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Individual Effects of Sodium, Potassium, Calcium, and Magnesium Chloride Salts on *Lactobacillus pentosus* and *Saccharomyces cerevisiae* Growth

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ABSTRACT

A quantitative investigation on the individual effects of sodium (NaCl), potassium (KCl), calcium ($CaCl_2$), and magnesium ($MgCl_2$) chloride salts against *Lactobacillus pentosus* and *Saccharomyces cerevisiae,* two representative microorganisms of table olives and other fermented vegetables, was carried out. In order to assess their potential activities, both the kinetic growth parameters and dose-response profiles in synthetic media (deMan Rogosa Sharpe broth medium and yeast-malt-peptone-glucose broth medium, respectively) were obtained and analyzed. Microbial growth was monitored via optical density measurements as a function of contact time in the presence of progressive chloride salt concentrations. Relative maximum specific growth rate and lag-phase period were modeled as a function of the chloride salt concentrations. Moreover, for each salt and microorganism tested, the noninhibitory concentrations and the MICs were estimated and compared. All chloride salts exerted a significant antimicrobial effect on the growth cycle; particularly, $CaCl_2$ showed a similar effect to NaCl, while KCl and $MgCl_2$ were progressively less inhibitory. Microbial susceptibility and resistance were found to be nonlinearly dose related.

Currently, there are large-scale fermentation processes for the commercial production of food and beverages. In particular, lactic acid bacteria (LAB) are used for a variety of dairy, vegetable, and meat fermentations *(29).* Among them, *Lactobacillus* spp. are present in cucumber, sauerkraut, and olives *(13, 29). Saccharomyces cerevisiae* is present in many food fermentations, has been identified as the most abundant yeast species in table olives *(14),* and can be related to practically any processing style *(13).* However, *Lactobacillus* and *Saccharomyces* spp. can also be usual contaminants in orange juice and responsible for the decreasing of shelf life of the product *(4).* The gram-positive bacteria, including diverse species of lactobacilli and pediococci, are the most hazardous organisms in beer *(16). Saccharomyces* spp. have been identified by Savard et al. *(30)* as spoilage yeasts in fermented vegetables, which were resistant to high levels of lactic acid. Malfeito Ferreira et al. *(21)* reported that *S. cerevisiae* was able to produce fermentation in laboratory media in the presence of weak organic acids and potassium sorbate. Thus, the study of the behavior of these microorganisms against diverse chloride salts is of interest not only for table olives, but also for food technology in general.

Fermented vegetables are traditionally prepared using common salt as a main ingredient, with the aim of flavoring and preserving the final products *(13, 15).* Common salt, initially consisting of sodium chloride, lowers the water activity, increases the ionic strength of the solution, reduces the solubility of oxygen in water, and renders the product less prone to spoilage *(20).* However, producers must also consider new concerns of the population with respect to the effect of common salt on cardiovascular diseases, and the fact that overall sodium intake has declined since the early 1980s *(10).* To improve consumers' opinion of high-salt, fermented vegetables, sodium chloride (NaCl) could be replaced, at least partially, with other chloride salts with more favorable effects on health such as potassium chloride (KCl), calcium chloride ($CaCl_2$), or magnesium chloride ($MgCl_2$) *(1),* whose cations are macroelements, and whose contents must be declared in nutritional labeling, according to the legislation of most countries *(6, 12).*

The effects of diverse chloride salts on the growth of *Lactobacillus plantarum* in cucumber extracts were studied by Naewbanij et al. *(24, 25).* Chavasti et al. *(5)* evaluated the effect of NaCl in low-salt cucumber juice on *Bifidobacterium, Lactobacillus, Leuconostoc, Lactococcus, Pediococcus,* and *Propionibacterium.* Guillou et al. *(15)* produced good quality pickles when moderate amounts of NaCl (5%) and $CaCl_2$ (0.2%) were present together with potassium sorbate (0.2%). Directly brined olives, containing $CaCl_2$ and KCl, were of comparable quality to those obtained by using only NaCl *(23).* Marsilio et al. *(22)* studied the sensory analysis of green table olives fermented in different saline solutions (NaCl, KCl, and their mixtures) and obtained acceptable products, although slightly bitter. Tassou et al. *(32)* followed the microbiological and physicochemical changes of naturally black olives at different temperatures and NaCl levels in brine and obtained the best

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conditions at 25°C and 6% NaCl. Tsapatsaris and Kotzekidou *(35)* studied the effects of a substitution of NaCl with 50% KCl on *L. plantarum* and *Debaryomyces hansenii* growth in olive juice obtained from the natural black Greek variety Kalamon. However, information about the individual effects of the different chloride salts (with nutritional interest for consumers) on *L. pentosus* growth, a bacteriocin-producing LAB *(9, 33),* and *S. cerevisiae,* a ubiquitous microorganism in table olive fermentations *(13),* is still scarce ($CaCl_2$ and KCl) or nonexistent ($MgCl_2$).

The characterization of the effects of progressive concentrations of the inhibitory compounds (different chloride salts in this work) used in fermentation against the microorganisms usually present in the process is essential. The optical density–time curves can be used to derive the kinetic growth parameters (the maximum specific growth rate [μ_{max}] and the lag-phase period [λ]) of the microorganisms under study. Furthermore, determination of the MIC and noninhibitory concentration (NIC) and the characterization of the growth parameters in the interval MIC to NIC is frequently the preferred method to study these effects. Lambert and Pearson *(18)* developed a simple method for the estimation of the MIC and NIC, using turbidimetry. The procedure relates the area under the optical density–time curves to the degree of inhibition observed, using the ratio of control to that of the test (fractional area [FA]). The plot of FA versus log inhibitor concentration produces a sigmoid-shaped curve, which can be fit by a modified Gompertz function *(18)* or by the exponential decay function *(17).* The great advantage to these procedures is that they permit the use of all the growth information to deduce the MIC, while the tube dilution series or its extension to the microtiter wells (based on the demarcation between growth–no growth and the concentration of inhibitor in the well with no growth) usually discards all the growth information below the MIC concentration. The entire sigmoid-shaped curve is then divided into three sections: points corresponding to concentrations from zero up to the NIC (concentrations at which no effect of the inhibitor is observed), concentrations between NIC and MIC (within which growth inhibition progressively occurs), and a third section above MIC (where no growth relative to the control is recorded).

