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Modeling the Efficacy of Triplet Antimicrobial Combinations: Yeast Suppression by Lauric Arginate, Cinnamic Acid, and Sodium Benzoate or Potassium Sorbate as a Case Study

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ABSTRACT

The growth of four spoilage yeasts, *Saccharomyces cerevisiae, Zygosaccharomyces bailii, Brettanomyces bruxellensis,* and *Brettanomyces naardenensis,* was inhibited with three-agent (triplet) combinations of lauric arginate, cinnamic acid, and sodium benzoate or potassium sorbate. The inhibition efficacy was determined by monitoring the optical density of yeast cultures grown in microtiter plates for 7 days. The relationship between the optical density and the sodium benzoate and potassium sorbate concentrations followed a single-term exponential decay model. The critical effective concentration was defined as the concentration at which the optical density was 0.05, which became an efficacy criterion for the mixtures. Critical concentrations of sodium benzoate or potassium sorbate as a function of the lauric arginate and cinnamic acid concentrations were then fitted with an empirical model that mapped three-agent combinations of equal efficacy. The contours of this function are presented in tabulated form and as two- and three-dimensional plots. Triplet combinations were highly effective against all four spoilage yeasts at three practical pH levels, especially at pH 3.0. The triplet combinations were particularly effective for inhibiting growth of *Z. bailii,* and combinations containing potassium sorbate had synergistic activities. The equal efficacy concentration model also allowed tabulation of the cost of the various combinations of agents and identification of those most economically feasible.

A common way to control microbial growth in foods is to add naturally occurring or synthetic antimicrobials as preservatives *(10)*. In recent years, synthetic antimicrobials have become less popular because of the desire to eliminate synthetic additives from the food supply. However, the effective concentration of many naturally occurring antimicrobials used to suppress food spoilage organisms and pathogens can be so high as to have adverse effects on food flavor or to exceed the allowed regulatory level *(5)*. The ability of certain yeasts to grow at low pH in the presence of traditional lipophilic weak-acid preservatives (e.g., benzoic acid, propionic acid, and sorbic acid) inflicts heavy losses on the food and beverage industries at costs that have been estimated to be in the billions of Euros per year *(22)*. Thus, more effective preservation systems are urgently needed, requiring increased testing of new preservatives and/or combinations of existing and alternative preservatives. However, the removal of the traditional preservatives such as sodium benzoate is difficult from food quality, food safety, and sensory perspectives.

The use of a combination (cocktail) of antimicrobials may be the solution to these problems and may be a way to control emerging resistant species or mutant forms of existing ones, which is a growing concern to many food manufacturers *(17)*. The control of spoilage yeasts in foods with a low pH and high sugar content is difficult. Davenport group 1 yeasts are particularly problematic because of their resistance to chemical preservatives and their high tolerance to osmotic stresses *(22)*. This Davenport group consists of 13 species: 3 *Brettanomyces* species, 3 *Saccharomyces* species, 4 *Zygosaccharomyces* species, *Hanseniaspora valbyensis, Schizosaccharomyces pombe,* and *Torulaspora delbruekii*. Genetic adaptations in some of these yeasts has allowed them to survive and grow in the presence of various traditional antimicrobials such as benzoic and sorbic acids, forcing food manufacturers to search for new and more effective preservatives *(2)*.

Theoretically, a mixture of antimicrobials can have a wider spectrum of activity than each antimicrobial alone because the mixture may act simultaneously on different species or may interrupt different metabolic elements within the same species *(17)*. Most of the antimicrobial combinations tested to date have been binary (i.e., composed of two antimicrobial agents). The effectiveness of these binary combinations has been widely studied, and methodologies have been developed to determine whether two antimicrobials are synergistic or antagonistic or whether their effects are only additive, as illustrated in Figure 1 *(17)*. In contrast, reports on the use of combinations of three antimicrobials are rare. In theory, these triplet combinations should be more effective than binary combinations because it is more difficult for the target organism to adapt to and resist a simultaneous attack on several targets in its cell wall and interior *(9)*. Determination of antimicrobial activity, especially when additional factors such as pH and salt or sugar concentration are involved, requires considerable labor,

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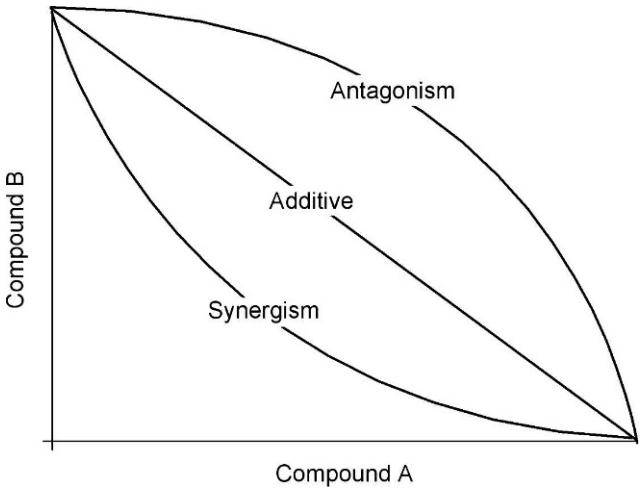

FIGURE 1. *Interpretation of effective agent combinations in terms of antagonism, additivity, and synergism (17).*

time, and laboratory materials. Therefore, if an appropriate model could be developed it could be used to help identify effective treatments, thus reducing costs without compromising food stability and quality.

