**Fermentation profile of green Spanish-style Manzanilla olives according to NaCl content in brine**

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**Running title:** Fermentation of Manzanilla olives in salt mixtures

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**Abstract**

This work studies the effects of the partial substitution of NaCl with potassium and calcium chloride salts on the fermentation profile of Spanish-style green Manzanilla olives. For this purpose, response surface methodology based in an enlarged simplex centroid mixture design with constrain (∑salts = 100 g/L) was used. Regarding to physicochemical characteristics, pH decreased when CaCl2 increased, titratable acidity was lower in presence of KCl while combined acidity increased as the contents of KCl and CaCl2 were close to the barycentre of the experiment (33.33% each salt). Regarding to microbiological profile, *Enterobacteriaceae* growth was slight stimulated in presence of high CaCl2 contents, yeast patterns were not linked to the initial brine compositions, while the maximum lactic acid bacteria population decreased slightly as KCl and CaCl2 increased in the proportion 1:1, although a moderate (equilibrated) content of both may be stimulating. Results obtained in this work show that Spanish-style green Manzanilla cv. can be fermented in diverse mixtures of chloride salts, albeit the initial CaCl2 should be limited to 20-30 g/L to prevent excessive *Enterobacteriaceae* growth; combining it with a similar proportion of KCl may also improve LAB predominance.

**Keywords:** calcium chloride; green table olives; mixture design; potassium chloride; sodiumchloride.

**1. Introduction**

Spanish-style green Manzanilla cv. is probably the most appreciated and marketable table olive product. The Spanish production was about 156.000 tons in the 2012 season (Agencia Española para el Aceite de Oliva, 2013) but it is also fairly popular in the rest of olive growing countries. Its processing is quite standard and is characterized by the use of an intermediate concentration of sodium hydroxide solution (20-25 g/L) during debittering. Then, the fruits are washed to remove the excess of alkali (18-24 h) and brined in a NaCl solution (110-120 g/L NaCl) where a spontaneous or inoculated lactic acid fermentation process is achieved (Garrido Fernández et al., 1997).

Fresh olive fruits (raw material) are low in Na content but on the contrary high in K and, in decreasing proportions, Ca, Mg, and P (Garrido Fernández et al., 1997). Most of the original elements in flesh, except Ca, are lost in great proportions during processing because the fruits’ immersion in successive aqueous solutions. Hence, after the fermentation and conditioning, the final products are poor in K, Mg and P and other micronutrient minerals but rich in Na which, according to an industrial survey, has an average content of 17 g Na/kg olive flesh (equivalent to 42.5 g salt/kg flesh) (López López et al., 2008). Therefore, in case of a 100 g olive flesh consumption, such level would supplied, approximately, 70% of the recommended daily intake for salt (WHO, 2003), established as 5 g/day (2000 mg Na+/day) (WHO, 2003; British Food Standard Agency (FSA), 2009).

The average intake of salt in Spain has been estimated in 9.8 g/day (Ortega et al., 2011); reduction of this level may, eventually, be achieved by limiting the salt content in the most contributing foods. In this survey, the impact of table olives on the overall salt intake was not considered of concern due to its low consumption (4 kg olives (equivalent to 2.8 kg flesh)/year). In spite of this, a reduction of NaCl concentration in table olives would be favorable for consumers. Following the suggestions of the National Salt Initiative (Council, 2010), the EU Commission made a called to Member States to develop coordinate national nutritional policies to reduce salt intake. In this framework, the “Agencia Española de Seguridad Alimentaria y Nutrición” (AESAN) implemented in Spain the so called NAOS strategy, which includes an initiative to reduce salt consumption (AESAN, 2010).

 Different studies have been carried out to evaluate the reduction or substitution of NaCl in table olive and other vegetable fermentations (Bautista-Gallego et al. 2013). They have shown that this possibility is becoming a reality but food reformulation must be checked product by product. Tassou et al. (2007) reported an increase in the cell wall breakage due to the presence of CaCl2. Kanovouras et al. (2005) produced natural black olives using mixtures of NaCl (12.8%) and CaCl2 (0.29%) without lost of acceptance by consumers. Di Silva (2000) also arrived to similar results after processing green olives in solutions containing KCl and CaCl2. Bautista Gallego et al. (2010) established that NaCl could be substituted with KCl and/or CaCl2 in diverse proportions in brined green olives. In addition, the composition of the initial brines caused a great impact on the Gordal cv. processing, decreasing sugar release and acidity production (Bautista-Gallego et al., 2011). However, the fermentation of Manzanilla cv. as green Spanish-style in low NaCl brines, supplemented with other mineral nutrients, has not been investigated yet. However, these studies are necessary because the potential microbial risk inherent to any food reformulation (Sleator and Hill, 2007).

Experimental design and Response Surface Methodology (RSM) are powerful tools to study the simultaneous effects of several variables (Myers and Montgomery, 2002) and has been widely used in experimental studies related to salt substitution (Guillou and Floros, 1993; Tsapatsaris and Kotzekidou, 2004; Bautista Gallego et al., 2010, 2011).