The aim of this work was the estimation of the effect of the progressive concentrations of NaCl, KCl, $CaCl_2$, and $MgCl_2$, all of them with potential use in table olive and vegetable fermentations, on the kinetic growth parameters of *L. pentosus* and *S. cerevisiae,* and for calculating the NICs and MICs of such salts. Results may have potential applications in the development of new fermentation and storage processes for cucumber, table olives, capers, and other vegetables and for the design of new, healthier final products.

MATERIALS AND METHODS

Origin and identification of microbial species. The species used in this study were *L. pentosus* and *S. cerevisiae,* previously isolated from table olive fermentations. They were identified using biochemical and molecular methods. *L. pentosus* was identified with API 50 CHL (bioMérieux, Inc., Marcy l'Etiole, France), together with PCR of the *recA* gene, with species-specific primers for *L. pentosus, L. plantarum,* and *Lactobacillus paraplantarum* *(34),* resulting in a size of amplicons of 218 bp. *S. cerevisiae* was identified with API 20C AUX (bioMérieux, Inc.), together with the restriction pattern generated with endonucleases *Cfo*I, *Hae*III, *Hin*fI, and *Scr*FI from PCR to the amplified 5.8.S rRNA gene and two internal transcribed spacers (ITS1 and ITS2) *(11).* These species belong to the current collection of microorganisms of the Department of Food Biotechnology (Instituto de la Grasa, Consejo Superior de Investigaciones Científicas, Seville, Spain).

Inoculum preparation and growth media. The inocula were prepared by introducing a single colony from pure cultures of each species into 5 ml of deMan Rogosa Sharpe broth medium (MRS; Merck, Darmstadt, Germany) (*L. pentosus*) or into 5 ml of yeast-malt-peptone-glucose broth medium (YM; Difco, Becton and Dickinson Company, Sparks, Md.) (*S. cerevisiae*). After 48 h of incubation at 30°C, the tubes were centrifuged at 9,000 × *g* for 15 min, the pellets washed with sterile peptone water (0.01% [wt/vol]), centrifuged, and resuspended in sterile peptone water to obtain a concentration of about 1.99×10^8 CFU/ml for *L. pentosus* and 2.51×10^7 CFU/ml for *S. cerevisiae,* which were confirmed by surface spread on MRS and YM agar plates, respectively.

The growth media selected for all experiments were MRS and YM broths, modified individually with different concentrations of chloride salts (ranging from 0 to 440 g/liter). NaCl, KCl, $CaCl_2$, and $MgCl_2$ (assay of 99.9%) were supplied by Panreac Química S.A. (Castellar del Vallés, Barcelona, Spain). After the addition of salts, media were dispensed into 10-ml tubes and sterilized at 115°C for 10 min.

Optical density measurements. Growth was measured with a Bioscreen C device (Labsystems, Helsinki, Finland) at 30°C, using a wide-band filter (420 to 580 nm) after preshaking for 10 s. Measurements were taken every 2 h for 7 days. The inocula were obtained from the first decimal dilution of the initial suspension for each microorganism. The wells of the Bioscreen plate were filled with 0.05 ml of inoculum and 0.35 ml of MRS or YM broth medium (according to the species) modified with the diverse salts, reaching an inoculum level of 1.52×10^6 CFU/ml for *L. pentosus* and 2.47×10^5 CFU/ml for *S. cerevisiae.* The inocula were just above the detection limit of the apparatus, which was determined by comparison with a previously established calibration curve. All experiments were carried out at least in duplicate. Simultaneously, an uninoculated well for each experimental series was also included in the plate.

Estimation of the kinetic growth parameters and their modeling. The growth parameters (μ_{max} and λ) of *L. pentosus* and *S. cerevisiae* in the presence of different salt concentrations were deduced by directly fitting optical density data versus time to the reparametized Gompertz equation proposed by Zwietering et al. *(38)*:

$$y = D \exp\{-\exp[(\mu_{max}e/D) \times (\lambda - t) + 1]\} \quad (1)$$

where $y = \ln(OD/OD_0)$, OD_0 is the initial optical density value; OD the optical density value at time *t,* $D = \ln(OD_\infty/OD_0)$ is the maximum optical density value reached with OD_∞ as the asymptotic maximum, μ_{max} is the maximum specific growth rate (h^{-1}), and λ the lag-phase period (h).

The relative change of μ_{max} as a function of the diverse salt concentration was modeled by the equation developed by Peleg *(27)* for survival curves having an "activation shoulder." The model took the following form:

$$\log\left[\frac{\mu_{max}(c)}{\mu_{max}(\text{positive control})}\right] = \frac{t\{1 - \ln[1 + \exp(bt)]^n\}}{k_1 + k_2 t} \quad (2)$$

where $\mu_{max}(c)$ is the maximum specific growth rate at a specific

salt concentration, μ_{max}(positive control) is the maximum specific growth rate in the absence of salt, and *b, n,* k_1, and k_2 are the four adjustable parameters. The model was constructed as a product of $t/(k_1 + k_2t)$, which is a general growth model with an initial growth rate of $1/k_1$, $1/k_2$ as an asymptote, and $1 - \ln[1 + \exp(bt)]^n$. This term assumes values very close to one for small values of *t.* For larger values of *t,* values become negative territory at a rate determined by *b* and *n* (shape parameter). To facilitate visualization of the effect of the salts, the growth rate versus concentrations was plotted and the equation transformed accordingly.

The onset of lag microbial growth in the presence of all salts started only when a certain concentration level was reached. This behavior can be described by the logistic equation *(7),* which takes the following form:

$$\lambda = L_0 + \ln\{1 + \exp[k(C - C_c)]\} \quad (3)$$

where λ is the lag-phase period, C_c marks the concentration where inactivation intensifies, and *k* is the rate at which λ increases with concentration once C_c has been largely exceeded. L_0 is a constant introduced in this case to account for the normal lag-phase period in the absence of any added salt. According to this model, when $C \ll C_c$, $\lambda = L_0$ (there is no inactivation at all due to the added salt, and only the lag period normally observed in the medium for the respective organism is observed). If $C \gg C_c$, then λ increases linearly with concentration [$\lambda = L_0 + k(C - C_c)$].