An example of one such model is that of Battey et al. *(2),* which can be used to estimate the probability of spoilage of cold-filled ready-to-drink beverages by *Saccharomyces cerevisiae, Zygosaccharomyces bailii,* and *Candida lipolytica* as a function of pH, titratable acidity, sugar content, and sodium benzoate or potassium sorbate levels. The authors first transformed plate count data into positive and negative growth responses over time, where a response of 1 meant that yeast growth was observed and a response of 0 meant that no detectable counts were observed, and then fitted the data to a second-order logistic regression model that included linear and quadratic terms for time. Another example is the model of Belletti et al. *(3),* which can be used to assess the probability of yeast growth in soft drinks during storage as a function of the concentration of added aroma compounds, the duration of thermal treatment, and the inoculum size. Similar to Battey et al., Belletti et al. first converted growth observations into positive and negative growth responses over time, with a value 0 assigned to the bottles in which yeast growth was not observed and a value of 1 assigned to bottles in which growth occurred. The data were then analyzed using a logistic regression model. Yet another example is the model developed by Lopéz-Malo and Palou *(16)* to predict the temperature needed to inhibit *Z. bailii* growth in mango puree (at different water activities in the presence of 1,000 ppm of potassium sorbate or sodium benzoate) to assure a minimum shelf life of 35 days.

In contrast to previous studies that focused on binary antimicrobial combinations and environmental factors, the main objective of our work was to develop a method to specifically identify triplet antimicrobial combinations of equal efficacy as yeast growth suppressors. As a test case, we determined and modeled the inhibitory effect of sodium benzoate or potassium sorbate alone and in combination with lauric arginate and cinnamic acid. Lauric arginate is a novel antimicrobial compound that is derived from lauric acid, L-arginine, and ethanol, all naturally occurring substances. Lauric arginate has been of great interest to food manufacturers because of its broad spectrum of antimicrobial activity, high water partition coefficient (>10), activity over a wide range of pH values (3 to 7), and extremely low toxicity. Lauric arginate is easily hydrolyzed in the human digestive system and thus has been granted the status of generally recognized as safe under U.S. regulation 21 CFR section 170.30(b) *(1)*. Sodium benzoate and potassium sorbate are traditional preservatives that have a wide spectrum of activity and are widely used in the food and beverage industries. Cinnamic acid and its derivatives can be extracted from plants and fruits, where they provide a natural protection against infections by pathogenic microorganisms. In fruit juices and wines, these compounds can prevent fermentation by *S. cerevisiae,* thus increasing the shelf life of these products *(6)*. Thus, a combination preservation system using these three antimicrobials may effectively inhibit growth of food spoilage organisms or foodborne pathogens in beverages while allowing lower concentrations of sodium benzoate and potassium sorbate to be used. The target yeast species in this study were *S. cerevisiae, Z. bailii, Brettanomyces bruxellensis,* and *Brettanomyces naardenensis,* which are all members of the Davenport group and have known resistance to weak organic acids.

MATERIALS AND METHODS

Microbial protocols: yeast strains. Four strains of acid-resistant spoilage yeasts, *S. cerevisiae* (SC), *Z. bailii* (ZB), *B. bruxellensis* (BB), and *B. naardenensis* (BN), were obtained from the Pepsico R&D Culture Collection (Valhalla, NY). Yeast cultures were kept frozen at −70°C in 20% glycerol. The yeast strains were refreshed on malt extract agar plates (Becton Dickinson, Sparks, MD) before treatments. A single yeast colony from the plate was inoculated into 5 ml of Sabouraud dextrose broth (SDB) medium adjusted with HCl to pH 3.0 (Sigma Chemicals, St. Louis, MO). The culture was incubated at 25°C under mild agitation (180 rpm in a rotary shaker) for 4 days. The cultures were then diluted to approximately 104 CFU/ml. As a guideline, at an optical density at 630 nm (OD_{630}) of 0.3, cultures of yeast strains contain approximately 106 organisms per ml.

Microbial protocols: preparation of antimicrobial stock solutions. Stock solutions of sodium benzoate, potassium sorbate, and cinnamic acid (Sigma) were prepared in double-distilled water at concentrations (wt/vol) of 1, 1, and 0.04%, respectively. Lauric arginate was commercially obtained as Mirenat-N (Vedeqsa, Grupo Lamirsa, Barcelona, Spain), a dispersion of 10% (wt/vol) active ingredient in propylene glycol. Lauric arginate was dispersed at 0.1% (wt/vol) active ingredient. All stock solutions were filter sterilized (0.22-μm pore size; Whatman Filter, Whatman PLC, Maidstone, England) and kept refrigerated until used. Combinations of antimicrobials were prepared by mixing individual stock solutions.

Microbial protocols: antimicrobial activity assay. A microbroth dilution assay in combination with a factorial design was used to determine the MIC of combinations of antimicrobials *(20)*. Tested concentrations were 0, 50, 100, 150, 200, 250, 300, and 350 ppm for sodium benzoate or potassium sorbate, 0, 2.5, 5,

7.5, 10, and 15 ppm for lauric arginate, and 0, 10, 20, 30, 40, and 50 ppm for cinnamic acid. Microtiter plate wells were filled with 120 μl of double-strength filter-sterilized antimicrobials that had been adjusted to pH 3.0, 3.5, or 4.0 with citrate phosphate buffer before the wells were filled and 120 μl of inoculated double-strength SDB ($\sim 10^4$ CFU/ml) adjusted to pH 3.0, 3.5, or 4.0 (see above). The pH in the wells was measured with an Orion microcombination pH electrode (Thermo Fisher Scientific, Waltham, MA) at day 0 and day 7 and deviated by less than ± 0.2 units from the target pH. Plates were sealed with NUNC sterilized film (Thermo Fisher Scientific) and incubated at 25°C. The OD_{630} was measured at day 7 using an absorbance microplate reader (ELx800, Bio-Tek Instrument Inc., Winooski, VT). The pH of wells was measured with an Orion microcombination pH electrode before and after incubation to ensure that buffer capacity of the medium was not exceeded. All plates were processed in duplicates, and critical concentrations used for the subsequent modeling step were means of the calculated values for the two plates.

