



Modelling mould spoilage in cold-filled ready-to-drink beverages by *Aspergillus niger* and *Penicillium spinulosum*

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Mathematical models have been developed to predict the probability of growth of spoilage moulds in response to various preservative systems in ready to drink beverages. A Box-Behnken experimental design included five variables, each at three levels: pH (2.8, 3.3, 3.8), titratable acidity (0.20%, 0.40%, 0.60%), sugar content (8.0, 12.0, 16.0 °Brix), and preservative concentrations (sodium benzoate and potassium sorbate, each 100, 225, 350 ppm). Duplicate samples were inoculated with a mould cocktail consisting of equal proportions of Aspergillus niger and Penicillium spinulosum spores (5.0 × 10⁴ spores/ml). The inoculated samples were plated on malt extract agar after 0, 1, 2, 4, 6, and 8 weeks. Logistic regression was used to create predictive models. The pH, titratable acidity, sugar content, sodium benzoate, and potassium sorbate levels were all found to be significant factors in predicting the probability of mould growth over time. Interactions between pH and sodium benzoate, pH and potassium sorbate, and pH and sugar content were also statistically significant. This logistic model was validated against 14 new conditions and predicted the growth of mould after 8 weeks with over 96% accuracy. Product developers can use these models to predict mould growth in ready to drink beverages.

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Introduction

Ready-to-drink beverages have high water activities (A_w) that permit microbial growth. Combinations of hurdles, such as pH, sugar content and chemical preservatives, prevent the growth of most organisms in ready to drink beverages (Leistner 1995). *Aspergillus niger* and *Penicillium spinulosum* are highly resistant to chemical preservatives such as sorbic and benzoic acids, and can tolerate both high acid and

lower A_w environments (Banwart 1979, De Boer and Nielsen 1995).

The food industry conducts challenge studies to assess the ability of organisms to grow in a particular foodstuff, but these studies require considerable labour, time, and materials, and the number of parameters that can be tested is often limited. Validated predictive models can provide rapid information about the microbial stability of a product that can reduce the time and effort needed for challenge studies.

The field of predictive microbiology has been focussed on creating polynomial regression equations to model foodborne pathogens, resulting in such models as the Food MicroModel

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(McClure et al. 1994), and the United States Department of Agriculture's Pathogen Modeling Program (Whiting and Buchanan 1997). Some mathematical models have been developed for food spoilage micro-organisms (Whiting 1995), including models for spoilage bacteria (Battey and Schaffner 2001, Cuppers and Smelt 1993, Llaudes et al. 2001, Ng and Schaffner 1997), yeasts (Battey 1999, Battey et al. 2001, Praphailong and Fleet 1997) and moulds (Gibson et al. 1994, Pitt 1993, Smith et al. 1988).

This research developed a predictive model for a cold-filled ready to drink beverage system to aid in product development. The resulting model will determine which factors have the most influence on the growth of spoilage moulds in ready to drink beverages. The model can be used to compare formulations (Whiting and Buchanan 1997) and identify alternative formulations with similar or enhanced stability (Cole et al. 1987, Whiting 1995).

Materials and Methods

Organisms and cocktail preparation

Isolated spores of *A. niger* and *P. spinulosum* were obtained from Kraft Foods, Inc., Microbiology Department, Tarrytown, New York, USA. The cultures were grown on potato dextrose agar for 5 days at 25°C. Spores were harvested by scraping the agar surface and then filtering the collected material through glass wool. Spores were washed several times with mineral salt solution and refrigerated (5°C). Organisms were counted by plating 1.0 ml of decimal dilutions on malt extract agar (MEA) (Oxoid Ltd., Basingstoke, Hampshire, UK) 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, Massachusetts, USA). The solutions were refrigerated (5°C) until counts were obtained. Once the individual solutions had been quantified, they were diluted with buffer to obtain a concentration of approx. 5.0×10^4 cfu ml⁻¹. The individual solutions were then blended together in equal amounts and mixed thoroughly to form the mould cocktail. The cocktail was held at 5°C until use.

Experimental design

A Box-Behnken design with five variables at three levels was created using JMP[®] software (SAS Institute, Cary, NC). Two points at the centre 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 (0.20%, 0.40%, and 0.60%), sugar content (8.0, 12.0, and 16.0 °Brix), sodium benzoate [100, 225, and 350 ppm (w/v)], and potassium sorbate [100, 225, and 350 ppm (w/v)].