In this context, the aim of this work was to study the effect of the partial replacement of sodium chloride by potassium and calcium chloride on the physicochemical and microbiological profile of green Spanish-style Manzanilla cv. fermentation, using RSM based on a simplex centroid mixture design (enlarged with central points).

**2. Material and methods**

*2.1. Olives and experimental design*

The experiments were carried out with Manzanilla olives (*Olea europaea pomiformis*), which is the most popular cultivar for preparing Spanish-style green table olives all over the world. Fruits were supplied by a local producer (JOLCA S.A., Huevar del Aljarafe, Seville, Spain). After a previous selection for removing deteriorated and low size fruits, the olives were introduced in the fermentation vessels (2.90 kg/container) and immersed in a 21 g/L sodium hydroxide solution (lye) (2.35 L solution/container). When the alkali had penetrated 2/3 of flesh, the lye was substituted with water. After washing for about 18 h, the liquid was removed and immediately substituted with different brine solutions. The fermentation vessels were let to equilibrate for 22 h and, then, carbon dioxide was bubbled until saturation (pH stabilization). The suspension was allowed to equilibrate for ~20 h and then all the fermentations vessels were inoculated with a 24 h *Lactobacillus* (*Lb.) pentosus* culture (strain IGLAC01) to reach an initial population of approximately 6 log10 CFU/mL. The growth of the rest of the microbiota (*Enterobacteriaceae* and yeasts) was left to be spontaneous.

The experimental design consisted of 15 runs composed of 10 independent treatments obtained from a simplex centroid mixture design, enlarged with some interior points and 5 replicates (Table 1). The composition of each brine was obtained using Design Expert v.6.06 software (StateEasy, INC., Minneapolis, USA). The total initial concentrations of the diverse salts was constrained to [NaCl]+[KCl]+[CaCl2]=100 g/L, with NaCl ranging from 40 to 100 g/L, KCl from 0 to 60 g/L and CaCl2 from 0 to 60 g/L. The overall sum of mixture concentrations (100 g/L) mimicked that of brines containing only NaCl, commonly used in this process.

*2.2. Physicochemical analyses*

The analyses of olive brines for pH, titratable and combined acidity were carried out using the methods described by Garrido Fernández et al. (1997). The changes in pH were modelled using the following first order decay equation:

y = a+b·exp(-c·x) (1)

where *x* is the time (h), *a* is the lower asymptote, *b* the total change in pH and *c* the kinetic constant unit (h-1).

The production of titratable acidity was modelled using a first order kinetic formation, according to the equation:

y=a·(1-exp(-b·x)) (2)

where x is the time (h), *a* is the total final acid content expressed as g lactic acid (upper asymptote), and *b* is the specific formation rate unit (h-1). In this case, the time to form half the amount of acid is given by x50=ln2/b. The areas for the evolution of the physicochemical characteristics *versus* time were estimated by integration using Origin 7.5 software (OriginLab Corporation, Northampton, USA).

*2.3. Microbiological analyses*

Brine samples or their decimal dilutions were plated using a Spiral System model dwScientific (Don Whitley Scientific Limited, England) on the appropriate media. Subsequently, plates were counted, using a CounterMat v.3.10 (IUL, Barcelona, Spain) image analysis system, and results expressed as log10 CFU/mL. *Enterobacteriaceae* were counted on VRBD (Crystal-violet Neutral-Red bile glucose)-agar (Merck, Darmstadt, Germany), LAB on MRS (de Man, Rogosa and Sharpe)-agar (Oxoid) with 0.02% (wt/vol) sodium azide (Sigma, St. Luis, USA), and yeasts on YM (yeast-malt-peptone-glucose medium)-agar (DifcoTM, Becton and Dickinson Company, Sparks, MD, USA) supplemented with oxytetracycline and gentamicin sulphate as selective agents for yeasts. Plates were incubated at 30ºC for 48-72 h.

Changes in the *Enterobacteriaceae* populations *versus* time were modelled using the two-term Gompertz equation proposed by Bello & Sánchez Fuertes (1995), which has the following expression:

log Nt=log(N0)+*k1*\*exp[−exp(−*k2*(t−*k3*))]−*k4*\*exp[−exp(−*k5*(t−*k6*))] (3)

where Nt is the population (log10 CFU/mL) at time t(days); N0 is the initial population (log10 CFU/mL); *k*1 is the increase in microorganisms from the initial level to the maximum (log10 CFU/mL); *k*2 is the relative growth rate (days-1); *k*3 is the time at which growth rate is maximum (days); *k*4 is the decrease from the maximum to a minimum level (log10 CFU/mL); *k*5 is the relative death rate (days-1) and *k*6 is the time (days) at which death rate is maximum.