Data analysis: determination of inhibitory concentrations. The MIC is commonly defined as the lowest concentration of an antimicrobial whose administration results in no visible growth after 7 days. In this study, the MIC was defined as the agent concentration above which the OD_{630} remained below 0.05. This MIC served as a criterion of treatment effectiveness and for comparison of the equivalency of the various triplet combinations. This critical concentration, C_{crit}, was calculated from the plot of the optical density of the spoilage yeast suspension versus the sodium benzoate or potassium sorbate concentration, as shown schematically in Figure 2. In principle, C_{crit} can be calculated from a plot of the optical density of the spoilage yeast suspension versus the concentration of any one of the antimicrobials in the triplet combinations. In this study, we selected sodium benzoate and potassium sorbate for the C_{crit} calculation because these compounds are traditionally used in beverage preservation. The C_{crit} in the absence of any of the other antimicrobials is equivalent to the MIC of the two organic salts and thus allows a direct comparison with published studies. To identify the C_{crit}, the experimental data, i.e., $OD(C)$ versus concentration C, were fitted with an ad hoc exponential decay or linear model (equation 1 or equation 2, respectively):

$$OD(C) = (OD_0 - OD_r)\exp(-kC) + OD_r \quad (1)$$

$$OD(C) = OD_0 - kC \quad (2)$$

where $OD(C)$ is the optical density of the yeast suspension containing an antimicrobial agent at concentration C, OD_0 is the optical density when no agent is present, and OD_r is the residual optical density at $C \rightarrow \infty$. From the linear regression, OD_0 and k were obtained, and from the exponential decay regression, OD_r, OD_0, and k were obtained. The C_{crit} value can be considered equivalent to the MIC, except that it was extracted from a continuous $OD(C)$ versus C relationship (Equations 1 and 2) rather than from a set of discreet data. Therefore, C_{crit} is a more accurate estimate of the needed agent concentration whose determination does not require trial and error and does not depend on the chosen dilution table.

The concept of C_{crit} was extended to triplet agent combinations by defining it as the lowest sodium benzoate or potassium sorbate concentration that would keep the optical density of the yeast suspension below 0.05 for at least 7 days in the presence of the other two components: lauric arginate and cinnamic acid. The dependence of C_{crit} on the concentrations of these two agents was described by the model

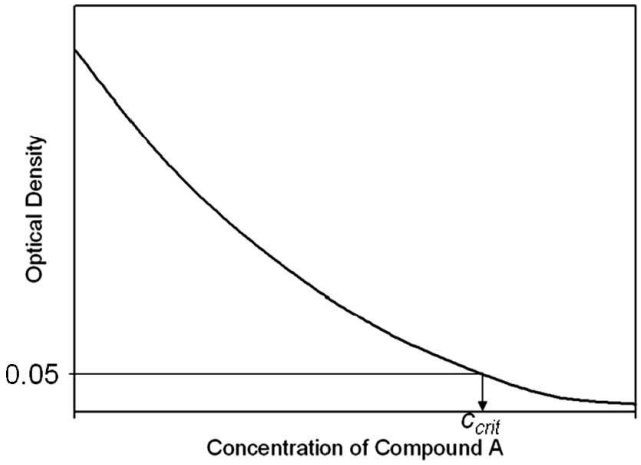

FIGURE 2. *Schematic graph illustrating our approach to calculate the critical concentration (C_{crit}) by fitting the optical density versus the concentration of added potassium sorbate or sodium benzoate.*

$$C_{crit}(x,y) = C_{crit}(0) - ax - by - cxy \quad (3)$$

where $C_{crit}(x, y)$ is the critical concentration of benzoate or sorbate when the concentration of lauric arginate in the combinations is x and the concentration of cinnamic acid is y, or vice versa. $C_{crit}(0)$ is the critical concentrations of benzoate or sorbate when applied alone (which emerges from a plot of the optical density of the yeast suspensions versus the benzoate or sorbate concentration, i.e., from equations 1 and 2, where the lauric arginate and the cinnamic acid concentrations were zero), and a, b, and c are constants, which can be pH dependent.

According to equation 3, the higher the coefficients a and b, the less benzoate or sorbate is needed to achieve the same degree of growth inhibition. The coefficient c is a measure of the interaction between lauric arginate and cinnamic acid. A positive c indicates synergism, and a negative c indicates antagonism with respect to the ability of these two compounds to lower the concentration of benzoate or sorbate needed. Theoretically, $c = 0$ indicates no interaction, i.e., the effect of lauric arginate and cinnamic acid was merely additive. However, because the experimental results had some scatter, which limited the model's precision, we treated c values in the range of -0.15 to 0.15 as indicating additivity, $c \geq 0.15$ as synergism, and $c \leq -0.15$ as antagonism. A more detailed explanation of this definition is presented below.

Equation 3 can be used to present the $C_{crit}(x, y)$ versus x and y relationship as a two- or three-dimensional plot. The contours of these plots mark the lauric arginate and cinnamic acid combinations that require the same sodium benzoate or potassium sorbate concentration to produce the same inhibitory effect in the particular environmental medium, e.g., at the same pH and sugar concentration. Equation 3 also can be used to tabulate triplet combinations of equal effectiveness. The resulting table can then be used to calculate the cost of each effective mixture, in cents per liter of product, using the price of the three agents. The formulae used to calculate the cost were

$$\text{Cost} = C_{crit}(x,y)\cdot P_B + x\cdot P_{LA} + y\cdot P_{CA} \quad (4)$$

for benzoate-based mixtures and

$$\text{Cost} = C_{crit}(x,y)\cdot P_S + x\cdot P_{LA} + y\cdot P_{CA} \quad (5)$$

for sorbate-based mixtures, where P_B and P_S are the unit cost of sodium benzoate ($7/kg at the time of this writing) and potassium