Preparation of beverages

The beverages were prepared with bottled water (Poland Springs[®], Poland, Maine, USA), high fructose corn syrup (HFCS 42) (Cargill, Edyville, Indiana, USA), granular citric acid (Cargill), potassium sorbate (pKa = 4.76) (Sorbistat[®]-K, Cultor Food Science Inc., Ardsley, New York, USA), and sodium benzoate (pKa = 4.18) (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 disposable filter units (Nalgene Company, Rochester, New York, USA), and cold-filled into sterile, 50 ml screw-top centrifuge tubes (Corning Inc. Corning, New York, USA). The tubes simulated the conditions of a sealed, bottled beverage.

Samples were tested to confirm proper preparation. The titratable acidity (TA) and pH were measured using a pH titroprocessor (Brinkmann, Herisau, Switzerland). Sugar concentration (°Brix) was determined using a RFM 340 refractometer (Bellingham and Stanley Ltd, London, UK). For consistency, °Brix reported here is that from HFCS 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 mould cocktail (100 µl 50 ml⁻¹) and im-

mediately plated on MEA medium. The inoculated samples were also stored at 25°C and sampled after 1, 2, 4, 6, and 8 weeks, using the same tube each time. Decimal dilutions were made using phosphate buffer to assure that the results would be countable. The dilutions were plated on MEA and enumerated after incubation for 3 days at 25°C.

Model development

Plate counts were transformed into positive and negative mould growth responses for 1, 2, 4, 6 and 8 weeks, where a response of '1' meant that mould growth was detected and '0' meant no detected counts were observed. Logistic regression analysis was conducted on the data using JMP[®] software. Few logistic regression models exist for food microbes, but this modelling method is becoming more important in predictive food microbiology (Zhao et al. 2001). The equation for the full second order logistic regression model that includes linear and quadratic time terms is:

$$\begin{aligned} \text{Logit}(P) &= \ln\left(\frac{P}{1-P}\right) \\ &= \sum_{i=1}^5 \beta_i X_i + \sum_{i,j=1,i>j}^5 \beta_{ij} X_i X_j \\ &\quad + \sum_{i=1}^5 \beta_{ii} X_i^2 + \text{time} + \text{time}^2 \end{aligned} \quad (1)$$

where P is the probability of no growth, β are the parameter estimates for each term, and Xs represent the individual five variables in the model.

Backward stepwise regression was used to develop a simplified model able to predict detectable mould growth. Models were developed from actual values of the variables and normalized values, where -1, 0, +1 were used to represent the three levels of each variable. The use of normalized terms was needed to compare terms on an equivalent basis and minimize the correlation between terms (Mantel 1970).

Model validation

Validation experiments with varying pH, TA, °Brix, potassium sorbate, and sodium benzoate

levels were collected using the same methods. Validation conditions were selected using a new combination of factors based on typical levels found in ready to drink beverages.

Results

The mould growth responses for each of the 42 duplicated experiments are shown in Table 1. Mould grew in 13 of the beverage conditions. The duplicate samples exhibited the same growth response in every instance. Mould growth was either detected on MEA plates or observed in the beverages as floating mycelia. The mycelia were predominately white, often with small black spots. The beverages remained colourless throughout the duration of the experiments.

Neither *A. niger* nor *P. spinulosum* grew in any samples at pH 2.8. At pH 3.3, mould was able to grow when the total preservative concentration was 325 ppm or fewer (one preservative at 100 ppm, and the other at 100 or 225 ppm), regardless of titratable acidity or °Brix. This was also true at pH 3.8, where mould grew in samples with 325 ppm combined preservatives or lower. No mould growth was observed at any other combination of preservatives at either pH 3.3 or 3.8.