Changes in the LAB populations *versus* time were fit using the Pruitt & Kamau (1993) model, which has the following expression:

Nt = (*Nmax*/[1+exp(-*μ*(t-*τ*))]+*Nd*\*exp(-*γ*\*t) (4)

where *Nt* is the population (log10 CFU/mL) at time t(days), *Nmax* is the maximum asymptotic population (log10 CFU/mL), *µ* is the maximum growth rate (days-1), *τ* is the time (days) for Nmax/2, *Nd* is the damage population (log10 CFU/mL) and *γ* is the maximum death rate (days-1). Model fits were achieved by using the non-linear module of the Statistica 7.1 software package (Statsoft Inc, Tulsa, USA).

Changes in the microbial populations were also assessed by estimating the area under the growth/decline curves (Bautista-Gallego et al., 2011). Areas were calculated by integration, using Origin 7.5 software.

*2.4. Molecular identification of microorganisms*

 Brine samples (100 mL) were collected in sterile conditions at the end of the fermentation (~ 135 days) and plated on the yeast and LAB selective media described above. A total of 300 isolates, 150 LAB and 150 yeasts (10 for each treatment) were randomly selected and purified by subsequent re-streaking on YM or MRS agar, respectively. The different LAB isolates were then identified at species level using multiplex PCR analysis of the *rec*A with species-specific primers for *Lb. pentosus*, *Lb. plantarum* and *Lb. paraplantarum*, following the protocol described by Torriani et al. (2001). Yeasts were identified by RFLP analysis of the 5.8S-ITS rDNA regionaccording to the procedure described by Esteve Zarzoso et al. (1999).

*2.5. Modelling of the effect of salt mixture composition on fermentation profile*

Physicochemical and microbiological parameters (responses) were subjected to multiple quadratic regression (secondary model) analysis (Myers and Montgomery, 2002; Bautista Gallego et al., 2010) to relate them to initial chloride salt concentrations in brine. First, the sequential sums of squares were estimated and the most appropriate model suggested. Then, the coefficients were calculated and the fit, lack of fit, and precision evaluated by the regression analysis of variance (ANOVA). Only significant models (p≤0.05) with not significant lack of fit (p≥0.05) and precision higher than 4 were considered. Terms for the model were selected by backwards elimination and only those significant at p≤0.05 or introduced in application of the hierarchical principle were retained.

Selected parameters of the *Enterobacteriaceae* (time to sprang, time to disappearance, maximum population, and area under growth curve), yeasts (time to sprang, maximum population, and area under growth curve) and LAB (maximum population and area under growth curve) growth were also subjected to PCA analysis to disclose relationships among variables and between them and cases.

*2.6. Statistical data analysis*

Statistica software version 7.1, Design Expert v6.06, Origin 7.5 and Sigma Plot version 11 (Systat Software, Inc.) and Table Curve 5.1 (Systat Software, Inc.) were used for data processing, analysis and plotting.

 **3. Results and discussion**

*3.1 Effect of the chloride salt mixtures on physicochemical fermentation profile*

The diverse salt mixtures caused some differences among the initial pH of the fermentation brines. The behaviour is in agreement with Bautista Gallego et al. (2011) who found that high CaCl2 and KCl contents led to low and high pH values respectively while NaCl did not have effect on this parameter. During fermentation, changes of pH versus time followed a similar trend, although with some peculiarities among treatments. Curves of pH changes could be appropriately modelled (all parameters were significant at p<0.001). An example of this fit is shown in Figure 1 (upper panel). The asymptotic final pH for treatments were within a narrow range (3.99-4.43) (Table 2). Apparently, there was a consistent trend for obtaining lower final pH levels in presence of high proportion of CaCl2, as already observed in green Spanish-style Gordal olives (Bautista Gallego et al., 2011). However, in this case, the major effect that the presence of CaCl2 caused was a delay in the diffusion of sugars (Rodríguez Gómez et al., 2012). No pH parameter from the fit models could be linked to the initial salt composition of the brines; however, the areas under pH (Table 2) could be related to the salt concentrations in the initial brines. The equation, in terms of the actual concentrations, was:

Area under pH =+130.023\*NaCl +137.54\*KCl +122.25\*CaCl2 (5)

The interpretation of the equations from the pH contour lines (Figure 1, panel B) showed that these were perpendicular to the base of the triangle (60 g/L KCl – 60 g/L CaCl2) and parallel among them. That is, the final pH was independent of the NaCl content in the initial brine while, according to the contour lines, addition of KCl and CaCl2 increased and decreased it, respectively. In this case, lowest areas mean most convinient pH evolution. The presence of CaCl2 in the fermentation of green Spanish-style Gordal olives led to similar conclusions (Bautista-Gallego et al., 2011). Then, CaCl2 can play an important role to decrease the risk of spoiling microorganisms due to its lowering pH effect.