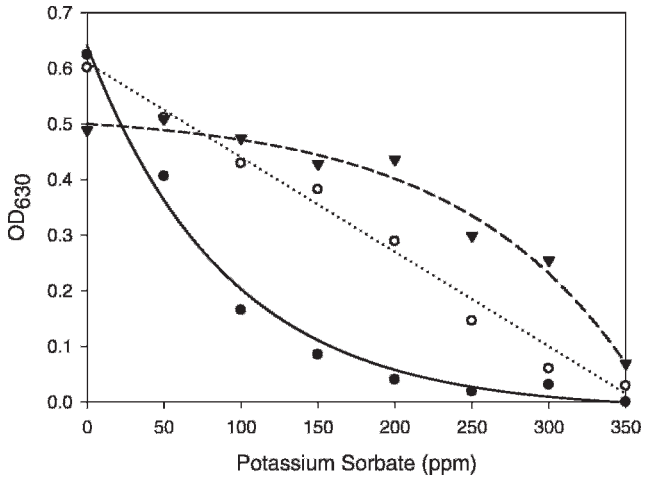

FIGURE 3. *Three examples demonstrating our approach to calculation of the* C_{crit} *by fitting of the optical density versus the concentration of added potassium sorbate.* ●*, Optical density of ZB in the presence of potassium sorbate combined with lauric arginate (5 ppm) and cinnamic acid (10 ppm) at pH 3.0.* ○*, Optical density of SC in the presence of potassium sorbate without lauric arginate (0 ppm) and with cinnamic acid (20 ppm) at pH 3.5.* ▼*, Optical density of BN in the presence of potassium sorbate without lauric arginate (0 ppm) and cinnamic acid (0 ppm) at pH 4.0.*

sorbate ($8.70/kg), respectively. P_{LA} and P_{CA} are the costs of lauric arginate ($55/kg) and cinnamic acid ($34.8/kg), respectively *(21)*. A table containing both the agent concentrations and the mixture costs per liter of product enables the user to identify mixtures of same or similar inhibitory effects and to find combinations that have the lowest cost. Such a table can be updated easily as market prices change, allowing the user to choose a less expensive alternative with the same efficacy.

In this work, we used OriginPro (version 7.5, OriginLab Corporation, Northampton, MA) to estimate C_{crit} and Mathematica 6.0. (Wolfram Research, Champaign, IL) to generate, plot, and tabulate the $C_{crit}(x, y)$ relationships using equation 3 as a model and to calculate the corresponding mixture costs using equation 4 or equation 5.

RESULTS

Critical concentrations. The C_{crit} of benzoate or sorbate was the concentration corresponding to an optical density of 0.05 as calculated from the fit of the measurements of the optical density versus the concentration of benzoate or sorbate to an exponential decay or linear model (equation 1 or equation 2). Figure 3 shows examples of measured data points and their corresponding fits. For example, at pH 3.0, when the optical density of ZB was plotted as a function of the potassium sorbate concentration at a lauric arginate concentration of 5 ppm and a cinnamic acid concentration of 10 ppm, equation 1 gave the best fit with $R^2 = 0.99$, and C_{crit} for potassium sorbate concentration was calculated as 202 ppm. At pH 3.5, the optical density of SC decreased linearly with the concentration of potassium sorbate at a lauric arginate concentration of 0 ppm and a cinnamic acid concentration of 20 ppm. Here, equation 2 yielded the best fit ($R^2 = 0.98$), and C_{crit} was calculated as 324 ppm. At pH 4.0, equation 1 provided the best fit ($R^2 = 0.99$) for the decrease in optical density of BN with added potassium sorbate in the absence of lauric arginate and cinnamic acid, and the C_{crit} was calculated as 356 ppm. Table 1 shows as an example the C_{crit} of sodium benzoate as a function of lauric arginate and cinnamic acid at pH 3, 3.5, and 4 for *S. cerevisiae*. Figure 3 illustrates the different curves (concave, linear, and convex) that are indicative of synergistic, additive, or antagonistic interactions among the three agents.

TABLE 1. *Critical concentrations of sodium benzoate for* S. cerevisiae *as a function of lauric arginate (LA) and cinnamic acid (CA) concentrations at pH 3, 3.5, and 4*

pH	LA concn (ppm)	CA concn (ppm): 0	10	20	30	40	50
3	0	354	308	250	166	143	31
	2.5	244	157	107	51	0	0
	5	154	109	22	0	0	0
	7.5	0	0	0	0	0	0
	10	0	0	0	0	0	0
	15	0	0	0	0	0	0
3.5	0	505	509	436	382	305	177
	2.5	347	345	316	284	217	0
	5	330	292	261	200	147	0
	7.5	268	222	144	36	0	0
	10	137	67	0	0	0	0
	15	0	0	0	0	0	0
4	0	661	691	676	574	431	439
	2.5	570	449	484	426	364	309
	5	401	347	386	325	295	234
	7.5	370	319	287	261	200	86
	10	250	228	210	164	73	0
	15	10	2	0	0	0	0

Inhibition surfaces and contour plots. Equation 3 was used to model the relationship between the C_{crit} of sodium benzoate or potassium sorbate and the concentrations of lauric arginate and cinnamic acid required to inhibit growth of yeasts for 7 days. Surface and contour plots generated by our model are shown in Figure 4 for the four strains of yeasts tested at pH 3.0. The three-dimensional surface plot (top of each part of Fig. 4) shows the inhibition surface, consisting of data points that represent a concentration triplet of three agents able to inhibit growth of yeasts at pH 3.0. Any combination consisting of concentrations of the three antimicrobials above this surface would completely inhibit the growth of spoilage yeasts for 7 days, whereas below the inhibitory surface growth may occur. The corresponding inhibitory concentrations (C_{crit}) are shown as points plotted within the three-dimensional coordinate system. Comparison of the fitted surface and C_{crit} obtained from measured data points demonstrates the high accuracy of the model. An alternative representation of the data set is shown in the bottom of each part of Figure 4. Contour plots are two-dimensional representation of three-dimensional data sets where the lines (isopleths) describe the intersection of the inhibition surface with one or more horizontal planes. In our case, horizontal planes are those where concentra-