Simplified logistic model

A full second-order logistic regression model utilizing all of the linear, quadratic and interaction terms along with the time and time² terms was generated. The postdictions of the model were 99.2% concordant with the data. Backward stepwise regression eliminated 12 insignificant terms from the full second-order logistic model, leaving a simplified model consisting of 11 terms (intercept, time, time², five linear, and three interaction terms), as detailed in Table 2. All five of the variables were found to be significant in predicting the growth of *A. niger* and *P. spinulosum*. This simplified model was 99% concordant with the experimental data, and had a coefficient of

Table 1. Experimental variables with logistic growth responses after 8 weeks for the growth of *Aspergillus niger* and *Penicillium spinulosum* cocktail in model cold-filled ready to drink beverages

pH	TA, %	Sugar, °Brix	Potassium sorbate, ppm	Sodium benzoate, ppm	Observed outcome of both replicates	
2.8	0.2	12	225	225	0	
			225	225	0	
			100	225	0	
			225	100	0	
	0.4	8	225	225	0	
			225	350	0	
			350	225	0	
			225	225	0	
	0.6	12	225	225	0	
			225	225	0	
			225	225	0	
			225	225	0	
3.3	0.2	8	225	225	0	
			100	225	1	
			225	100	1	
			225	350	0	
			350	225	0	
			225	225	0	
	0.4	8	100	225	1	
			225	100	1	
			225	350	0	
			350	225	0	
			100	100	1	
			100	350	0	
	0.6	12	225	225	0	
			225	225	0	
			350	100	0	
			350	350	0	
			100	225	1	
			225	100	1	
	3.8	0.2	12	225	225	1
				225	225	1
				100	225	1
				350	225	0
		0.4	8	225	100	1
				225	350	0
225				225	0	
225				225	0	
0.6		12	225	225	0	
			225	225	0	
			225	225	0	
			225	225	0	

determination (R^2) of 0.814:

$$\begin{aligned} \text{logit}(P) = & 130.367597 + 6.34482382 (\text{Time}) \\ & - 0.5499497 (\text{Time}^2) - 50.809871 (\text{pH}) \\ & - 100.73612 (\text{TA}) - 3.9819013 (^\circ\text{Brix}) \\ & - 0.1155358 (\text{PS}) + 0.03875187 (\text{SB}) \\ & + 31.6499345 (\text{pH} * \text{TA}) \\ & + 1.3004905 (\text{pH} * ^\circ\text{Brix}) \\ & + 0.04898558 (\text{pH} * \text{PS}) \end{aligned} \quad (2)$$

where P is the probability of growth, PS is potassium sorbate concentration and SB is sodium benzoate concentration.

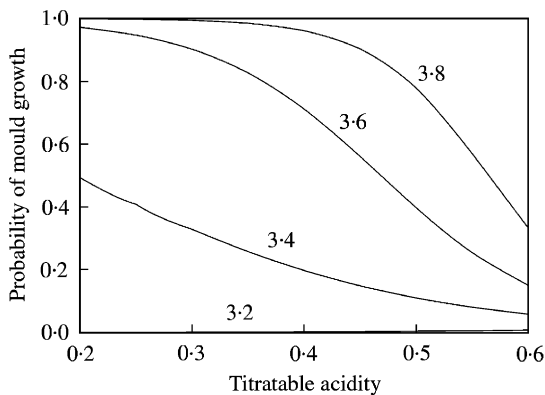
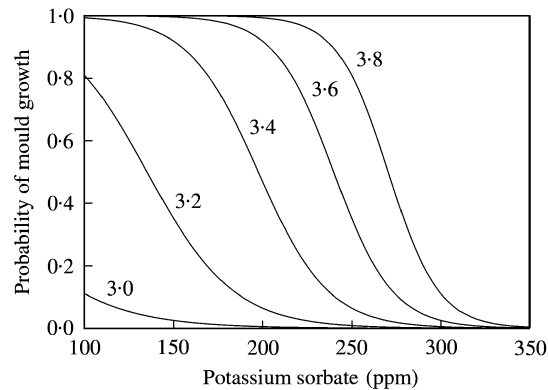
Several interaction terms were statistically significant. Though there are no quadratic terms for any of the five environmental variables, the non-linear effect of TA can nonetheless be observed in Fig. 1. As the pH increases, TA becomes increasingly important in controlling spoilage mould growth. There is a similar synergistic relationship between pH and potassium sorbate shown in Fig. 2. The same concentration of potassium sorbate (225 ppm) that keeps the probability of growth of moulds below 5% at pH 3.2, is very ineffective at pH 3.8, where the probability of growth is nearly 90%. Titratable acidity, sugar concentration and potassium sorbate were much more effective at inhibiting mould growth at low pH values, where large increases in their concentrations did not dramatically decrease the probability of *Aspergillus* and *Penicillium* growth in ready to drink beverages. The efficacies of both preservatives at pH 3.3 are shown in Fig. 3. Lower concentrations of potassium sorbate are required to achieve the same probability of mould growth as sodium benzoate, indicating that sorbic acid is more inhibitory than benzoic acid in this study.