Estimation of the MICs and NICs. The basis of the technique used for estimating the MIC and NIC was the comparison of the area under the optical density–time curve of a positive control (absence of chloride salt, optimal conditions) with the areas of the tests (presence of chloride salts). As the amount of inhibitor in the well increases, the effect on the growth of the organism also increases. This effect on the growth is manifested by a reduction in the area under the optical density–time curve relative to the positive control at any specified time. The area under the optical density–time curves was calculated by integration, using OriginPro 7.5 software (OriginLab Corporation, Northampton, Mass.). The relative amount of growth, FA, was obtained using the ratios of the test area ($area_{test}$) to that of the positive control ($area_{cont}$), according to the following formula:

$$FA = (area_{test} - area_{uni})/(area_{cont} - area_{unicont}) \quad (4)$$

where $area_{uni}$ is the area under the optical density–time curve corresponding to the uninoculated well of the experience tested, and $area_{unicont}$ is the area under the optical density–time curve corresponding to the uninoculated well of the control (negative control).

The FA-versus-concentrations (expressed in log or ln) plot gives sigmoid-shaped curves. The estimations of the MIC and NIC values from these curves were achieved by fitting a modified Gompertz function *(18)* and the exponential decay function developed by Lambert *(17).* The modified Gompertz function, which relates FA versus log concentrations, can be represented by the following equation:

$$FA = A + C\exp[-\exp B(x - M)] \quad (5)$$

where *A* is the lowest asymptote of FA (approximately zero), *B* is a slope parameter, *C* is the distance between the upper and lower asymptote (approximately 1), and *M* is the log concentration of the inflexion point. The values of NIC and MIC are defined as the intersection of the lines $FA = A + C$ and $FA = A$, with the equation of the line tangential to the point M, $A + C\exp(-1)$, respectively. The NICs and MICs can then be estimated as:

$$NIC = 10^{M-(e/B)} \quad (6)$$

$$MIC = 10^{M-(1/B)} \quad (7)$$

The exponential decay function developed by Lambert *(17)* takes the following form:

$$FA = \exp\left[-\left(\frac{x}{P_1}\right)^{P_2}\right] \quad (8)$$

where the meaning of FA has already been explained, *x* is the inhibitor concentration in ln (concentration units), P_1 is the concentration of the preservative at $FA = 1/e$ ($e \approx 2.7118$), and P_2 is a slope parameter. The MICs and NICs can be estimated by the following equations, also developed by Lambert *(17)*:

$$\ln(NIC) = P_1\exp\left(\frac{1 - e}{P_2}\right) \quad (9)$$

$$\ln(MIC) = P_1\exp\left(\frac{1}{P_2}\right) \quad (10)$$

The main disadvantage of using the above approaches to a susceptibility study is the impossibility of estimating the confidence interval of both NIC and MIC, since they do not compare explicitly in the respective equations. In this work, the Gompertz (equations 9 and 10) and Lambert (equation 12) equations have been reparametized as follows.

Gompertz equation:

$$FA = A + C\exp\left[-\exp\left\{\frac{e}{\log MIC - \log NIC} \times\left[x - \frac{(e-1)\log MIC + \log NIC}{e}\right]\right\}\right] \quad (11)$$

In addition, the values of *A* and *C* were considered to be 0 and 1, the respective theoretical asymptotes for no growth and optimum growth (absence of inhibitors), similar to the assumptions made in the Lambert *(17)* equation.

Lambert equation was reparametized as:

$$FA = \exp\left\{-\left[\frac{x}{\ln MIC} \div\left\{\exp\left[-\left(\ln\frac{\ln NIC}{\ln MIC}\right)\Big/ e\right]\right\}\right]^{-[e/\{\ln[(\ln NIC)/(\ln MIC)]\}]}\right\} \quad (12)$$

This transformation has already been used by Conte et al. *(8).* The meaning of the parameters of the new equations has been defined in preceding definitions.

The new reparametized equations estimate the NICs and MICs, their standard errors, and their confidence limits directly from their nonlinear fits.

Statistical analysis. Nonlinear fits and graphs were performed by TableCurve 2D version 5 (SYSTAT Software, Inc., Chicago, Ill.) and Statistica version 6 (StatSoft, Inc., Tulsa, Okla.). The comparison of the goodness of fits between Gompertz and Lambert equations, used for the estimation of NICs and MICs, was achieved by their respective mean square errors, since both equations have the same number of adjustable parameters in the conditions assumed in this work. Comparison among other results was made, when appropriate, by one-way analysis of variance (ANOVA) and/or the confidence limits of their respective estimated values.

RESULTS

Effect of chloride salts on kinetic growth parameters. The growth data of *L. pentosus* and *S. cerevisiae* in relation to chloride salt concentrations and contact time

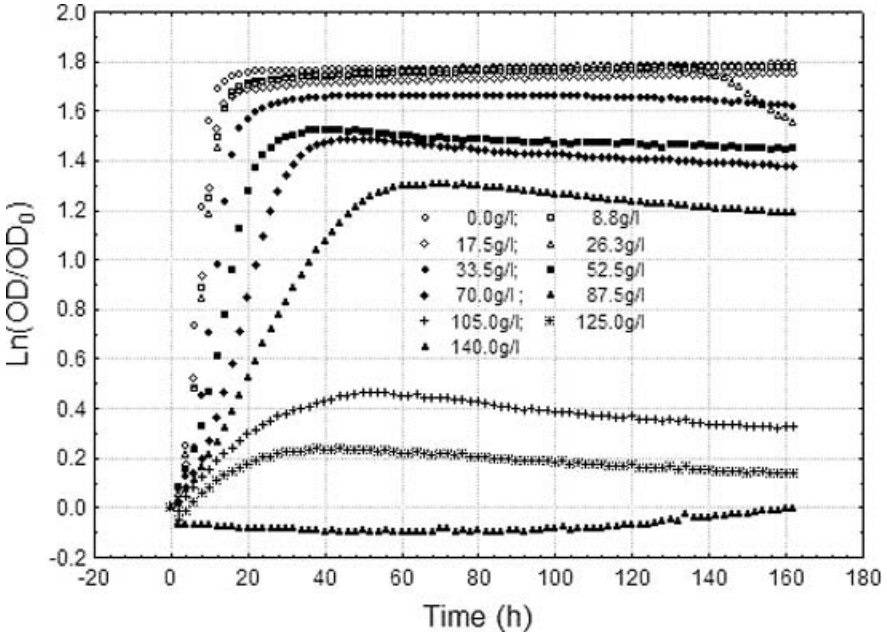

FIGURE 1. S. cerevisiae *optical density growth curves at $CaCl_2$ between 0 and 140 g/liter. Values are the average of at least duplicated experiments.*