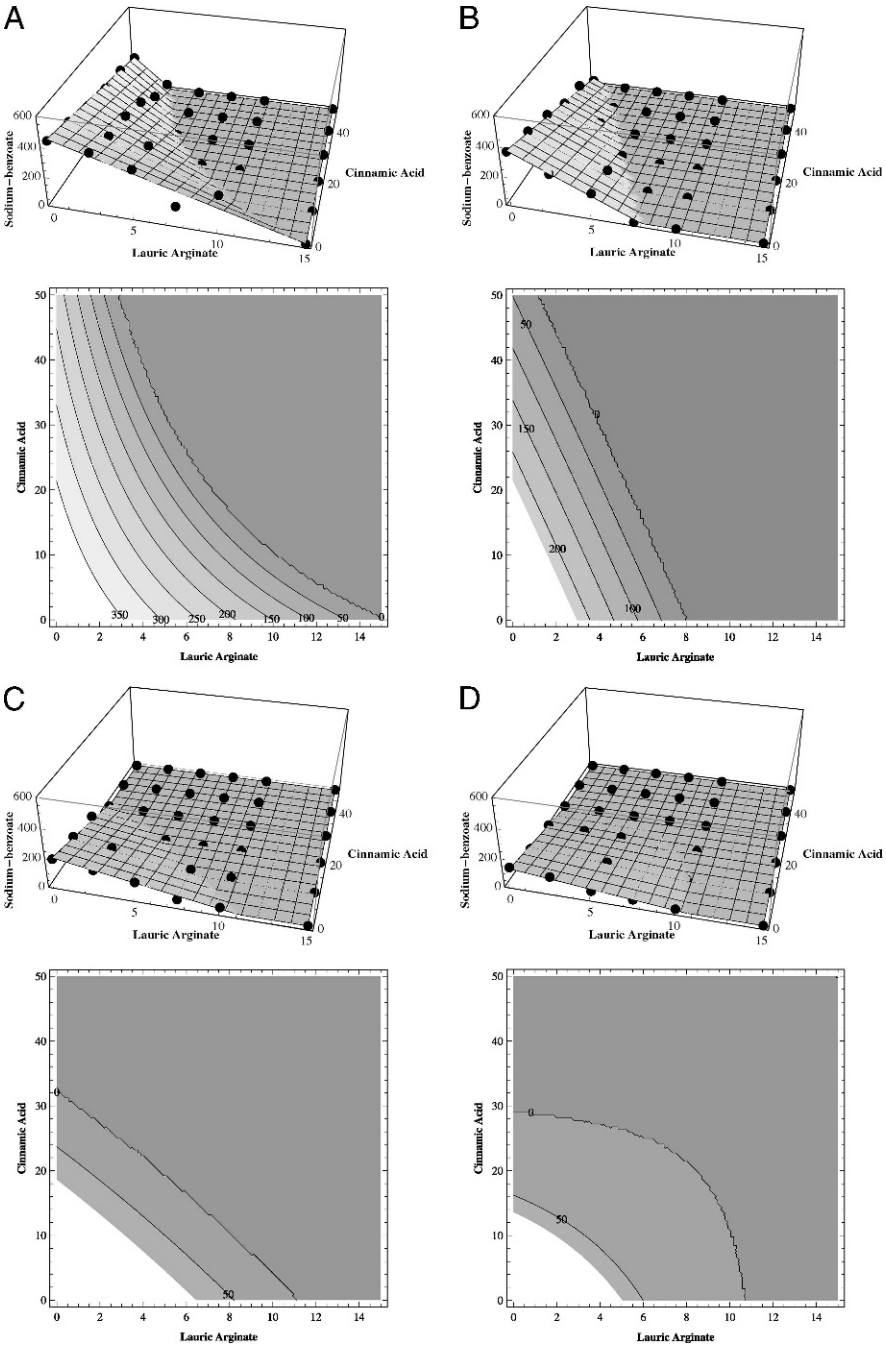

FIGURE 4. *Modeling of inhibition of spoilage yeasts by application of triplet combinations of lauric arginate, cinnamic acid, and sodium benzoate at pH 3.0 (equation 3). Top: Three-dimensional representation of fitted inhibition surfaces representing effective concentrations that inhibit growth of yeasts for 7 days. Measured critical concentrations of combinations are shown in black. Bottom: Contour plots of inhibition surfaces. (A)* Z. bailii *(ZB). Fit:* $C_{crit}(0) = 441$, a = *29.18*, b = *4.27*, c = *1.03*. *(B)* S. cerevisiae *(SC). Fit:* $C_{crit}(0) = 362$, a = *45.41*, b = *6.26*, c = *−0.04*. *(C)* B. bruxellensis *(BB). Fit:* $C_{crit}(0) = 192$, a = *17.27*, b = *5.99*, c = *−0.09*. *(D)* B. naardenensis *(BN). Fit:* $C_{crit}(0) = 114$, a = *10.70*, b = *3.95*, c = *−0.33*.

tions of cinnamic acid are constant (e.g., 0, 10, 20, 30, 40, and 50 ppm). The contour plots illustrate that isopleths can be linear (Fig. 4B and 4C), concave (Fig. 4A), or convex (Fig. 4D).