Model validation

Predicted mould growth responses at 8 weeks were compared to observed growth responses for the same beverage formulations after 8 weeks' incubation. The simplified model predicted mould growth successfully (Table 3). There was concordance between the predicted and observed responses in 13 of the 14 samples. In the discordant sample, one tube showed mould growth and the other did not. It is inter-

Table 2. Simplified model parameters for the growth of *Aspergillus niger* and *Penicillium spinulosum* cocktail in model cold-filled ready to drink beverages

Term	Native estimate ^a	Normalized estimate	P value ^b
Intercept	130.36760	-13.009503	<0.0001
Time	6.34482	6.344824	<0.0001
Time ²	-0.54995	-0.549950	<0.0001
pH	-50.80987	-5.761128	<0.0001
Titrateable acidity	-100.73612	0.741733	0.0004
°Brix	-3.98190	1.238870	0.0038
Potassium sorbate	-0.11554	5.764573	0.0173
Sodium benzoate	0.03875	4.843983	<0.0001
pH* Titrateable acidity	31.64993	3.164993	0.0003
pH* °Brix	1.30049	2.600981	0.0020
pH* Potassium sorbate	0.04899	3.061599	0.0014

^a $R^2 = 0.814$;^bUsing normalized terms.**Figure 1.** The effect of titrateable acidity (TA) and pH on probability of *Aspergillus niger* and *Penicillium spinulosum* growth in a ready to drink beverages model at 12 °Brix, 225 ppm sodium benzoate, and 225 ppm potassium sorbate.**Figure 2.** The influence of pH and potassium sorbate on the probability of *Aspergillus niger* and *Penicillium spinulosum* growth in a ready to drink beverages model at 225 ppm sodium benzoate, 0.4% titrateable acidity, and 12 °Brix.

esting to note that the model prediction in this instance was 0.74, closest to equivocal (0.5) of any prediction in the data set.

Comparison of models

There was not a significant predictive difference between the full and simplified logistic models. Both models were 100% accurate at postdicting the growth of spoilage moulds in the 8-week data subset for all 42 formulations (data not shown). Also both models predicted approximately the same probabilities of growth for the validation conditions, making

them equally acceptable descriptors of mould growth in cold-filled ready to drink beverages.

Discussion

Explanation of parameters and interactions in simplified model

The pH of the beverage was found to have a very significant effect on mould growth. The range of pH values studied in these experiments was highly acidic (2.8–3.8). *Aspergillus niger* and *Penicillium spinulosum* are able to survive at low pH, but their growth is impeded (Banwart

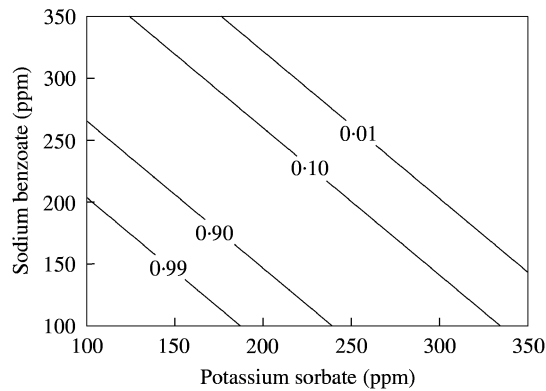


Figure 3. The effect of potassium sorbate and sodium benzoate on the probability of *Aspergillus niger* and *Penicillium spinulosum* growth in a ready to drink beverages model at pH 3.3, 0.25% titratable acidity, and 12 °Brix.

1979). It is well established that low pH environments are harmful to the homeostasis of micro-organisms (Brown and Booth 1991). At low pH values, undissociated citric acid is thought to permeate the cell wall altering the internal pH of the micro-organisms, causing denaturation and inactivation of enzymes (Brown and Booth 1991).

The titratable acidity of the model beverages had a significant effect ($P=0.0004$) on the growth of spoilage mould (Table 2); as the titra-

table acidity of the beverage increased, the likelihood of growth decreased slightly. This may be due to the chelating properties of citric acid and potassium citrate, which bind the heavy metals required for enzyme activity of micro-organisms (Davidson 1997, Young and Foegeding 1993).