High titratable acidy production is the main objective of green Spanish-style green table olive fermentation because pH, combined acidity, color, texture, and product stability depend on it (Garrido Fernández et al., 1997). The formation of titratable acidity was fit by a first order kinetic model (Figure 2, upper panel). Most of the parameters were significant at p<0.001 and only asymptotic maximum value and production rate for run 5 were not significant and have non realistic values (Table 3). However, none of the kinetic parameters (significant for all runs) were related to the salt contents in the initial brines. However, the areas under the titratable acidity curves was appropriate to measure the overall trends. Apparently, parameters related to overall pH and titratable acity changes could be more sensitive to initial brine mixtures.

The equation of the area under titratable acidity curves, in terms of the actual concentrations, was:

Area under titratable acidity =+16.38\*NaCl+13.04\*KCl+23.53\*CaCl2 (6)

The countour lines (Figure 2, lower panel) were quite similar to those of pH, although with the lines slightly inclined. Overall, presence of CaCl2 led to greater areas (associated to higher acid formation) while addition of KCl produced, in general, curves that were below (lower titratable acidity contents) than those containing CaCl2. As in the case of pH, NaCl content (within the range used in this work) apparently played a neutral role in the acid production. The similarities observed between the trends of pH and titratable acidity production with respect to the effects of KCl and CaCl2 on the fermentation process is due to the strong association between them (Garrido Fernández et al., 1997). The behavior found in this work is also in agreement with results reported from the fermentation of green Gordal Spanish-style olives (Bautista Gallego et al., 2011) and constitutes a solid support of the strong effects of salt mixtures on the Spanish-style processing.

It is known that brine buffer capacities influence pH changes during fermentation and packaging (Garrido Fernández et al., 1997). Initial combined acidities in treatments ranged from 30 to 90 mEq/L but as fermentation progressed the values were approaching (between 70 to 100 mEq/L). The final value of this physicochemical parameter was related to the initial salt mixture concentrations. The equation was:

Final combined acidity (mEq/L) = +0.881\*NaCl + 0.226\*KCl + 1.007\* CaCl2 + 0.012\* NaCl\*KCl + 0.018\*KCl\*CaCl2 (7)

The graph for this equation is shown in Figure 3. The countour lines corresponded to an inclined hill which climbs as the KCl and CaCl2 contents are higher. In fact, the line of maximum slope was parallel to the NaCl-CaCl2 base and corresponded, approximately, to a fixed relatiosnip NaCl:KCl of 2/3:1/3. The highest value of final combined acidity was reached around the barycentre of the triangle, where the composition corresponds to an initial brine concentration of 33.33% in each salt. The area under combined acitidity curves were related to initial salt mixtures, showed a similar trend, and led to the same conclusions (data not shown).

*3.2. Effect of the chloride salt mixtures on the fermentation microbiological profile*

In most of the treatments, the presence of *Enterobacteriaceae* was detected from the very beginning of the fermentation but in others there was a slight delay in their detection or were completely absent (run 4). The growth of these microorganisms was very fast and reached the maximum at around 200 h, followed by a declining trend, with marked differences among runs, until their total disappearance (counts under the detection limit). This behaviour could be modeled using the Bello and Sánchez (1995) equation (Table 4). Figure 4 (upper panel) shows an example of this fit for run 11. In general, the fit was fairly good (p<0.001) but in some treatments, the estimation of certain parameters was not significant and the model was not interpretable (Table 4). The greatest increase in *Enterobacteriaceae* from the initial level to maximum population (*k1*) was observed in run 9 (9.51 log10 CFU/mL). On the contrary, the lowest values, apart from the absence in run 4, were in runs 12 and 14. These cicunstances made unreliable the use of most these parameters as responses for the experimental design. On the contrary, the maximum population (assuming no presence in run 4) was related to the initial brine composition. The equation, in terms of actual concentrations, was:

*k*1 (log10 CFU/mL) = + 0.06019\*NaCl + 0.32827\*KCl + 0.03202\*CaCl2 -0.00639\*NaCl\*KCl + 0.00081\*NaCl\*CaCl2 - 0.01933\*KCl\*CaCl2 + 0.00049\*NaCl\*KCl\*CaCl2 (8)

 From the graphic of contour lines (Figure 4, lower panel), it is observed that the greatest *Enterobacteriaceae* population levels were found at CaCl2 concentrations above a proportion higher than 20 g/L. On the contrary, the lowest population levels were situated around the central point of the NaCl-KCl base, where such salts were in the ratio 5:3. Thus, it is apparent that CaCl2 plays an essential role in the growth of *Enterobacteriaceae* populations which were favoured by the presence of this chloride salt, particularly when its content in the initial brine was higher than 20g/L. However, in the case of green Gordal cv., the most important influence on the process was related to the sugar leakage into the brine (and the subsequent effect on titratable acidity formation) (Rodriguez-Gómez et al., 2012). The different behavior observed for CaCl2 presence on the fermentacions of Manzanilla and Gordal may be caused by the differences in size between both cultivars; in general, olives from the first cultivar are smaller than those from the second and, as result, the sugar diffusion (and the subsequent acid production) are less affected. The effect of CaCl2 on *Enterobacteriaceae* growth is interesting and suggests further experiments at industrial scale to prevent the risk of gas-pocket spoilage and safety issues due to an eventual excesive growth on this group of microorganisms (Garrido Fernández et al., 1997).

