

Modeling Yeast Spoilage in Cold-Filled Ready-To-Drink Beverages with *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, and *Candida lipolytica*

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Mathematical models were developed to predict the probability of yeast spoilage of cold-filled ready-to-drink beverages as a function of beverage formulation. A Box-Behnken experimental design included five variables, each at three levels: pH (2.8, 3.3, and 3.8), titratable acidity (0.20, 0.40, and 0.60%), sugar content (8.0, 12.0, and 16.0 °Brix), sodium benzoate concentration (100, 225, and 350 ppm), and potassium sorbate concentration (100, 225, and 350 ppm). Duplicate samples were inoculated with a yeast cocktail (100 µl/50 ml) consisting of equal proportions of *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, and *Candida lipolytica* (~5.0 × 10⁴ CFU/ml each). The inoculated samples were plated on malt extract agar after 0, 1, 2, 4, 6, and 8 weeks. Logistic regression was used to create the predictive models. The pH and sodium benzoate and potassium sorbate concentrations were found to be significant factors controlling the probability of yeast growth. Interaction terms for pH and each preservative were also significant in the predictive model. Neither the titratable acidity nor the sugar content of the model beverages was a significant predictor of yeast growth in the ranges tested.

The high water activity (A_w) of most ready-to-drink beverages typically allows microbial growth. Hurdles such as pH, sugar content, and chemical preservatives prevent the growth of most organisms in ready-to-drink beverages (1). Spoilage yeasts, such as *Saccharomyces cerevisiae*, *Candida lipolytica*, and *Zygosaccharomyces bailii*, are sometimes able to overcome these hurdles. These organisms tolerate acidic environments and are resistant to chemical preservatives, such as potassium sorbate and sodium benzoate (1, 12).

Challenge studies are conducted in order to assess the ability of organisms to grow in a particular foodstuff. Challenge studies require considerable labor, time, and materials, and the number of parameters that can be tested is often limited. Validated predictive models, however, can provide rapid information about the microbial stability of a product and can be used in conjunction with challenge studies to improve product stability and reduce costs.

The focus of predictive microbiology has been in the creation of pathogen models with polynomial regression, such as the Food MicroModel (25) and the U.S. Department of Agriculture's Pathogen Modeling Program (40). Spoilage models are less prevalent in the literature (28, 39) but include models for yeasts (31), molds (18), and bacteria (2, 9, 22). Logistic regression models are also less prevalent in predictive food microbiology but are gaining importance (42).

Our research applies the techniques of predictive food microbiology to the spoilage of a model cold-filled beverage system. Several models for *Z. bailii* have been developed (5, 7, 11), but the focus of our research was to create a single mathematical model to describe spoilage by three common spoilage yeasts, in order to aid in the product development of ready-

to-drink beverages. The resulting model demonstrates which factors most influence spoilage yeast growth and allows comparison of product formulations (40) and the identification of possible alternative formulations with similar or enhanced resistance to growth (6).

MATERIALS AND METHODS

Organisms and cocktail preparation. Cultures of *S. cerevisiae*, *C. lipolytica*, and *Z. bailii* isolated from spoiled ready-to-drink beverages were obtained from Kraft Foods, Inc., Microbiology Department, Tarrytown, N.Y. These cultures were grown in malt extract broth solutions (Difco Laboratories, Detroit, Mich.) for 2 days at 30°C at 140 rpm on a Lab-Line Orbit Environ-Shaker (Melrose Park, Ill.). Malt extract broth was chosen because it is commonly used in the beverage industry for spoilage microorganism enumeration (14). Organisms were counted by plating 1.0 ml of decimal dilutions on malt extract agar (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) and incubating at 25°C for 5 days. Decimal dilutions of the cultures were made using phosphate buffer solution (Butterfield's buffer; Nutramax Products, Inc., Gloucester, Mass.). The solutions were held refrigerated (5°C) until enumerated (5 days). Once the individual solutions had been quantified, they were diluted with buffer to obtain a concentration of 5.0 × 10⁴ CFU/ml. The individual solutions were then blended together in equal amounts and mixed thoroughly to form the yeast cocktail. The cocktail was held at 5°C until used.