There was also a significant ($P=0.0003$) interaction between pH and TA. The growth and survival of micro-organisms can be influenced by both the pH and the type and concentration of the acids, particularly when the pH is as low as in this study (Brown and Booth 1991, Buchanan et al. 1993). At low pH levels, the micro-organism is already stressed and is not impacted by an increase in titratable acidity (Fig. 1). The chelating properties of citric acid are most effective when the pH (and corresponding potassium citrate levels) of the beverage are higher (Praphailong and Fleet 1997), as in the results at pH 3.8 (Fig. 1). These results show that titratable acidity is more important to formulating stable ready to drink beverages at higher pH levels (3.8), and less important at very low pH levels (2.8).

The °Brix of the beverage also has a significant effect on mould growth. As the sugar content increased, the moulds were less likely to grow. This is consistent with Gibson et al. (1994) who observed the dependence of mould growth on water activity (A_w). The effect of

Table 3. Comparison of observed and predicted mould growth responses after 8 weeks to validate the simplified model of the growth of *Aspergillus niger* and *Penicillium spinulosum* in cold-filled ready to drink beverages

pH	TA	°Brix	Potassium sorbate, ppm	Sodium benzoate, ppm	Observed outcomes	Probability of growth	Predicted most likely outcome
2.8	0.2	8	100	225	0,0	0.000083	0
2.8	0.3	12	350	225	0,0	0.000005	0
2.8	0.4	12	350	225	0,0	0.000016	0
2.9	0.2	12	100	225	0,0	0.003554	0
2.9	0.6	12	100	225	0,0	0.113482	0
3.0	0.3	16	100	350	0,0	0.000759	0
3.1	0.5	8	350	100	0,0	0.016430	0
3.2	0.4	12	100	100	1,1	0.998152	1
3.2	0.5	8	225	225	0,0	0.045641	0
3.3	0.4	16	350	225	0,0	0.000071	0
3.3	0.4	16	225	100	0,1	0.741313	1
3.4	0.4	8	100	100	1,1	0.999991	1
3.5	0.2	12	100	225	1,1	0.999841	1
3.5	0.6	12	100	225	1,1	0.991282	1

water activity on fungal osmoregulation and growth has been the subject of many reviews (Andrews and Pitt 1987, Leistner and Russell 1991). Increasing the sugar concentration of the beverages from 8 to 16 °Brix causes the A_w to decrease slightly (Lueck 1980). *Aspergillus niger* and *P. spinulosum* are xerophilic and have enzyme systems that produce compatible solutes (Leistner and Russell 1991) that allow them to grow (albeit more slowly) at decreased A_w .

Potassium sorbate has a significant impact on mould growth ($P = 0.0173$). Higher levels of potassium sorbate decrease the likelihood of mould growth. Sorbic acid inhibits both *Aspergillus* and *Penicillium* (Davidson 1997), although some strains of *A. niger* and some species of *Penicillium* are resistant to sorbic acid (De Boer and Nielsen 1995). The antifungal activity of sorbic acid is due to its inhibition of sulphhydryl enzymes and amino acid uptake, which disrupts the proton motor force on the cell membrane (Davidson 1997).

Sodium benzoate also has a very significant inhibitory impact on mould growth ($P < 0.0001$). Our results indicate, however, that potassium sorbate is more effective at inhibiting mould growth than sodium benzoate (Fig. 3). This is consistent with Chichester and Tanner (1972) who reported that sorbic acid is more effective than benzoic acid against mould growth. This is in contrast to Baird-Parker and Kooiman (1980) who reported that sorbic and benzoic acids were equally effective against spoilage moulds. Benzoic acid inhibits fungal growth by uncoupling substrate transport and oxidative phosphorylation from the electron transport system and by inhibiting enzymes involved in acetic acid metabolism and oxidative phosphorylation (Davidson 1997).