Yeasts changes in brine could not be modelled by any primary model. Their final populations in brine had a wide range (from 1.9 to 5.0 log10 CFU/mL). It was noticed a slight trend to observe lower yeast population levels in the brines with high initial CaCl2 concentrations. There was also a tendency to find the highest values of area under yeast growth curves as the NaCl content increased and the lowest values in curves from runs with high levels of CaCl2 (data not shown). However, there was no a significant relationship with the concentrations of the salt mixtures in the initial brines.

The differences among treatments in the LAB populations in the brines were reduced due to the inoculation after a few days of brining. As usual, the habitual initial decrease of the inoculum was followed by a rapid increase to a maximum and a slight decrease up to the end of the process, which was modeled using the Pruit & Kamau model (1993) (Figure 5, upper panel). However, among the parameters obtained, only the estimated maximum growth was significantly linked to the initial brine compositions. The model suggested was quadratic and the equation, in terms of the actual concentrations, was:

Estimated maximum population (log10CFU/mL) = +0.0699\*NaCl + 0.0735\*KCl + 0.0725\*CaCl2-0.0004\*KCl\*CaCl2 (9)

The interpretation of the equation through their countour lines (Figure 5) showed that the maximun population of LAB decreased as the NaCl moved from 100 to 60 g/L; the steepest line was observed when the contents of KCl and CaCl2 were aproximatelly in the 1:1 ratio, being the minimun for the mixture represented by 40:30:30 g/L (of NaCl, KCl and CaCl2, respectively). However, in absolute terms, the effect was limited because the differences was of only 0.14 log10 CFU/mL.

*3.3. Characterization of the yeast and LAB populations*

Molecular studies (data not shown) carried out on the yeasts and LAB populations from the different treatments indicated that the biodiversity of the microbial populations in the fermentation brines at the final sampling was very limited. The only yeast species found in all treatments was *Pichia galeiformis*; however, in runs 7 (50:40:10), 11 (40:30:30) and 13 (70:0:30), it was also found *Candida tropicalis* with frequencies of 20, 50 and 50%, respectively, while the presence of *Wickerhamomyces anomalus* was detected in runs 2 (50:10:40), 3 (40:30:30) and 12 (40:0:60)*,* with frequencies of 30, 50 and 50%, respectively. This behavior of Manzanilla was similar to that already reported for Gordal in which *P. galeiformis* was the most abundant species, in combination with *W. anomalus* and *Candida boidinii* in some runs (Bautista Gallego et al., 2011). With respect to LAB, only the species *L. pentosus* was present at the end of the fermentation process.

3.4. Multivariate analysis of *Enterobacteriaceae*, LAB and yeast growth

It was also considered of interest the simultaneous analysis of all microbial growth varaibles simultaneously to disclose possible relationships among them. There was a strong negative correlation (-0.729) between yeast and LAB maximun populations, indicating a competition between them during fermentation and a close relationship among all *Enterobacteriaceae* parameters. However, the overall relationships may be better displayed by the projection of variables and cases on the two first PCs (Figure 6). Factor 1 was mainly related to the *Enterobacteriaceae* parameters (positively with time to sprang and negatively with the rest) and can be generically called “*Enterobacteriaceae*” (Figure 6, upper panel). The Factor 2 was related to LAB (negatively) and yeast (positively) populations, as correspond to their inverse relationship, and yeast time to detection (Figure 6, upper panel). Interestly, yeast and LAB areas are not related to their respective maximum populations (angle close to 90 ). Then, the multivariate analysis has been useful to establish an overall relationship among the main growing characteristics of the diverse microbial populations.

The projection of cases on the first two Factors showed that some cases (13, characterized by their high yeast population and long time to detection; and 4, absense of *Enterobacteriaceae* growth) had singular behaviors (Figure 6, lower panel) while most of the rest had average scores and were gouped around the origin. However, runs 6 (60,20,20), 2 (50, 10, 40) and, mainly, 5 (80. 10, 10) had high LAB maximum populations; which means the convenience of the presence of both KCl and CaCl2 to improve LAB imposition during fermentation. On the contrary, KCl absence (run 13) may lead to excessive yeast growth while an equilibrate concentration (30 g/L) may prevent *Enterobacteriaceae* progress, indicating the convenience of the presence of this salt on the initial brines; however, high proportions of KCl could not be convinient because may promote maximum population and area under growth curve of *Enterobacteriaceae*, mainly in absence of CaCl2 (run 8).