Effectiveness of individual antimicrobials and binary combinations. In addition to showing the effectiveness of triplet combinations, Figure 4 also allows estimation of the activities of each individual antimicrobial. For example, intersects of the isopleths with the *x* axis suggest that at pH 3.0, SC was more sensitive to lauric arginate than were any of the other tested yeasts. However, comparison of the isopleth intersects at the *y* and *z* axes revealed that BN and BB were more sensitive to organic acids than were ZB and SC. In agreement with previously conducted studies, ZB was the yeast most resistant to application of individual antimicrobials *(4, 22)*. In binary systems, significantly higher concentrations of sodium benzoate and cinnamic acid are needed to inhibit the growth of ZB and SC strains than to inhibit the growth of BB and BN.

Effectiveness of triplet combinations. At pH 3.0, triplet combinations of lauric arginate, cinnamic acid, and sodium benzoate effectively inhibited spoilage yeasts, and the concentrations of each antimicrobial in the triplet combination that were required to inhibit the growth of yeasts were lower than those in single treatments or binary combinations. BB and BN strains were more sensitive to triplet combinations than were ZB and SC strains; lower concentrations of antimicrobials were needed in effective combinations (Fig. 4). For example, the triplet combination of lauric arginate, cinnamic acid, and sodium benzoate at

TABLE 2. *Combined inhibitory effect of lauric arginate (LA) and cinnamic acid (CA) against four yeasts in the presence of sodium benzoate and potassium benzoate*[a]

		LA + CA + sodium benzoate			LA + CA + potassium sorbate		
Strain[b]	pH	Synergism	Additivity	Antagonism	Synergism	Additivity	Antagonism
SC	3.0		−0.04		0.4		
	3.5		−0.04			0.07	
	4.0			−0.24	0.3		
BB	3.0		−0.09		1.1		
	3.5		−0.11		0.62		
	4.0	0.46			0.33		
BN	3.0			−0.32	0.29		
	3.5			−0.35			−0.18
	4.0			−0.15			−0.41
ZB	3.0	1.03			1.05		
	3.5	0.24				0.06	
	4.0	0.5			0.47		

[a] Values are for parameter c in equation 3 after fit, where $-0.15 < c < 0.15$ indicates that the effects of the triplet compounds were additive, $c \geq 0.15$ indicates synergism, and $c \leq -0.15$ indicates antagonism.
[b] SC, *S. cerevisiae*; BB, *B. bruxellensis*; BN, *B. naardenensis*; ZB, *Z. bailii*.

concentrations of 5, 10, and 45 ppm, respectively, completely inhibited BN, whereas at a concentration combination of 5, 10, and 200 ppm growth of ZB strains was completely inhibited. As previously indicated, the contour graphs showed three different types of behaviors, similar to those described by Lopéz-Malo et al. *(17)* (Fig. 1):

(i) Isopleths of equal critical sodium benzoate concentration and various cinnamic acid and lauric arginate concentrations were linear (Fig. 4B and 4C). The values for the interaction parameter c calculated from equation 3 were −0.04 and −0.09 for SC and BB, respectively (Table 2). These findings suggest a simple additive effect of the three active agents.

(ii) Isopleths of equal critical sodium benzoate concentration at various cinnamic acid and lauric arginate concentrations were concave (Fig. 4A). The value for the interaction parameter c calculated from equation 3 was 1.03 for ZB. These findings suggest a synergistic effect of the three active agents, which may be attributed to one or more compounds enhancing the effect of the other agent(s).

(iii) Isopleths of equal critical sodium benzoate concentration at various cinnamic acid and lauric arginate concentrations were convex (Fig. 4D). The value for the interaction parameter c calculated from equation 3 was negative (−0.33) for BN. This finding suggests antagonistic activity among the three active agents, which among other factors could be attributed to an unfavorable interaction (e.g., aggregation or phase separation) between one or more agents or one compound desensitizing the cell against the other compounds, rendering them less effective.

Table 2 shows an overview of the interaction parameter c calculated from equation 3 for all experimental data obtained in this study. For example, for ZB, c was 1.03 at pH 3.0 (Fig. 4A) and decreased to 0.24 at pH 3.5 (Fig. 5A) and 0.5 at pH 4.0 (Fig. 5B), suggesting that combinations of lauric arginate, cinnamic acid, and sodium benzoate acted synergistically at all three pH values tested.

The binary combination of lauric arginate and cinnamic acid acted antagonistically, as indicated by a change of the curvature of the isopleths at $C_{\text{crit,benzoate}} = 0$ from linear to convex (Fig. 4 and Table 2). For example, for *B. bruxellensis* (Fig. 4D) at a sodium benzoate concentration of 50 ppm, the curvature decreased, suggesting a decrease in antagonism between the two antimicrobials, i.e., the addition of the third compound apparently reduced unfavorable interactions between the two agents. Isothermal titration calorimetry studies of solutions of binary or triplet combinations are required to further investigate the nature of these interactions. Isothermal titration calorimetry is a technique that can quantitatively determine the binding affinity (Ka), enthalpy changes (ΔH), and binding stoichiometry (n) of the interaction between two or more molecules in solution and thus provide insights into a potential complex formation between the agents *(18)*. Formation of such complexes also potentially could be observed using dynamic light scattering, a technique that can be used to determine the size distribution profile of small particles in solution. In addition to the interaction between the individual agents, one compound may improve or inhibit the permeation of another into the cell membrane or interior. Fluorescence studies may help to determine the location and concentration of these agents.