Experimental design. A Box-Behnken design with five variables at three levels was created using JMP software (SAS Institute, Cary, N.C.). Two points at the center of the design were used for a total of 42 experiments. The variables and levels were pH (2.8, 3.3, and 3.8), titratable acidity (TA) (0.20, 0.40, and 0.60%), sugar content (8.0, 12.0, and 16.0 °Brix), sodium benzoate concentration (100, 225, and 350 ppm), and potassium sorbate concentration (100, 225, and 350 ppm).

Preparation of beverages. The beverages were prepared with bottled water (Poland Springs, Poland, Maine), high-fructose corn syrup 42 (Cargill, Edyville, Iowa), granular citric acid (Cargill), potassium sorbate (Sorbistat-K; Cultor Food Science, Inc., Ardsley, N.Y.), and sodium benzoate (Cultor). Desired pH levels were obtained by buffering the beverages with potassium citrate (Cultor). Samples were mixed thoroughly on a magnetic stirrer, filtered through sterile, 0.20-µm-pore-size, disposable filter units (Nalgene Co., Rochester, N.Y.), and cold filled into sterile, 50-ml centrifuge tubes (Corning Inc., Corning, N.Y.). The tubes simulated the conditions of a sealed, bottled beverage.

The TA and pH were confirmed using a pH titroprocessor (Brinkmann, Herisau, Switzerland). Sugar concentration (°Brix) was determined using a RFM 340 refractometer (Bellingham and Stanley Ltd., London, United Kingdom). For

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consistency, °Brix reported here is that from high-fructose corn syrup alone. Actual °Brix measurements were slightly higher due to the presence of other solids (citric acid, potassium citrate, and preservatives). Because the resulting predictive model will be used for product formulation, changes in °Brix and pH over the course of the experiment were not measured.

Experimental methods. Duplicate beverage samples were inoculated with the yeast cocktail (100 µl/50 ml) and were immediately plated on malt extract agar medium. The inoculated samples were stored at 25°C and were sampled after 1, 2, 4, 6, and 8 weeks. Decimal dilutions were made using phosphate buffer solution, and colonies were enumerated after a 5-day incubation at 25°C. The goal of this study was to determine if any spoilage yeast growth occurred, so total plate counts rather than enumeration for specific species were used.

Model development. Plate count data were transformed into positive and negative growth responses over time where a response of 1 meant that yeast growth was observed and 0 meant that no detectable counts were observed. Logistic-regression analysis was conducted on the data using SAS software (SAS Institute). The equation for the full second-order logistic-regression model that includes linear and quadratic terms for time is

$$\text{logit}(P) = \ln\left(\frac{P}{1-P}\right) = \sum_{i=1}^5 \beta_i X_i + \sum_{ij=1}^5 \beta_{ij} X_i X_j + \sum_{i=1}^5 \beta_{ii} X_i^2 + \text{time} + \text{time}^2 \quad (1)$$

where P is the probability of growth, β 's are the parameter estimates for each term, and X 's are the five variables (pH, TA, °Brix, potassium sorbate concentration, and sodium benzoate concentration) in the model.

The simplified model was generated using backward stepwise regression ($P \leq 0.10$). Simplified models were developed using both actual values and normalized terms (-1, 0, +1) to describe the three levels of each variable. The normalized terms were used to facilitate comparison and to minimize correlation between terms (24).

A simplified logistic-regression model was generated using the terms from the backward stepwise regression with the actual values for the variables. The parameter estimates and the corresponding prediction equation for yeast growth were generated.

