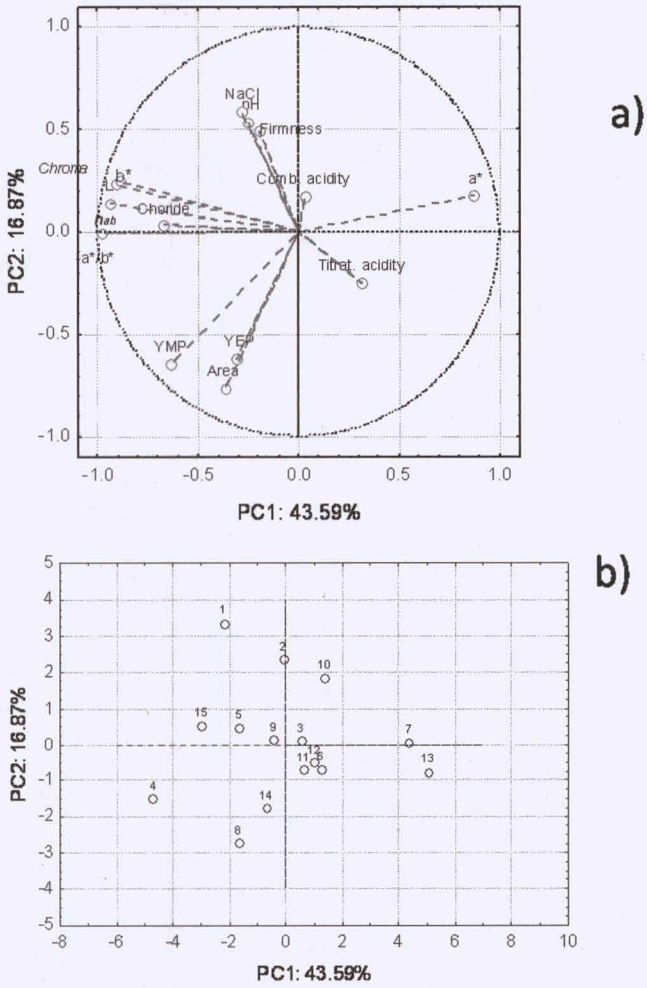

FIGURE 5. *Principal components analysis based on standardized data from microbiological and physicochemical parameters of brines and firmness and color of olives. (a) Projection of the original variables onto the plane formed by the two first PCs. (b) Projection of cases (using run number as labeling variable) onto the plane formed by the first two PCs.*

previously mentioned variables) revealed three different clusters (see characteristics and runs linked to each one in Table S2 in the supplementary material). Cluster 3 included only run 8 (characterized by a high concentration of NaCl (4.167 g/100 ml) and moderate concentrations of KCl and $CaCl_2$ (0.417 g/100 ml). The second cluster grouped runs 4, 6, 9, 12, and 14 with the lowest NaCl concentrations in brine. The third cluster included runs with various concentrations of NaCl. Thus, treatments could be grouped into three profiles, apparently based on the sodium concentration; the peculiar characteristics of run 8, with equilibrated proportions of KCl and $CaCl_2$, were particularly apparent.

The principal components analysis resulted in only four eigenvalues greater than 1. The proportion of variance explained was 43.59, 16.87, 11.13, and 9.39%, respectively, for these four PCs, accounting for 81% of the total. The projection of the original variables onto the plane of the first two PCs (Fig. 5) revealed the close relationship among all the microbiological variables (maximum and minimum populations and area under the respective curves) and those related to color, including a* with a relationship that was opposite that of the rest of the color parameters. Physicochemical variables were also very close to each other, including titratable acidity (opposite sign from pH, as expected). As result, the new PCs were linked to these groups (see Fig. 5 and factor loadings in Table S3 in supplementary material). PC1 could represent original variables related to color, i.e., positive for a* (0.874) and negative for the rest of the color parameters (L*, −0.9415; b*, −0.9056; h_{ab}, −0.9753; c*, −0.9807; −a*/b*, −0.9751), and could be renamed color quality. PC2 was related to microbiological variables (area below curve for yeasts, −0.7670; yeast final population, −0.6217; yeast minimum population, −0.6438; Cl^- concentration expressed as NaCl, 0.5947) and could be renamed microbial stability. In contrast, the physicochemical variables (pH, −0.6245; titratable acidity, 0.6622; combined acidity, 0.7228) were more closely related to PC3 (see Table S3), a relationship that would have been unable to be determined from Figure 5. PC4 was related to only olive firmness (0.5039), but with a limited relationship.

Principal components analysis can also be used to understand relationships among the new variables (factors) and runs (Fig. 5). Runs 7 and 13 (clearly distinguished from the rest) can be related to high values of PC1 (low values for L*, b*, h_{ab}, c*, −a*/b*, and color index but high values for a*) and average values for PC2 (microbial stability). Runs 1, 2, and 10 are characterized by low minimum and ending yeast populations, low values for areas under the curves (because of the negative correlation with PC2), and high NaCl concentrations. These runs were also in the same group in the dendrogram. Run 8 was distinguished by approximately opposite values of these variables (high minimum and ending yeast populations); its position is in agreement with the formation of a separate group in the dendrogram. Runs 9, 3, 12, 11, and 6 have an intermediate position for both factors. However, the relationship among

run positions in the new PC axes cannot be related to the concentrations of the various salts in the packing brine because runs 7 and 13 and runs 1 (similar to 2) and 10 have different brine compositions. The scores of runs for the new variables also were not related to initial salt mixtures in the packing brines.

In summary, the use of different salt mixtures in the packaging brines of seasoned table olives produced slight changes in the survival of contaminating yeasts, possible selection for particular yeast species, and significant changes in pH and chloride concentration. Other characteristics of these mineral-fortified products were similar to those of products currently on the market. These results suggest the usefulness of a detailed microbiological and physicochemical study when developing new specific product types.

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