showed a similar behavior, although with different specific values according to microorganism and salt. Microbial growth followed a sigmoidal trend and $\ln(OD/OD_0)$ increased with time. Figure 1 is a representation of the growth kinetic of *S. cerevisiae* according to $CaCl_2$ levels, as an example of the data obtained with the diverse chloride salts essayed. At fixed contact time, the $\ln(OD/OD_0)$ decreased as a function of concentrations. The growth parameters μ_{max} and λ were obtained by fitting the reparametized Gompertz function *(38).* Results suggested that, in addition to NaCl, the other chloride salts also exert a significant antimicrobial effect on each phase of the *L. pentosus* and *S. cerevisiae* growth cycle. These effects caused a μ_{max} decrease, a lag-phase increase, as well as a maximum cell-load decrease. Results also point out that growth kinetics seems to be a complex function of chloride salt concentrations.

The plot of the relative growth of *L. pentosus* versus chloride salt concentrations (Fig. 2) showed that, at low concentrations, the diverse chloride salts always produced an increase in the relative μ_{max}. The highest increases corresponded to NaCl, KCl, and $MgCl_2$ (Fig. 2). Further salt concentrations always caused a μ_{max} decrease; NaCl and $CaCl_2$ showed the strongest effects (steeper curves), while KCl was less inhibitory and $MgCl_2$ had the lowest effect. The whole curves with activation shoulder were well modeled by the Peleg *(27)* equation, whose estimated parameters are shown in Table 1. The adjusted R^2 for the different salts ranged from 0.9307 and 0.9937, and the ANOVA probability of fit was always ≤ 0.00001. The highest initial relative growth (as measured by $1/k_1$) was observed in the presence of KCl (1.134 h^{-1}), while the other chloride salts showed only slightly lower rates, which ranged from 1.025 h^{-1} (NaCl) to 1.059 h^{-1} ($CaCl_2$).

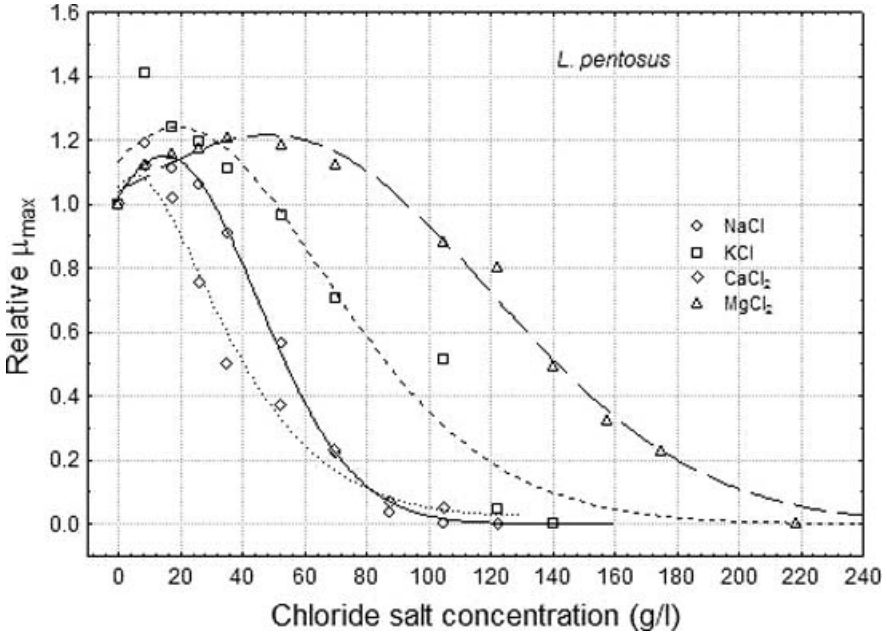

FIGURE 2. *Observed relative μ_{max} decay of* L. pentosus *versus salt concentration and fitted Peleg (27) model for the diverse chloride salts.*

TABLE 1. *Adjustable parameters of the Peleg model (27) fit to the relative μ_{max} versus chloride salt concentrations*

Microorganism, salt	b $(h^{-1})^a$	n^a	k_1 $(h)^a$	$k_2{}^a$	Adjusted R^2	$P > F$
L. pentosus						
NaCl	0.0399 ± 0.0029	0.0397 ± 0.0033	0.9754 ± 0.0382	0.0174 ± 0.0016	0.9937	<0.00001
KCl	0.0179 ± 0.0018	0.0090 ± 0.0013	0.8815 ± 0.1017	−0.0016 ± 0.0009	0.9307	0.00007
$CaCl_2$	0.1160 ± 0.0564	0.0075 ± 0.0012	0.9511 ± 0.051	−0.0067 ± 0.0023	0.9804	0.00001
$MgCl_2$	0.0032 ± 0.0003	0.0276 ± 0.0066	0.9624 ± 0.0397	0.0176 ± 0.0086	0.9846	<0.0
S. cerevisiae						
NaCl	0.0618 ± 0.0161	0.0073 ± 0.002	1.0010 ± 0.0322	−0.0006 ± 0.0005	0.9941	<0.00001
KCl	0.0043 ± 0.0016	0.0122 ± 0.0074	0.9543 ± 0.036	0.0121 ± 0.0087	0.985	<0.00001
$CaCl_2$	0.0681 ± 0.0002	0.0036 ± 0.0017	1.0039 ± 0.0274	0.0111 ± 0.0075	0.9951	<0.00001
$MgCl_2$	0.0033 ± 0.0029	0.0052 ± 0.0005	0.9442 ± 0.0351	0.0053 ± 0.0003	0.977	<0.00001

[a] Values are ± standard error.