Model simplification is useful for several reasons. Models with many terms can have very high correlation coefficients and predict the data used to create the model with great accuracy. This prediction accuracy can come at the expense of postdiction accuracy (where postdiction is the ability to predict responses in experiments not used to create the model). This occurs because factors in these overparameterized models are actually fitting noise in the data rather than the data themselves (17). Model simplification can also aid in formulating hypotheses consistent with underlying biological mechanisms.

Model validation. Validation experiments with various pH, TA, °Brix, potassium sorbate, and sodium benzoate levels were conducted in the model beverages using the same methods. Fourteen sets of validation conditions were selected using a new combination of factors based on typical levels found in ready-to-drink beverages, and each was tested in duplicate.

RESULTS

The yeast growth responses for all of the 42 duplicated experiments are shown in Table 1. Yeast grew in only 12 samples despite being inoculated with 100 CFU/ml. There were four instances where the duplicate samples did not exhibit the same growth response, even though they were inoculated with the same level of yeast and had the same formulation. No visual signs of growth were noted in any of the 84 samples inoculated with the yeast cocktail; all beverages remained clear and colorless throughout the 8 weeks of the experiments.

S. cerevisiae, *C. lipolytica*, and/or *Z. bailii* were unable to grow in any of the beverages with a pH of 2.8. At the highest pH (3.8), yeasts grew in the samples with pH 3.8, 0.4% TA, 12 °Brix, and a combined preservative concentration of 325 ppm. The yeasts were not able to grow at pH 3.8 under any other conditions tested, which all had higher total preservative concentrations. Yeast did not grow at any pH when the maximum level (350 ppm) of either preservative was used. At pH 3.3

TABLE 1. Experimental variables with logistic growth responses^a of spoilage yeast cocktail after 8 weeks in model cold-filled ready-to-drink beverages

pH	TA (%)	°Brix	Potassium sorbate concn (ppm)	Sodium benzoate concn (ppm)	Growth response	
2.8	0.2	12	225	225	0.0	
	0.4	8	225	225	0.0	
	0.4	12	100	225	0.0	
	0.4	12	350	225	0.0	
	0.4	12	225	100	0.0	
	0.4	12	225	350	0.0	
	0.4	16	225	225	0.0	
	0.6	12	225	225	0.0	
	3.3	0.2	8	225	225	0.0
		0.2	12	225	100	1.0
		0.2	12	225	350	0.0
		0.2	12	100	225	0.0
		0.2	12	350	225	0.0
		0.2	16	225	225	0.0
		0.4	8	100	225	0.0
		0.4	8	350	225	0.0
0.4		8	225	100	1.0	
0.4		8	225	350	0.0	
0.4		12	100	100	1.1	
0.4		12	100	350	0.0	
0.4		12	350	100	0.0	
0.4		12	350	350	0.0	
0.4		12	225	225	0.0	
0.4		12	225	225	0.0	
3.8	0.4	16	100	225	1.0	
	0.4	16	350	225	0.0	
	0.4	16	225	100	1.0	
	0.4	16	225	350	0.0	
	0.6	8	225	225	0.0	
	0.6	12	225	100	1.1	
	0.6	12	225	350	0.0	
	0.6	12	100	225	0.0	
	0.6	12	350	225	0.0	
	0.6	16	225	225	0.0	
	3.8	0.2	12	225	225	0.0
		0.4	8	225	225	0.0
		0.4	12	100	225	1.1
		0.4	12	225	100	1.1
		0.4	12	225	350	0.0
		0.4	12	350	225	0.0
0.4		16	225	225	0.0	
0.6		12	225	225	0.0	

^a 0 = no growth; 1 = growth.

several sets of conditions showed yeast growth. Two sets of conditions (0.4% TA, 12 °Brix, and 100 ppm of both preservatives; and 0.6% TA, 12 °Brix, 225 ppm of potassium sorbate, and 100 ppm of sodium benzoate) evidenced growth in both samples. Other conditions at pH 3.3 supported yeast growth in one of the two replicates, including the two conditions of 0.4% TA and 16 °Brix and a total of 325 ppm of both preservatives.