Effect of pH on effectiveness of triplet combinations. Although triplet combinations of lauric arginate, cinnamic acid, and sodium benzoate acted synergistically at all three pH values tested, higher critical concentrations of sodium benzoate were required to achieve complete inhibition at pH 3.5 and 4.0 than at pH 3.0, and the degree of synergism as indicated by the interaction parameter c decreased with increasing pH (Table 2). Sodium benzoate is the salt of an organic acid and has a pK_a of 4.2. The activity of organic

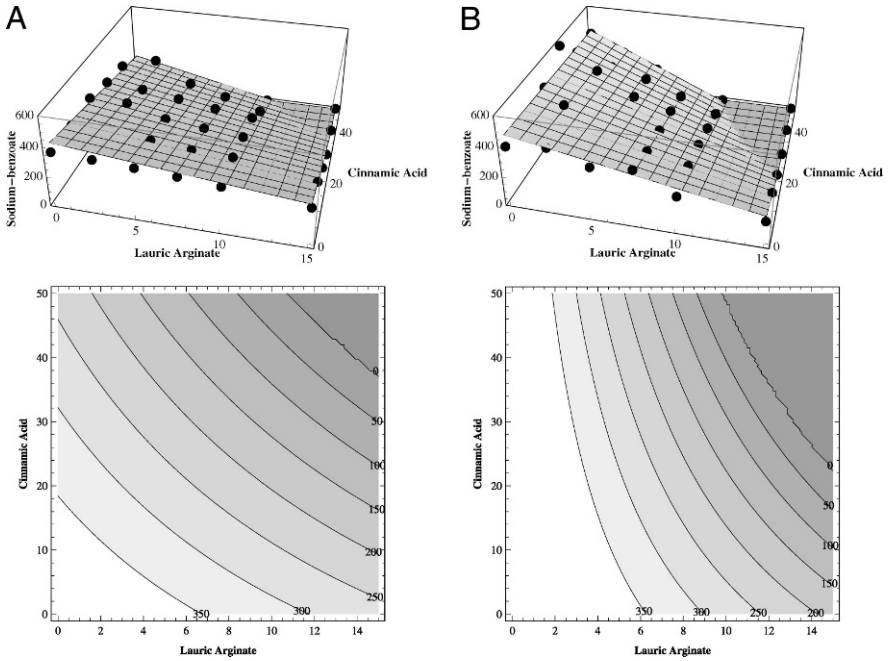

FIGURE 5. *Modeling of inhibition of* Z. bailii *by combinations of lauric arginate, cinnamic acid, and sodium benzoate as influenced by pH (equation 3). Top: Three-dimensional representation of fitted inhibition surfaces representing concentrations required to inhibit growth of yeast for 7 days. Measured critical concentrations of combinations are shown in black. Bottom: Contour plots of inhibition surfaces. (A) pH 3.5. Fit:* $C_{crit}(0) = 417$, a = *9.95*, b = *3.63*, c = *0.24*. *(B) pH 4.0. Fit:* $C_{crit}(0) = 467$, a = *18.69*, b = *0.68*, c = *0.51*.

acid–based antimicrobials is highly dependent on the pH because only undissociated organic acids can permeate the membrane of cells. The closer the pH is to the pK_a, the higher the concentration of the undissociated part of the organic acid becomes. Thus, weak acids have higher activity at pH 3 (manifested by the smaller amount of weak acids needed) than at pH 4, a fact associated with the higher concentration of undissociated organic acid at the lower pH *(13)*. This fact may explain why the synergistic activity in the triplet combination decreased as the pH increased.

Price calculations of preservative cocktails as an example of the practical use of inhibition models. Our modeling approach presented above may be used to generate a table of concentrations for effective antimicrobial cocktails that inhibit yeast growth and to calculate the cost of these cocktails using equations 4 and 5. Table 3 shows the result of such a calculation for triplet antimicrobial formulations of potassium sorbate, lauric arginate, and cinnamic acid that effectively inhibit the growth of BN at pH 4.0. The price calculations suggest that although lauric arginate is more expensive than the other two compounds, the price of the cocktail decreased when lauric arginate was added. The lowest price was obtained at a ratio of 15:10:11 of lauric arginate–cinnamic acid–potassium sorbate.

DISCUSSION

The mode of action of combined antimicrobials may be additive, synergistic, or antagonistic *(17)*. To date, few antagonistic effects of combinations of antimicrobials have been reported. Dai and Weiss *(7)* reported antagonism between potassium sorbate and saponins in the inhibition of spoilage yeasts; higher concentrations of both antimicrobials were required when used in combinations than when used individually. More often, synergies have been reported; combinations of two compounds were more effective for inhibiting target microorganisms than were the individual compounds alone *(19)*. Fyfe et al. *(11)* reported that combinations of plant oils and derivatives of benzoic acid synergistically inhibited growth of *Listeria monocytogenes* and *Salmonella* Enteritidis. Nazer et al. *(19)* used a factorial design to investigate the activity of combinations of five aromatic compounds and four acidic compounds for inhibiting the growth of *Salmonella* Typhimurium. These authors reported that more than one of those combinations efficiently prevented growth at lower doses, but no real synergy (or additivity) was found between compounds. Use of combinations of preservatives has become more frequent in recent years. When agents in combination act synergistically, concentrations of individual agents can be reduced, and overuse of a single antimicrobial agent may be prevented *(17)*. The use of combined preservatives theoretically provides a greater spectrum of activity and increases antimicrobial activity against the pathogenic or spoilage organisms if synergism occurs *(8, 17)*.