The effect of the chloride salts on the λ of *L. pentosus* showed a range of concentrations without any appreciable effect on lag, and then was followed by a rapid increase of this period as the contents increased (Fig. 3). NaCl and $CaCl_2$ had a stronger influence on λ and, above 60 to 70 g/liter, and there was a marked λ-period increase, which led to a rapid practical inhibition (λ > 170 h); the slope of NaCl was steeper than that of $CaCl_2$. KCl did not have an appreciable effect up to about 100 g/liter; after this concentration, λ increased rapidly and led to inhibition at around 130 g/liter (Fig. 3). Finally, $MgCl_2$ increased λ after about 120 g/liter and showed a practical inhibition just above 220 g/liter. The log-logistic equation always fit the changes in λ versus salt concentration of *L. pentosus* well, and the parameters obtained are shown in Table 2. The adjusted R^2 values ranged from 0.9964 and 0.9990 and the probability for the F obtained in the ANOVA was always below 0.00001. The constant of the log-logistic equation ranged from 3.70 and 5.60 h; values that should be considered as the normal lag phase of *L. pentosus* in the MRS broth medium at the incubation conditions. The C_c values (at which the increase might be initiated) ranged from 66 ($CaCl_2$) and 56 (KCl) g/liter. The values were then similar among the diverse salts and always lower than the corresponding MICs (see below). The values of *k,* related to the slopes of the curves for the diverse chloride salts were also close, except for $CaCl_2$. In the relative μ_{max}–versus–chloride salt concentrations graphs of *S. cerevisiae* (data not shown), the activation shoulders were only observed for KCl and $MgCl_2$ and were slightly pronounced. All these curves were also modeled successfully with the same combined model of Peleg *(27).* The effect of NaCl and $CaCl_2$ concentrations on the relative μ_{max} of *S. cerevisiae* was similar to that observed against *L. pentosus,* and the respective curves practically overlapped (data not shown). The fit characteristics for *S. cerevisiae* were similar to those observed for *L. pentosus* (adjusted R^2 from 0.9770 to 0.9941 and ANOVA probability of regression below 0.00001). The kinetic constant b for NaCl and $CaCl_2$ were fairly close (Table 1); b values for KCl and $MgCl_2$ were lower than those for the other two salts.

The plot of the lag period of *S. cerevisiae* versus chloride salt concentrations also showed a phase of no effect, and was followed by another of rapid increase, similar to

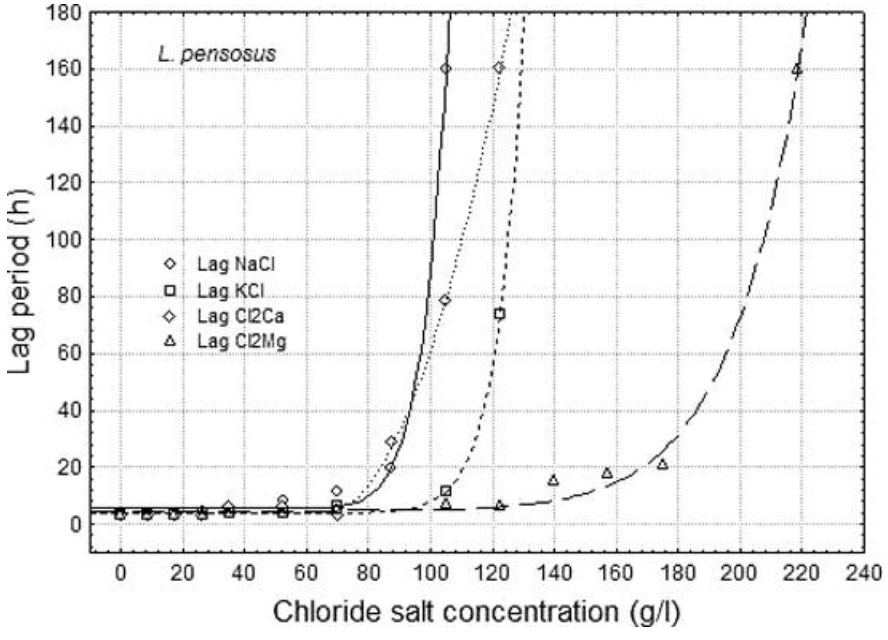

FIGURE 3. *Observed lag-phase period (h) of* L. pentosus *versus salt concentration and fitted log-logistic model for the diverse chloride salts.*

TABLE 2. *Adjustable parameters of the log-logistic model (27) fit to the curve λ versus chloride salt concentrations*

Microorganism, salt	k (1/g × 1)[a]	C_c (g/liter)[a]	M[a]	L_0 (h)[a]	Adjusted R^2	$P > F$
L. pentosus						
NaCl	0.0414 ± 0.0049	62 ± 0.1	8.7 ± 1.4	5.6 ± 1	0.9971	<0.00001
KCl	0.0158 ± 0.0008	56 ± 0.1	14 ± 1.3	3.9 ± 0.5	0.9982	<0.00001
$CaCl_2$	0.24 ± 0.0901	66 ± 4	1.9 ± 0.2	3.7 ± 0.6	0.999	<0.00001
$MgCl_2$	0.0101 ± 0.0008	64 ± 0.1	9 ± 1.1	4.7 ± 1.2	0.9964	<0.00001
S. cerevisiae						
NaCl	0.494 ± 0.021	84 ± 5.6	1.3 ± 0.1	5.7 ± 2.8	0.9486	<0.00001
KCl	13.22 ± 0.9332	173 ± 3	0.7 ± 0.1	6.1 ± 2.5	0.9441	<0.00001
$CaCl_2$	102.6 ± 5.601	121.5 ± 0.6	0.6 ± 0.2	4.9 ± 2.4	0.974	<0.00001
$MgCl_2$	0.044 ± 0.0002	239 ± 33	3.1 ± 1.5	5.1 ± 0.7	0.991	<0.00001

[a] Values are ± standard error.