**4. Conclusions**

The partial substitution of NaCl with KCl and CaCl2 affected the characteristics of the Spanish-style green Manzanilla olive fermentation. The presence of CaCl2 led to slightly lower pH values, while treatments containing KCl tend to have less titratable acidity levels. The *Enterobacteriaceae* spontaneous growth was strongly affected by the brine composition, and greater proportions of CaCl2 in the initial brines led to higher populations of this microorganisms during a part of the fermentation process*.* Yeast growth was also spontaneous, and apparently, did not followed a pattern that could be expressed as a function of the initial brine composition. After inoculation, LAB suffered an initial decrease but their presence increased rapidly and were always abundant without important differences in the evolution pattern. The maximum LAB population decreased slightly as the proportions of KCl and CaCl2 increased in the proportion of 1:1 aproximately. Apparently, this behavior is another argument in favour of using a moderate calcium proportion in the fermentation of Manzanilla olives. As demonstrated by PCA, a moderate equilibrated presence of both KCl and CaCl2 could be convinient for promoting LAB growth. Therefore, Spanish-style green Manzanilla olive fermentation can be achieved in presence of mixtures of diverse chloride salts in the initial brine; however, the composition of these brines may have significant effects on the evolution of the process and the CaCl2 should not exceed 20-30 g/L to preven excesive *Enterobacteriaceae* growth. At the same time, this concentration, in combination with a similar proportion of KCl may improve LAB imposition.

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**Table 1.** Expanded simplex centroid mixture design used in the present study for the fermentation of Manzanilla olives. Constrains: NaCl+KCl+CaCl2=100 g/L, with NaCl ranging from 40 to 100 g/L, KCl from 0 to 40 g/L and CaCl2 from 0 to 60 g/L.

|  |  |  |  |
| --- | --- | --- | --- |
| **Treatments** | **NaCl (g/L)** | **KCl (g/L)** | **CaCl2 (g/L)** |
| 1a | 100 | 0 | 0 |
| 2 | 50 | 10 | 40 |
| 3 b | 40 | 30 | 30 |
| 4 | 70 | 30 | 0 |
| 5 | 80 | 10 | 10 |
| 6 c | 60 | 20 | 20 |
| 7 | 50 | 40 | 10 |
| 8d | 40 | 60 | 0 |
| 9 a | 100 | 0 | 0 |
| 10 e | 40 | 0 | 60 |
| 11 b | 40 | 30 | 30 |
| 12 e | 40 | 0 | 60 |
| 13 | 70 | 0 | 30 |
| 14d | 40 | 60 | 0 |
| 15 c | 60 | 20 | 20 |

Note: Runs with the same superscript correspond to duplicate experiments.

**Table 2.** Fit of the changes of pH *versus* time for the different treatments included in the experimental design.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Treatments** | **Low asymptote** **(pH units)** | **Overall decrease (pH units)** | **Decrease rate (h-1)** | **R2** | **Area under pH** |
| **Estimation** | **SE** | **Estimation** | **SE** | **k** | **SE** |
| 1 | 4.14 | 0.11 | 4.91 | 0.49 | 0.00491 | 0.00077 | 0.958 | 13183 |
| 2 | 4.03 | 0.08 | 3.96 | 0.46 | 0.00656 | 0.00106 | 0.952 | 12472 |
| 3 | 4.05 | 0.07 | 4.33 | 0.46 | 0.00684 | 0.00099 | 0.961 | 12523 |
| 4 | 4.10 | 0.07 | 7.86 | 0.56 | 0.00825 | 0.00074 | 0.984 | 12901 |
| 5 | 4.04 | 0.11 | 4.73 | 0.51 | 0.00522 | 0.00085 | 0.954 | 12802 |
| 6 | 4.17 | 0.08 | 4.69 | 0.61 | 0.00857 | 0.00139 | 0.952 | 12759 |
| 7 | 4.27 | 0.08 | 2.87 | 0.28 | 0.00385 | 0.00065 | 0.955 | 13393 |
| 8 | 4.20 | 0.20 | 4.02 | 0.39 | 0.00443 | 0.00070 | 0.959 | 13344 |
| 9 | 4.17 | 0.09 | 6.83 | 0.55 | 0.00685 | 0.00075 | 0.977 | 13161 |
| 10 | 3.99 | 0.06 | 3.66 | 0.27 | 0.00460 | 0.00054 | 0.976 | 12555 |
| 11 | 4.43 | 0.04 | 2.18 | 0.11 | 0.00293 | 0.00030 | 0.985 | 13846 |
| 12 | 4.06 | 0.05 | 3.92 | 0.30 | 0.00639 | 0.00067 | 0.979 | 12553 |
| 13 | 4.11 | 0.07 | 3.51 | 0.28 | 0.00452 | 0.00058 | 0.972 | 12898 |
| 14 | 4.29 | 0.10 | 3.94 | 0.42 | 0.00431 | 0.00075 | 0.950 | 13596 |
| 15 | 4.18 | 0.05 | 2.49 | 0.20 | 0.00425 | 0.00057 | 0.971 | 12964 |

Note: Fit with a first order decay kinetic model with intercept (see material and method section for equation). All coefficients were significant at p<0.001.