Various models to describe the inhibition of spoilage yeasts by combinations of environmental factors have been reported *(2, 3, 12, 15, 16)*. Most of these models predict the growth–no-growth interface via a logical regression method. At every tested concentration, two principal outcomes are possible: 0, indicating no growth, or 1, indicating growth. Thus, concentrations or environmental conditions can be established where growth is suppressed. In this study, we used continuous changes in the optical density measured in microtiter plates as a function of concentration of an active agent to calculate C_{crit} to predict the inhibition–no-inhibition interface of combinations of three antimicrobials. Both methods can be used to predict combinations that completely inhibit growth of spoilage yeasts, which every year cause large economic losses to food companies. Nevertheless, the method presented in this study also allows generation of a tolerance surface by simply changing the criterion used to obtain C_{crit}. For example, C_{crit} may instead be calculated at an OD of 0.075, yielding a triplet

TABLE 3. *Concentrations of potassium sorbate required to achieve the same degree of inhibition of* Brettanomyces naardenensis *at pH 4.0 in the presence of lauric arginate and cinnamic acid as calculated from equation 3*[a]

Lauric arginate concn (ppm)	Cinnamic acid concn (ppm):										
	0	5	10	15	20	25	30	35	40	45	50
0	**489**	**434**	**379**	**324**	**269**	**214**	**159**	**104**	**49**	**0**	**0**
Cost[b]	4.25	3.95	3.65	3.34	3.04	2.73	2.43	2.12	1.82	1.57	1.74
1	**460**	407	354	301	249	196	143	90	37	**0**	**0**
Cost	4.06	3.77	3.48	3.20	2.92	2.63	2.34	2.06	1.77	1.62	1.80
2	**432**	381	330	279	228	177	127	76	25	**0**	**0**
Cost	3.87	3.60	3.33	3.06	2.79	2.52	2.26	1.99	1.72	1.68	1.85
3	**403**	354	305	257	208	159	110	62	13	**0**	**0**
Cost	3.67	3.42	3.17	2.92	2.67	2.42	2.17	1.92	1.67	1.73	1.91
4	**374**	327	281	234	187	141	94	47	1	**0**	**0**
Cost	3.47	3.24	3.01	2.78	2.54	2.32	2.08	1.85	1.62	1.79	1.96
5	**345**	301	256	212	167	123	78	33	**0**	**0**	**0**
Cost	3.28	3.07	2.85	2.64	2.42	2.22	2.00	1.78	1.67	1.84	2.02
6	**317**	274	232	189	147	104	62	19	**0**	**0**	**0**
Cost	3.09	2.89	2.70	2.50	2.30	2.10	1.91	1.71	1.72	1.90	2.07
7	**288**	248	207	167	126	86	46	5	**0**	**0**	**0**
Cost	2.89	2.72	2.53	2.36	2.18	2.00	1.83	1.65	1.78	1.95	2.13
8	**259**	221	183	144	106	68	29	**0**	**0**	**0**	**0**
Cost	2.69	2.54	2.38	2.21	2.06	1.90	1.74	1.66	1.83	2.01	2.18
9	**231**	194	158	122	86	49	13	**0**	**0**	**0**	**0**
Cost	2.50	2.36	2.22	2.08	1.94	1.79	1.65	1.71	1.89	2.06	2.24
10	**202**	168	134	99	65	31	**0**	**0**	**0**	**0**	**0**
Cost	2.31	2.19	2.06	1.93	1.81	1.69	1.59	1.77	1.94	2.12	2.29
11	**173**	141	109	77	45	13	**0**	**0**	**0**	**0**	**0**
Cost	2.11	2.01	1.90	1.80	1.69	1.59	1.65	1.82	2.00	2.17	2.35
12	**145**	115	85	55	25	**0**	**0**	**0**	**0**	**0**	**0**
Cost	1.92	1.83	1.75	1.66	1.57	1.53	1.70	1.88	2.05	2.23	2.40
13	**116**	88	60	32	4	**0**	**0**	**0**	**0**	**0**	**0**
Cost	1.72	1.65	1.59	1.52	1.45	1.59	1.76	1.93	2.11	2.28	2.46
14	**87**	61	36	10	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Cost	1.53	1.47	1.43	1.38	1.47	1.64	1.81	1.99	2.16	2.34	2.51
15	**59**	35	11	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Cost	1.34	1.30	1.27	1.35	1.52	1.70	1.87	2.04	2.22	2.39	2.57

[a] Potassium sorbate concentrations in table body are given in ppm. Bold values are for effective single treatments or binary combinations.
[b] Cost, estimated cost (cents per liter) of product calculated with equation 5.

combination concentration surface where potentially tolerable growth occurs.

Results of this study demonstrate that triplet combinations tested were highly effective preservative systems able to inhibit the growth of spoilage yeasts and that these triplet combination were particularly effective in inhibiting growth of ZB. Effects obtained with combinations containing potassium sorbate showed evidence of synergistic activity.

Determination of C_{crit} from inhibition assays offers a convenient means of gauging the effectiveness of a particular antimicrobial, similar to the traditionally used MIC, but improves accuracies in subsequent modeling steps. A similar approach recently has been suggested by Lambert and Pearson *(14)* but has not been applied to model the efficacies of triplet combinations of antimicrobials.

Inhibition surfaces and contour plots provide simple-to-understand insights into the ability of triplet combinations to inhibit the growth of food spoilage organisms. The contour plots illustrate that isopleths can be linear, concave, or convex, a fact that may be used to interpret the effectiveness of the triplet combinations in the conventional terms of additivity, synergism, or antagonism, respectively. Although initially a large number of experiments were required to obtain the data for modeling, the model also provides a basis for minimization of required experiments. For example, food manufacturers wishing to employ a triplet antimicrobial preservation system in a specific food product should initially establish the axis intercepts that indicates the effectiveness of the individual systems and then select binary and triplet combinations. Because linear, concave, or convex isopleths can be expected, food manufacturers may then generate a dilution scheme that yields the minimum amount of information needed to provide an accurate generation of the entire inhibition surface. Once the surface has been generated, validation may be conducted by randomly picking concentrations on, below, or above the inhibition surface. On or above the inhibition surface, growth of microorganisms should be completely inhibited, while at subsurface concentrations, growth should be observed. As shown in this study, the

concentrations of individual agents may be used to generate additional helpful data such as price and sensory impact.

The approach presented in this study can easily be translated to antimicrobial assays conducted by agar dilution assays that yield absolute cell numbers. In that case, minimum acceptable cell numbers should be set as the critical concentration calculated at this cell number.

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