L. pentosus. The transition was particularly rapid for KCl and $CaCl_2$. All the curves could be fit by the modified log logistic equation. The characteristics of their fits (Table 2) were similar to that for *L. pentosus* (adjusted R^2, from 0.9441 to 0.9910, and $P < 0.00001$ always). The value of the constant L_0 of the log-logistic equation ranged from 5.10 and 6.10 h, which should correspond to the normal lag phase of *S. cerevisiae* when grown, at the same incubation temperature, in the YM broth medium.

Susceptibility testing. The wide range of concentrations assayed allowed development of a dose-response curve of *L. pentosus* and *S. cerevisiae* for the diverse chloride salts. Time (t) was set at 170 h, a period necessary to reach the stationary phase for both microorganisms at all tested salt concentrations. The areas under the diverse growth curves corresponding to *L. pentosus* and *S. cerevisiae* were converted into FA and plotted against the log or ln of the concentrations of chloride salts. Results proved that the microbial susceptibility to chloride salts were nonlinearly dose related. Furthermore, growing and no-growing status were accurately individualized by determining the NICs and MICs, respectively. In general, the curves showed the typical sigmoid-shape behavior of a decay function. The dose-response curve could be subdivided into three primary regions to describe microbial susceptibility to chloride salts. The first region lying below the NIC (highlighted as growth region in Fig. 4) defines a range of chloride salt concentrations that did not significantly affect microbial growth. The nonlinear dose-dependent increase of the antimicrobial activity was proven within an intermediate region bounded by NICs and MICs, where the observed sigmoid trend of the inhibitory profile allows one to hypothesize high-order kinetics for *L. pentosus* and *S. cerevisiae* inhibition within this boundary. As can be inferred from FA data, measurable

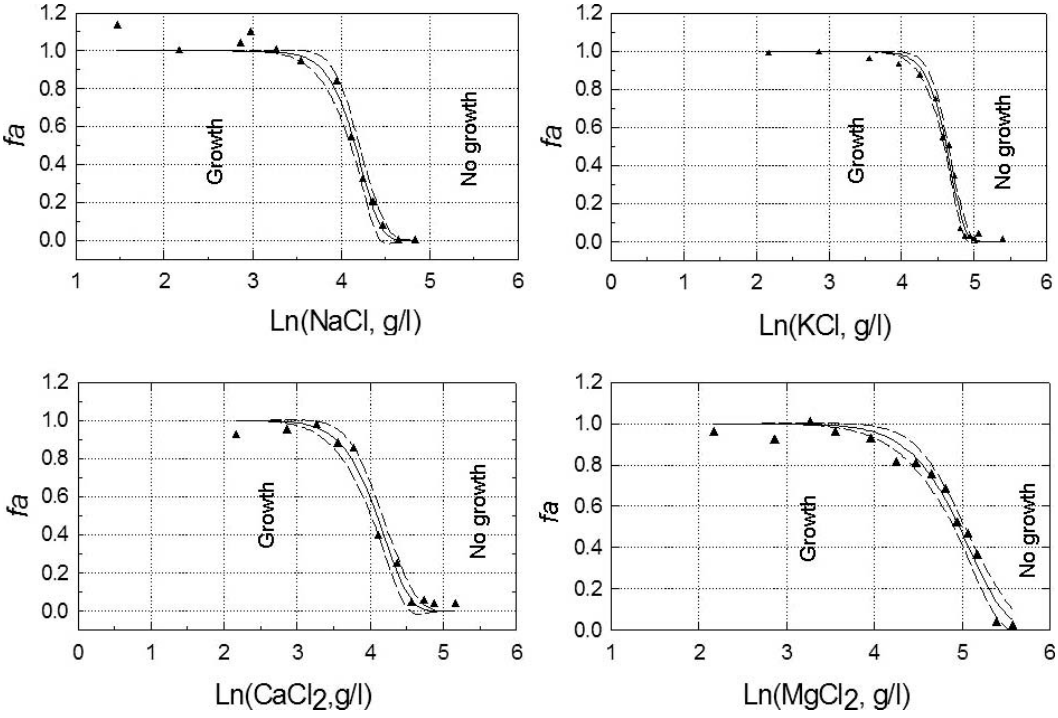

FIGURE 4. *Inhibitory profile (FA versus ln concentration) of* L. pentosus *against chloride salt levels and the curved (and confidence limits) fit by the Lambert model (17). FA is the fractional area calculated at a contact time of 170 h.*

TABLE 3. *MICs and NICs and mean square errors estimated from the Gompertz and Lambert reparametized equations*[a]

Microorganism, salt	Gompertz			Lambert		
	NIC (g/liter)	MIC (g/liter)	MSE (g/liter)[b]	NIC (g/liter)	MIC (g/liter)	MSE (g/liter)
L. pentosus						
NaCl	52 ± 2 A a[c]	82 ± 3 A a	0.0036	52 ± 2 A a	83 ± 3 A a	0.0035
KCl	84 ± 2 B a	126 ± 2 B a	0.0021	85 ± 2 B a	126 ± 3 B a	0.0022
CaCl b	44 ± 3 A a	87 ± 4 A a	0.0031	44 ± 2 A a	88 ± 5 A a	0.0029
MgCl b	98 ± 5 B a	228 ± 11 BC a	0.0028	99 ± 5 B a	231 ± 13 BC a	0.0034
S. cerevisiae						
NaCl	31 ± 2 A b	122 ± 8 A b	0.0031	31 ± 2 A b	120 ± 7 A b	0.0032
KCl	68 ± 5 B b	220 ± 14 B b	0.0036	66 ± 5 B b	216 ± 10 B b	0.0044
CaCl b	36 ± 4 A a	124 ± 11 A b	0.0053	36 ± 4 A a	121 ± 9 A b	0.0061
MgCl b	105 ± 7 C a	354 ± 28 C b	0.0039	104 ± 7 C a	354 ± 24 C b	0.0044

[a] Values are ± standard deviation.
[b] MSE, mean square error.
[c] Values followed by different capital letters, within the same column and microorganism, are significantly different. Values followed by different lowercase letters for the same chloride salt and different microorganism are significantly different.

microorganism growth was not detectable within a third region of the inhibition profile above the MIC (no growth region in Fig. 4). Particularly in the case of *L. pentosus,* there was an initial increase of FA up to about 26 g/liter NaCl concentrations.