Simplified logistic model. The full second-order logistic-regression model utilizing all of the linear, quadratic, and interaction terms along with the time and time² terms was 97.8% concordant with the data set. Backward stepwise regression eliminated 14 statistically insignificant terms from the full second-order logistic model, including the linear terms for potassium sorbate and sodium benzoate concentrations. The preservatives were significant as quadratic terms and in interaction terms, and so the linear potassium sorbate and sodium benzoate terms were returned to the simplified model. This simpli-

TABLE 2. Simplified model parameters for predicting the probability of growth of the *S. cerevisiae*, *C. lipolytica*, and *Z. bailii* cocktail in model cold-filled ready-to-drink beverages^a

Term	Parameter estimate		P
	Native terms	Coded terms	
Intercept	44.225919	-2.459741	0.0004
Time	3.876881	3.876880	<0.0001
Time ²	-0.366265	-0.366265	<0.0001
pH	-24.530336	-2.005873	<0.0001
Potassium sorbate	-0.035051	2.340813	0.3591
Sodium benzoate	-0.007356	3.414137	0.8544
Potassium sorbate × pH	0.039128	2.445490	0.0002
Potassium sorbate ²	-0.000079	-1.241274	0.0038
Sodium benzoate × pH	0.052066	3.254118	<0.0001
Sodium benzoate × potassium sorbate	-0.000176	-2.749729	0.0002
Sodium benzoate ²	-0.000217	-3.387250	<0.0001

fied model consisted of 11 terms: intercept, time, time², three linear, two quadratic, and three interaction terms (Table 2). This simplified model was 97.3% concordant with the data set, and the equation is

$$\begin{aligned} \text{logit}(P) = & 44.225919 + 3.87688063(\text{time}) \\ & - 24.530336(\text{pH}) - 0.0350506(\text{PS}) \\ & - 0.0073555(\text{SB}) + 0.03912785(\text{PS} \times \text{pH}) \\ & + 0.05206589(\text{SB} \times \text{pH}) - 0.000176(\text{SB} \times \text{PS}) \\ & - 0.3662647(\text{time}^2) - 0.0000794(\text{PS}^2) \\ & - 0.0002168(\text{SB}^2) \end{aligned} \quad (2)$$

where *P* is the probability of growth and PS and SB are potassium sorbate and sodium benzoate concentrations (in parts per million), respectively.

Logistic-regression models predict the probability of growth between 0 and 1. Model predictions are shown in Fig. 1 and 2. In Fig. 1, it is clear that sodium benzoate has a greater inhibitory effect on spoilage yeast growth than does potassium sorbate, as more potassium sorbate than sodium benzoate must be used to achieve the same probability of spoilage yeast growth. The pH-dependent efficacy of sodium benzoate is shown in Fig. 2. At lower pH values, less sodium benzoate must be used to achieve equal probability of yeast growth.

Predicted yeast growth responses from the simplified model were compared to the growth responses of a new set of experiments, as detailed in Table 3. The model predicts growth if its output is greater than 0.5 (50 to 100% chance of growth). If the output is lower than 0.5 (less than a 50% chance of growth), the model predicts no growth at that condition. The model correctly predicted the observed growth responses in all 28 samples of the 14 beverage formulations tested. It should be noted that if many replicate experiments were performed under a single set of conditions, one would expect the predicted probability to be equal to the fraction of observed cases (i.e., *P* = 0.25, one in four samples positive for growth).