There is a small, but significant interaction between pH and potassium sorbate ($P = 0.0014$), but there is no such interaction between pH and sodium benzoate. This emphasizes the efficacy of potassium sorbate on inhibition of *Aspergillus* and *Penicillium* as compared to sodium benzoate, and also the increasing importance of potassium sorbate concentration at higher pH values, when pH alone fails to control the growth of spoilage moulds. Organic acids, such as sorbic acid and benzoic acid, are most effective at inhibiting micro-

organisms in their undissociated forms, which increase in concentration as the pH decreases (Davidson 1997). Decreasing the pH of the beverage, therefore, allows a beverage developer to utilize less potassium sorbate and/or sodium benzoate to achieve the same probability of mould growth in the finished product. Conversely, increasing preservative levels provides microbial stability at increased pH levels (Praphailong and Fleet 1997). This is consistent with the well-documented effect of the correlation of pH with the antimicrobial effects of sorbic acid and benzoic acid (Lueck 1980, Sofos and Busta 1993). Eklund (1985) 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. Whether the stress of low pH or the increased level of the undissociated acid causes the synergy between pH and potassium sorbate, it is an important relationship for inhibiting micro-organisms in food and of great use to product developers.

An interaction between pH and °Brix was also significant in the model ($P = 0.0020$). In several studies, pH has been shown to be a more effective inhibitor of growth at higher solute concentrations (Baird-Parker and Kooiman 1980, Brown and Booth 1991). Sugars can also act synergistically with organic acids to inhibit microbial growth by increasing the concentration of undissociated acid (Baird-Parker and Kooiman 1980, Sofos and Busta 1993), however this effect was not observed in our model beverage system, as interactions between potassium sorbate and °Brix and sodium benzoate and °Brix were not significant in the model.

Implications

Mould growth causes spoilage in beverages, which can be detected by the consumer by the emergence of off-flavors or visible growth. *Aspergillus* and *Penicillium* species possess lipolytic enzymes which can degrade the fatty acids of sodium benzoate and potassium sorbate (Mossel et al. 1995) which can allow other spoilage organisms to grow as well (Banwart 1979).

The pH, titratable acidity, sugar content, potassium sorbate, and sodium benzoate levels were all found to have an impact on mould growth in ready to drink beverages. Each of these variables can be adjusted to balance microbial stability with other quality attributes (Baird-Parker and Kooiman 1980); while pH had a dramatic inhibitory impact on spoilage mould growth, there are challenges associated with formulating acceptable beverages at very low pH levels. The sugar content of the beverage can be balanced against the titratable acidity to achieve desired Brix/acid ratios, but the microbial impacts of both the titratable acidity and the °Brix of a beverage must be considered when formulating ready to drink beverages.

Potassium sorbate appeared more effective than sodium benzoate at controlling *Aspergillus* and *Penicillium* in this study. This can be an advantage for product developers who exploit differences between the preservatives. Potassium sorbate tends to be more expensive than sodium benzoate (De Boer and Nielsen 1995) and is more prone to oxidation and degradation (Sofos 1989), but it doesn't cause the burning after-taste associated with benzoic acid (De Boer and Nielsen 1995).

We have developed other models to predict the growth of spoilage bacteria (*Acinetobacter calcoaceticus* and *Gluconobacter oxydans*) (Battey and Schaffner 2001) and yeasts (*Saccharomyces cerevisiae*, *Candida lipolytica*, and *Zygosaccharomyces bailii*) (Battey 1999, Battey et al. 2001) in cold-filled ready to drink beverages, and all of these models can be taken into consideration when formulating a microbiologically stable beverage. In all three models, pH, preservative concentrations and a synergy between pH and potassium sorbate were statistically significant, however, the similarities end with those three terms. The mould spoilage model presented here contains no quadratic terms, whereas the yeast and bacterial models have statistically significant quadratic terms for both preservatives, and the bacterial growth rate model has a significant quadratic pH term. Linear terms for TA and °Brix were significant in the bacteria and mould models but not in the yeast model, meaning that interaction terms including either TA or sugar content (sorbate*Brix in the bacteria

model, pH*Brix in the mould model, and pH*TA in both) are also absent in the spoilage yeast model. Beverage developers can evaluate which organisms pose the greatest spoilage risk in their product and select one appropriate model to guide product development, or can combine one or more of the models to create a more stable beverage. Clearly low pH and higher preservative concentrations cause inhibition of all the spoilage microflora tested, and these factors can guide future beverage formulation.

This model provides a tool that food microbiologists and product developers can use to help assess the microbial stability of a beverage. The model should increase productivity by reducing the need for time-consuming microbiological testing procedures currently practiced, although a predictive model is never a substitute for challenge testing and the expertise of a trained microbiologist (Ross and McMeekin 1994).

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