Microbial sensitivity to each compound was related to the NICs, whereas the microbial resistance was related to the MICs. The results (NICs and MICs) of the nonlinear fitting of the reparametized Gompertz and Lambert equations to data from *L. pentosus* and *S. cerevisiae,* with their respective standard errors and confidence limits, were obtained (Table 3). Confidence limits of the fitted curve were also recorded (Fig. 4). The goodness of fit was always significant ($P < 0.0001$). The comparison between the goodness of fit of both models, achieved through their corresponding mean square errors of the ANOVA of fits, showed that results obtained by Gompertz and Lambert equations were similar (Table 3). Then, the application of both procedures led to similar goodness of fit and parameter values.

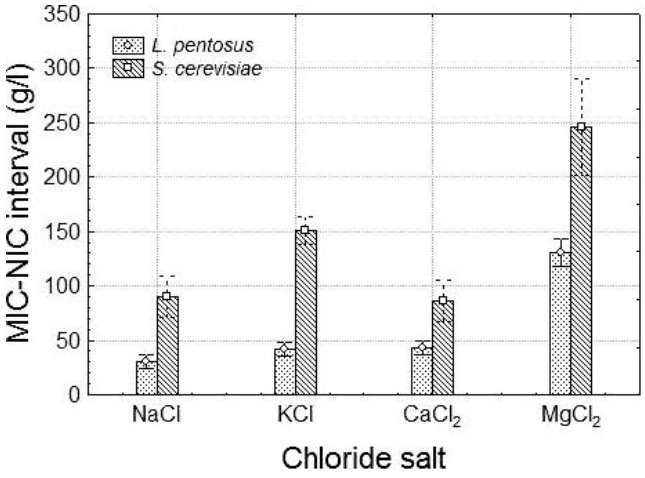

FIGURE 5. *Distribution of the MIC–NIC intervals (±confidence limits) according to microorganism and chloride salts.*

In the case of NaCl, the NIC for *L. pentosus* was 52 g/liter regardless of the estimation procedure, but the value for *S. cerevisiae* was 31 g/liter. *L. pentosus* MIC for this salt was 82 to 83 g/liter, whereas it was approximately 120 to 122 g/liter for *S. cerevisiae.* The NICs and MICs for the rest of the salts against *L. pentosus* and *S. cerevisiae* are also shown in Table 3. The behavior of *L. pentosus* and *S. cerevisiae* against the rest of the chloride salts assayed followed the same trend (higher NICs and lower MICs of the first), although the absolute values were different. $CaCl_2$ did not have a significantly different NIC than NaCl had (44 versus 52 g/liter) on *L. pentosus* or on *S. cerevisiae* (36 versus 31 g/liter). KCl, which was followed by $MgCl_2$, had progressively less effect on the growth of both microorganisms, and their NICs and MICs were significantly different between them and higher with respect to the NaCl and $CaCl_2$. The order of effects, as observed by the NICs (sensitivity), was slightly different for *L. pentosus* ($CaCl_2 \approx NaCl > KCl > MgCl_2$) than for *S. cerevisiae* ($NaCl \approx CaCl_2 > KCl > MgCl_2$) but followed the same trend ($NaCl \approx CaCl_2 > KCl > MgCl_2$) when assessed by the MICs (microbial resistant). The high MICs of the last two chloride salts against both microorganisms make their use difficult for microbial control in fermented products.

Lower MIC concentrations do not necessarily correspond to a lower NIC level for the same microorganism. Thus, the difference between MIC and NIC levels could allow for better understanding of microbial behavior in the presence of the diverse chloride salts. This range (MIC to NIC) can be used to quantify the differences in microbial susceptibility to salts. As can be inferred from the data reported in Figure 5, in which the error bars represent the confidence limits of the experimental data ($P = 0.05$), significant differences appear between *L. pentosus* and *S. cerevisiae* and among salt within the same microorganism. MIC-to-NIC ranges for *L. pentosus* were always lower than those of *S. cerevisiae* (Fig. 5).

DISCUSSION

This work was aimed at obtaining information about the individual influence of NaCl, KCl, $CaCl_2$, and $MgCl_2$ on *L. pentosus* and *S. cerevisiae,* two representative microorganisms of vegetable fermentations. The specific chloride salts assayed were chosen not only because of their probable inhibitory effects, but also due to their nutritional value. Currently, some minerals like potassium and magnesium are in low proportion in table olives *(13),* so the addition of these chloride salts during fermentation could improve the nutritional characteristics of the commercial presentations. A first step is to study the effects of these chloride salts on microbial growth since a better understanding of the microbiological food safety issues associated with product reformulation is necessary to prevent risks *(31).*

Previous works aimed at reducing the NaCl level of salt, or to substitute part of it in fermented products by other chloride salts are available. Among others, Tsapatsaris and Kotzekidou *(35)* investigated the effect of NaCl, calcium acetate, and calcium lactate in concentrations equivalent to 20 to 100 g/liter NaCl as well as the 50% replacement of NaCl by KCl on the growth of *L. plantarum* and *D. hansenii* in black olive juice. Both strains had higher individual maximum specific growth rates when the medium was supplemented with NaCl-KCl, calcium acetate, and calcium lactate. Yumani et al. *(37)* fermented cucumbers and turnips in various NaCl concentrations and found that 40 g/liter NaCl was the lowest concentration to produce acceptable products, but 30 g/liter NaCl in combination with 5 g/liter KCl and 5 g/liter $CaCl_2$ was also acceptable. The use of a starter of *L. plantarum* in the brine gave a pH similar to that of natural fermentation (≈3.0). However, information about the effects of the replacement of NaCl by other chloride salts in vegetable fermentations is still scarce.