Comparison of full and simplified logistic models. The accuracy of the logistic model decreased only slightly from the full to simplified models, indicating that the simplified model

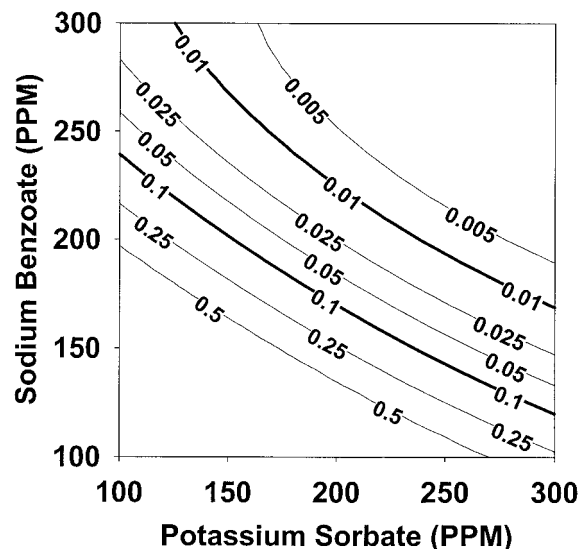


FIG. 1. The effects of sodium benzoate and potassium sorbate concentrations on the probability of growth of a *C. lipolytica*, *S. cerevisiae*, and *Z. bailii* cocktail after 8 weeks at initial pH of 3.3.

has roughly the same postdictive and predictive ability as the full second-order model. Neither could postdict the divergent growth responses of the four sets of conditions where the two replicates did not agree (one growth and one no-growth response were used in model development). For these four discordant sets of conditions, both models postdicted one false negative (occurrence where growth was observed but not postdicted) and three false positives (occurrence where growth was postdicted but not observed). However, the simplified logistic model predicted the growth responses of the validation experiments with complete success, validating the model.

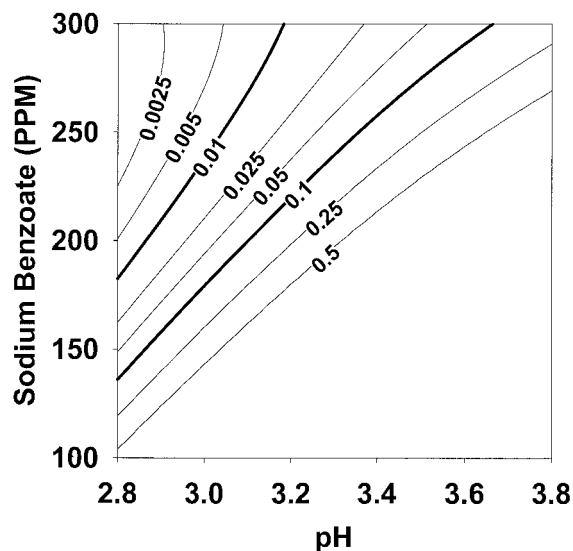


FIG. 2. The effects of pH and sodium benzoate on probability of growth of a *C. lipolytica*, *S. cerevisiae*, and *Z. bailii* cocktail after 8 weeks when potassium sorbate concentration was 100 ppm.

TABLE 3. Comparison of predicted and observed outcomes after 8 weeks to validate the simplified model of the growth of *S. cerevisiae*, *C. lipolytica*, and *Z. bailii* in cold-filled ready-to-drink beverages

pH	TA (%)	°Brix	Potassium sorbate (ppm)	Sodium benzoate (ppm)	Observed outcome ^a	Model prediction	Predicted outcome
2.8	0.2	8	100	225	0	0.0025	0
2.8	0.3	12	350	225	0	0.0031	0
2.8	0.4	12	350	225	0	0.0031	0
2.9	0.2	12	100	225	0	0.0061	0
2.9	0.6	12	100	225	0	0.0061	0
3.0	0.3	16	100	350	0	0.0065	0
3.1	0.5	8	350	100	0	0.0360	0
3.2	0.4	12	100	100	1	0.9985	1
3.2	0.5	8	225	225	0	0.0040	0
3.3	0.4	16	225	100	1	0.8438	1
3.3	0.4	16	350	225	0	0.0020	0
3.4	0.4	8	100	100	1	0.9999	1
3.5	0.2	12	100	225	1	0.5618	1
3.5	0.6	12	100	225	1	0.5618	1

^a *, outcome of both replicates.