**Table 3.** Fit of the titratable acidity changes *versus* time for the different treatments included in the experimental design.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Treatments** | **Maximum concentration (g/L)** | **Constant rate(h-1)** | **R2** | **x50 (h)** | **Area under curve** |
| **Estimation** | **ES** | **Estimation** | **SE** |
| 1 | 8.08 | 0.69 | 0.00093 | 0.00020 | 0.936 | 745 | 1617 |
| 2 | 9.92 | 0.61 | 0.00080 | 0.00011 | 0.976 | 866 | 1864 |
| 3 | 10.87 | 0.76 | 0.00086 | 0.00014 | 0.962 | 806 | 2110 |
| 4 | 7.84 | 0.65 | 0.00120 | 0.00028 | 0.896 | 577 | 1709 |
| 5 | 23.6\* | 11.53\* | 0.00021\* | 0.00013\* | 0.947 | 3300\* | 1862 |
| 6 | 10.58 | 0.87 | 0.00087 | 0.00017 | 0.942 | 797 | 2046 |
| 7 | 7.76 | 0.34 | 0.00112 | 0.00014 | 0.970 | 619 | 1661 |
| 8 | 10.14 | 1.95 | 0.00049 | 0.00016 | 0.927 | 1414 | 1453 |
| 9 | 6.21 | 0.40 | 0.00107 | 0.00019 | 0.948 | 648 | 1307 |
| 10 | 9.59 | 0.54 | 0.00107 | 0.00016 | 0.957 | 648 | 2014 |
| 11 | 6.22 | 0.26 | 0.00178 | 0.00025 | 0.940 | 389 | 1521 |
| 12 | 7.93 | 0.39 | 0.00161 | 0.00026 | 0.933 | 430 | 1896 |
| 13 | 7.92 | 0.39 | 0.00137 | 0.00020 | 0.949 | 506 | 1823 |
| 14 | 4.92 | 0.44 | 0.00116 | 0.00029 | 0.874 | 597 | 1043 |
| 15 | 7.42 | 0.17 | 0.00183 | 0.00014 | 0.982 | 379 | 1824 |

Note: Fit of data with a first order formation kinetic model (see material and method section for equation). Coefficients were all significant at p<0.001, except those for Run 5 which were not significant.

**Table 4.** Parameters deduced from the Bello and Sánchez (1995) model fit to the spontaneous growth of *Enterobacteriaceae* in the different treatments included in the experimental design.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Treatments** | ***k1*** **(log10 CFU/mL)** | ***k2*** **(days-1)** | ***k3*** **(days)** | ***k4*** **(log10 CFU/mL)** | ***k5*** **(days-1)** | ***k6*** **(days)** | **Area** |
| 1 | 6.68 (0.93) | 0.0276 (0.0066) | 196 (7) | 6.71 (0.93) | 0.0274(0.0035) | 338 (8) | 883 |
| 2 | 6.54 (0.20) | 0.0568 (0.0294)\* | 85 (3) | 6.65 (0.26) | 0.0082 (0.0011) | 471 (11) | 2723 |
| 3 | 5.10 (0.06) | 0.0491 (0.0049) | 64 (1) | 5.11 (0.07) | 0.2150 (17)\* | 326 (14) | 1259 |
| 4 | nd | nd | nd | nd | nd | nd | 0 |
| 5 | 6.45 (0.27) | 0.0496 (0.0149) | 59 (4) | 6.60 (0.36) | 0.0073 (0.0014) | 487 (24) | 2988 |
| 6 | 7.47 (0.13) | 0.0570 (0.0112) | 25 (3) | 7.54 (0.17) | 0.0125 (0.0013) | 409 (7) | 3056 |
| 7 | 4.68 (0.35) | 0.0536 (0.0205) | 36 (4) | 7.90 (0.40) | 0.0065 (0.0007) | 372 (19) | 3264 |
| 8 | 6.18 (0.26) | 0.3342 (39)\* | 35 (317)\* | 6.41 (0.47) | 0.0040 (0.0011) | 761 (48) | 4894 |
| 9 | 9.51 (2.08) | 0.0053 (0.0014) | 35 (47)\* | 9.45 (2) | 0.0140 (0.0035) | 316 (11) | 1834 |
| 10 | 6.46 (0.18) | 0.1010 (0.0377) | 52 (4) | 6.55 (0.24) | 0.0119 (0.0020) | 452 (13) | 2752 |
| 11 | 4.59 (0.16) | 0.0574 (0.0157) | 21 (6) | 7.64 (0.21) | 0.0079 (0.0008) | 431 (11) | 3546 |
| 12 | 2.51 (0.40) | 0.0213 (0.0119)\* | 26 (15)\* | 3.02 (0.57) | 0.0152 (0.0053) | 296 (26) | 2531 |
| 13 | 7.02 (0.22) | 0.0595 (0.0107) | 21 (3) | 7.05 (0.24) | 0.0148 (0.0014) | 260 (7) | 1853 |
| 14 | 2.77 (0.22) | 0.3935 (124)\* | 62 (11)\* | 6.19 (0.30) | 0.0081 (0.0016) | 519 (21) | 3316 |
| 15 | 417 (0.09) | 0.0540 (0.0087) | 22 (3) | 7.55 (0.12) | 0.0140 (0.0009) | 324 (4) | 2567 |

Notes: The biological meaning of the different parameters is explained in the Material and Methods section; \* the estimation was not significant.