The behavior of the diverse chloride salts essayed against *L. pentosus* and *S. cerevisiae* followed the same trend but with different sensitivities (NICs) and resistances (MICs). Progressive concentrations of salts always led to a μ_{max} decrease and changes, especially rapid within the MIC-to-NIC interval. The ranges were always lower for *L. pentosus* than for *S. cerevisiae,* which means that the effect of the salts was always stronger on the first microorganism than on the second within such limits. For *L. pentosus,* the intervals of NaCl, KCl, and $CaCl_2$ were similar, but that of $MgCl_2$ was significantly wider (Fig. 5), and changes less sharp than in the presence of the other salts. However, for *S. cerevisiae,* only NaCl and $CaCl_2$ intervals were the same ($P < 0.05$), while that of KCl was wider, and the one for $MgCl_2$ was the widest (Fig. 5). Unexpectedly, *S. cerevisiae* was more sensible to chloride salts (except $MgCl_2$) than *L. pentosus* was, but the yeast was significantly more resistant, particularly to KCl and $MgCl_2$ than was *L. pentosus.* The effect of NaCl on the growth of the yeast could be noticed above approximately 3.1% (wt/vol), while on the LAB, it would be observed above 5.2% NaCl. As a result, this strain of *L. pentosus* can be considered fairly well adapted to olive fermentations, in which the equilibrium NaCl levels are initially established at about 5.5% *(13),* but not the yeast species that can be partially controlled at this NaCl content. On the contrary, *S. cerevisiae* has been found to be more resistant, and its inhibition would always require higher levels than *L. pentosus* would; thus, concentrations of approximately 8.5% NaCl (enough to inhibit the LAB) will not completely control the yeast growth, which would require concentrations above 12.2% (Table 3). Querol and Fleet *(28)* reported on the great resistance of yeasts to high-salt concentrations in vegetable fermentations. Panagou *(26)* also showed the presence of yeasts in natural black olives with high levels of salts. Our results are in agreement with these observations.

The diverse chloride salts had scarce effect on the lag phase of *L. pentosus* and *S. cerevisiae* during a wide range of levels, except when approaching the inhibition concentration (Fig. 3). The C_c values were always below the corresponding MIC values for both microorganisms (Table 2), particularly in the case of *L. pentosus* and KCl and $MgCl_2$. Apparently, chloride salts are more efficient in controlling the growth and total population than the lag phase, and their use in vegetable fermentations would not cause additional delay in the initiation of the processes.

The FA of *L. pentosus* and *S. cerevisiae* versus the diverse chloride salt concentrations showed the characteristic sigmoid-shaped curve described by Lambert and Pearson *(18).* Some FA values of *L. pentosus* for NaCl concentrations below the NIC limit of this salt were above 1, the upper asymptote of the sigmoid curve. This means that the MRS broth medium could be eventually improved by slightly increasing its current NaCl level *(17).* Activation shoulders were also observed in the plots of relative μ_{max} versus salt concentration for *L. pentosus* (Fig. 2). Thus, not only an increase in NaCl, as observed in its FA versus concentration curve, can improve *L. pentosus* growth, but also the addition to the MRS medium of relatively low amounts of KCl, $CaCl_2$, and $MgCl_2$ (approximately 8.75, 8.75, 8.85, and 17.5 g/liter, respectively). In contrast, the YM broth would require only a slight increase in KCl and $MgCl_2$ concentrations to reach the highest μ_{max} for *S. cerevisiae.* A similar behavior observed during the inactivation of certain spores was attributed to recovery improvement due to the heat treatment *(27),* which, apparently, is not the case for the *L. pentosus* shoulder. The FA plot only revealed the activation effect of NaCl but was unable to show the influence of the rest of the salts. Then, growth kinetic and susceptibility testing were complementary for the description of the behavior of both microorganisms in the presence of the diverse chloride salts.

According to the kinetic growth parameters and NICs and MICs, there was an apparent similar behavior between the NaCl and $CaCl_2$ against *S. cerevisiae* and *L. pentosus,* while KCl was less inhibitory, and $MgCl_2$ had the lowest effect against these two microorganisms. These differences in the inhibitory effects might permit diverse uses in vegetable fermentations: $CaCl_2$ might be used in combination with NaCl for controlling the growth of LAB and yeasts, while KCl and $MgCl_2$ could be added to NaCl and/or $CaCl_2$ just for increasing the nutritional value of the final products.

The effects on *L. pentosus* and *S. cerevisiae* (and possibly other LAB and yeasts) by $CaCl_2$ may be particularly useful for the stabilization of products in which the spoilage is provoked by acidification during shelf life, as observed in seasoned Aloreña table olives *(3).* KCl had higher MICs and lower effects on μ_{max} and λ. Thus, obtaining a similar effect to that produced by NaCl or $CaCl_2$ will require high concentrations of KCl. Another alternative could be the addition of KCl simply to increase the nutritional value of fermented vegetables, without interfering with the usual fermentation/storage process. $MgCl_2$ had very high MICs and low inhibitory effects on relative μ_{max} and λ. As a result, it could be used in combination with NaCl in the same conditions as KCl. Eventually, a moderate level of $CaCl_2$, KCl, and $MgCl_2$ in the initial brine could also be useful for increasing the μ_{max} of *L. pentosus* (and possibly other LAB) and accelerate the fermentative process.

Information about how the individual chloride salts interact with the food matrices is scarce. Protein solubility of defatted *Colocynthis citrullus* increased with salt concentration, but partial replacement of NaCl for KCl did not increase protein solubility *(2).* Calcium added to food increased the palatability for hemodialysis patients *(19),* but addition of KCl to juice decreased all ratings of palatability *(36).* In addition, research on the interactions of these chloride salts with the food matrices is also convenient. Industrial application of these modifications will still require further studies on real fermentations and final products.

This work has provided individualized information related to NaCl, KCl, $CaCl_2$, and $MgCl_2$ effect on the kinetic growth parameters (μ_{max} and λ) and NICs and MICs of *S. cerevisiae* and *L. pentosus,* two of the most representative microorganisms of table olive fermentation. A rather similar behavior between NaCl and $CaCl_2$ on all the growth parameters has been found. KCl had a lower effect, and $MgCl_2$ had the lowest influence. One can consider the possibility of the use of a combination of NaCl with the other chloride salts studied in the fermentation and storage process of table olives as well as in the final products. However, further information on the combined effect of these chloride salts on μ_{max} and λ as well as on their NICs and MICs and their interactions with the diverse food matrices is still necessary before results can be applied to real fermentation processes.

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