DISCUSSION

Explanation of parameters and interactions in simplified model. The microbial stability of foods can be thought to be based upon a combination of several factors (hurdles) which, when acting in concert, should inhibit the growth of microorganisms (20). This "hurdle concept" can be applied to ready-to-drink beverages by using the simplified model (Table 2). The terms in this model and their degree of statistical significance can be employed to understand the possible mechanisms affecting yeast spoilage of beverage products. Only three of the variables (pH, potassium sorbate, and sodium benzoate) were found to be significant predictors for the growth of the spoilage yeasts. TA and sugar content were not significant factors in spoilage yeast growth. The magnitude of each term can be observed by comparing the normalized parameter estimates (Table 2).

The pH values used in these experiments were highly acidic (2.8 to 3.8). Many yeasts are able to survive but not grow at these low pH values (37), but *S. cerevisiae*, *Z. bailii*, and *C. lipolytica* can grow at pH levels as low as 2.0 (31). The effects of external pH on microbial homeostasis are well understood (4). As the pH of the beverage decreases, the amount of undissociated citric acid increases and can permeate the cell wall, altering the internal pH of the microorganisms (8).

The quadratic term for pH in the logistic model was not significant and was not included in the simplified yeast model. This is consistent with several previously published papers on the time to detection and time to growth of *Z. bailii* (11, 19). Quadratic pH terms, however, are included in other models for *Z. bailii* (5, 7) and *Kluyveromyces marxianus* (38), although the level of statistical significance of the quadratic pH terms was not provided.

The sugar content of the beverages in this study (8 to 16 °Brix, corresponding to A_w values from 0.95 to 0.99) was not a factor in predicting growth of these spoilage yeasts. *S. cerevisiae*, *C. lipolytica*, and *Z. bailii* have shown the ability to grow at 50 °Brix (31, 37), and they have enzyme systems that produce compatible solutes that allow them to grow at decreased A_w (21, 41). Due to the resistance of these spoilage yeasts to low

A_w , it is not surprising that the sugar concentrations in these experiments did not affect the yeast growth. However, these results were inconsistent with Cole and Keenan (7) and Cole et al. (5), who included the °Brix in their models predicting the growth of *Z. bailii* in beverages. The sugar contents of the beverages in those studies were 20 to 55 and 5 to 15 °Brix, respectively, but the models in these studies were not simplified to exclude the statistically insignificant terms in the model. Fructose content was determined to be a significant ($P < 0.0001$) factor for a *Z. bailii* time-to-growth model (19), but the range of sugar content was higher (up to 32% [wt/vol] fructose) than the range used in our experiments.

Both preservatives were significant in quadratic and interaction terms and were thus included in the simplified model. Higher levels of potassium sorbate or sodium benzoate decrease the likelihood of yeast growth. Sorbic acid affects yeast growth by inhibiting the uptake of amino acids and the function of sulfhydryl enzymes (10). Benzoic acid uncouples the electron transport system and destroys the proton motor force by increasing the internal proton level of the microorganism (10). This study showed that sodium benzoate, when used in combination with potassium sorbate, was a better inhibitor of spoilage yeasts than potassium sorbate (Fig. 1). This is consistent with Beuchat (3), who reported that sodium benzoate was more lethal towards *S. cerevisiae* than was potassium sorbate. Others (31), however, found that sorbic acid is more inhibitory than benzoic acid against spoilage yeasts, including *C. lipolytica*, *S. cerevisiae*, and *Z. bailii*.

There is a significant interaction between potassium sorbate and sodium benzoate in the simplified model ($P = 0.0002$), indicating that the two preservatives do not act independently of each other. The parameter estimate is very small and negative (-0.000176), indicating that there could be a slight antagonism (27) between potassium sorbate and sodium benzoate. This is consistent with Osman and El-Mariah (29), who reported that higher combined concentrations of sorbic acid and benzoic acid were required to prevent the growth of *S. cerevisiae*. It should be noted that other researchers have found evidence of synergistic inhibition of spoilage yeasts when both preservatives were used (6, 34).