**Table 5.** Parameters deduced using the Pruit and Kamau model (1993) for the lactic acid bacteria population in the brines of the different treatments included in the experimental design.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Treatments** | ***k1*****(log10 CFU/mL)** | ***k2*****(days-1)** | ***k3*****(days)** | ***k4*****(log10 CFU/mL)** | ***k5*****(days-1)** | **Area** |
| 1 | 7.03 (011) | 0.3088 (0.0354) | 9.07 (0.84) | 5.69 (0.33) | 0.1215 (0.0246) | 21133 |
| 2 | 6.72 (0.11) | 0.9674 (0.2294) | 3.48 (0.26) | 5.54 (0.45) | 0.1752 (0.0243) | 20619 |
| 3 | 6.91 (0.08) | 0.7552 (0.1794) | 2.73 (0.31) | 5.15 (0.55) | 0.2418 (0.0423) | 21477 |
| 4 | 6.91 (0.10) | 0.3561 (0.0416) | 6.87 (0.48) | 5.22 (5.25) | 0.1004 (0.0137) | 20999 |
| 5 | 7.22 (0.08) | 0.4626 (0.0430) | 5.78 (0.39) | 5.35 (0.32) | 0.1366 (0.0174) | 21985 |
| 6 | 6.88 (0.11) | 0.5985 (0.1483) | 3.10 (0.47) | 4.97 (0.66) | 0.1718 (0.0364) | 21223 |
| 7 | 7.07 (0.04) | 0.9493 (0.0931) | 3.13 (0.12) | 5.65 (0.21) | 0.2515 (0.0176) | 21579 |
| 8 | 7.29 (0.08) | 0.3648 (0.0360 ) | 6.12 (0.62) | 5.18 (0.36) | 0.1543 (0.0277) | 21764 |
| 9 | 6.88 (0.09) | 0.2744 (0.0255) | 9.57 (0.70) | 5.30 (0.26) | 0.0999 (0.0159) | 20579 |
| 10 | 7.19 (0.09) | 0.4850 (0.1155) | 2.81 (0.61) | 4.41 (0.64) | 0.2118 (0.0633) | 21742 |
| 11 | 6.74 (0.14) | 0.3142 (0.0552) | -0.097 (1.36)\* | 2.21 (0.79) | 0.0520 (0.0226) | 21254 |
| 12 | 7.14 (0.07) | 0.5572 (0.1126) | 2.82 (0.41) | 4.69 (0.53) | 0.2176 (0.0466) | 21860 |
| 13 | 7.17 (0.05) | 0.6080 (0.0542) | 3.77 (0.43) | 5.28 (0.27) | 0.2893 (0.0528) | 21655 |
| 14 | 7.15 (0.11) | 0.4466 (0.0511) | 9.19 (0.44) | 5.89 (0.28) | 0.1185 (0.0176) | 21505 |
| 15 | 7.04 (0.08) | 0.4990 (0.0996) | 2.63 (0.49) | 4.27 (0.63) | 0.1641 (0.338) | 21754 |

Notes: The biological meaning of the different parameters is explained in the Material and Methods section; \* the estimation was not significant. Model fit was always significant at p<0.001.

**Figure Legends**

**Figure 1.** Fit of the first order decay model to the pH experimental data in run 9 (upper panel), and two dimensions contour lines of the overall changes in pH (area under curves) related to chloride salt mixtures (lower panel). Duplicate design points are indicated by a 2 close to them.

**Figure 2.** Fit of the first order formation model to the titratable acidity values obtained in run 10 (upper panel), and two dimension contour lines of the overall changes in titratable acidity (area under curves) related to chloride salt mixtures (lower panel). Duplicate design points are indicated by a 2 close to them.

**Figure 3.** Two dimensions contour lines of the final combined acidity value as a function of the diverse NaCl, KCl, CaCl2 concentrations in the initial brines. Duplicate design points are indicated by a 2 close to them.

**Figure 4.** Fit of the Bello and Sánchez (1995) model to the *Enterobacteriaceae* counts (log10cfu/mL) in run 11 (upper panel), and two dimensions contour lines of *Enterobacteriaceae* maximum population as a function of the diverse NaCl, KCl, CaCl2 concentrations in the initial brines.

**Figure 5.** Fit of the Pruit and Kamau model to the LAB growth (upper panel) and two dimensions contour lines of LAB maximum population of the model parameter as a function of the NaCl, KCl, CaCl2 concentrations in the initial brines (lower panel).

**Figure 6.** PCA of mainparameters related to *Enterobacteriaceae*, yeast and LAB growth. Projection of variables (upper panel) and case scores (lower panel) on the first two components. Ent., *Enterobacteriaceae*; max, maximum; popul., population; disap, disappearance.