There are also significant interactions between pH and potassium sorbate ($P = 0.0002$) and pH and sodium benzoate ($P < 0.0001$). The synergy between pH and sodium benzoate is clearly illustrated in Fig. 2. This is consistent with the idea that undissociated benzoic acid is more inhibitory than dissociated benzoic acid because undissociated organic acids are more lipophilic and can therefore pass through the cytoplasmic membrane and affect intracellular pH (10, 23).

Decreasing the pH of the beverage would allow a beverage developer to use less potassium sorbate and/or sodium benzoate to achieve the same probability of yeast growth. Conversely, increasing preservative levels provides microbial stability at increased pH levels (31). Eklund has suggested that the increased microbial action of sorbic and benzoic acid at low pH may be due to the increased susceptibility of the organism and not due to increased activity of the undissociated forms (15, 16); however, it is difficult to ascertain whether the stress of low pH or the increased level of the undissociated acid causes the interaction between pH and organic acids. Regardless, our simplified model reflects the synergy between pH and

preservative efficacy and can be used to show different beverage conditions that provide equal microbial stability.

Interactions between potassium sorbate and °Brix and sodium benzoate and °Brix were not significant, although it has been shown that sugars can act synergistically with organic acids to inhibit microbial growth (1, 36). As noted above, sugar content may become a significant factor only at higher concentrations than those used in our study.

An interaction between pH and °Brix was expected but was not found to be significant in the model. Cole and Keenan (7) and Cole et al. (5) reported synergy between pH and °Brix when describing the influence of these two variables on the growth of *Z. bailii* in model fruit drink systems, but these studies, as previously mentioned, did not use any method to simplify their models. Also, Cole and Keenan used much higher °Brix values than those used in this study, so it is possible that sugar content is more influential and acts synergistically with pH at larger concentrations. Other studies have also found that increasing the sugar level allows the effects of pH to be more pronounced (4).

Implications. The growth of spoilage yeasts in ready-to-drink beverages can cause off-flavors to develop in the beverage and can cause containers to explode from a buildup of carbon dioxide (30, 33). Spoilage yeasts can also alter the environment of the beverage by changing the pH or degrading preservatives, allowing other spoilage organisms to grow (26). *Candida*, *Saccharomyces*, and *Zygosaccharomyces* also possess lipolytic enzymes which can degrade the fatty acids of benzoate and sorbate (26). The control of any growth of spoilage yeasts is, therefore, essential for quality assurance.

The pH, potassium sorbate, and sodium benzoate levels of a cold-filled ready-to-drink beverage were all found to have an impact on yeast growth. However, it can be challenging to formulate beverages of an acceptable sensory quality at very low pH levels (4). Sodium benzoate was found to be a better inhibitor of spoilage yeast growth than potassium sorbate. This is an advantage for beverage developers since sodium benzoate tends to be less expensive than potassium sorbate (12) and is less prone to oxidation and degradation (35). Benzoic acid, however, imparts a burning aftertaste while sorbic acid tends to be neutral (13).

This predictive model provides an important tool for beverage product developers. While the pH, sodium benzoate, and potassium sorbate levels can be adjusted to provide desired cost and flavor profiles, the developers can consider each formulation's microbial stability. While a predictive model cannot replace microbial testing or the judgment of a trained and experienced microbiologist (39), this model should reduce the need for time-consuming and invasive microbiological testing procedures (32).

Conclusion. A mathematical model predicting the growth of *S. cerevisiae*, *C. lipolytica*, and *Z. bailii* in cold-filled ready-to-drink beverages has been presented. The model includes factors that can be controlled by a product developer, such as pH, sodium benzoate, and potassium sorbate concentration. The TA and °Brix of a beverage did not significantly impact spoilage yeast growth. This model can predict microbial stability of ready-to-drink beverages while allowing beverage developers to consider the ingredient costs, quality, and flavor implications of various preservation systems.

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