Journal of Applied Microbiology 2001, **91**, 237–247

Modelling bacterial spoilage in cold-filled ready to drink beverages by *Acinetobacter calcoaceticus* and *Gluconobacter oxydans*

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664/12/00: received 1 December 2000, revised 9 March 2001 and accepted 9 March 2001

A.S. BATTEY AND D.W. SCHAFFNER. 2001.

Aims: Mathematical models were created which predict the growth of spoilage bacteria in response to various preservation systems.

Methods and Results: A Box-Behnken design included five variables: pH (2·8, 3·3, 3·8), titratable acidity (0·20%, 0·40%, 0·60%), sugar (8·0, 12·0, 16·0 °Brix), sodium benzoate concentration (100, 225, 350 ppm), and potassium sorbate concentration (100, 225, 350 ppm). Duplicate samples were inoculated with a bacterial cocktail (100 μl 50 ml^{-1}) consisting of equal proportions of *Acinetobacter calcoaceticus* and *Gluconobacter oxydans* (5×10^5 cfu ml^{-1} each). Bacteria from the inoculated samples were enumerated on malt extract agar at zero, one, two, four, six, and eight weeks.

Conclusions: The pH, titratable acidity, sugar content, sodium benzoate, and potassium sorbate levels were all significant factors in predicting the growth of spoilage bacteria.

Significance and Impact of the Study: This beverage spoilage model can be used to predict microbial stability in new beverage product development and potentially reduce the cost and time involved in microbial challenge testing.

INTRODUCTION

The high water activity (A_w) of most ready to drink beverages provides conditions that are favourable for microbial growth. Hurdles, such as low pH and chemical preservatives, prevent the growth of most organisms in ready to drink beverages (Sand 1975). Though few organisms are able to overcome this combination of hurdles, both *Acinetobacter calcoaceticus* and *Gluconobacter oxydans* have been shown to be able to grow in still beverages, in part because of their resistance to potassium sorbate and sodium benzoate preservatives (Sand 1975; Eyles and Warth 1989).

Acinetobacter are gram-negative, nonmotile rods that are common soil and water organisms. They are mesophilic and strictly aerobic. *Acinetobacter* possess lipolytic enzymes that degrade fatty acids such as benzoic and sorbic acid (Mossel 1971). *Acinetobacter* have no perceivable effect on beverage taste, odour or appearance, but can alter the pH and preservative levels, allowing other spoilage organisms to grow (Mossel 1971).

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Gluconobacter oxydans are gram-negative, strictly aerobic, polarly flagellated rods. *Gluconobacter* are characterized by their acid tolerance, ability to grow in low-nutrient conditions and their resistance to benzoic and sorbic acids (Eyles and Warth 1989). *Gluconobacter* can cause off-flavours in ready to drink beverages (Sand 1975).

Challenge studies are commonly conducted in order to assess the ability of particular microbes to grow in a particular foodstuff. The degree of risk associated with a particular formula is determined by observing whether the spoilage organism will grow when added to the product. Challenge studies require considerable time, labour and materials and the number of parameters that can be tested are often limited. Validated predictive models, on the other hand, can provide rapid information about the microbial stability of a newly developed product.

Predictive microbiology research has typically focused on the creation of pathogen models, such as the Food MicroModel (McClure *et al.* 1994) and the United States Department of Agriculture's Pathogen Modeling Program (Whiting and Buchanan 1997). Spoilage models are less prevalent in literature, but include models for spoilage yeasts (Membre *et al.* 1999), molds (Smith *et al.* 1988) and bacteria

(Cuppers and Smelt 1993; McClure *et al.* 1993; Ng and Schaffner 1997). There are currently no models available to predict the growth of *Gluconobacter oxydans* and *Acinetobacter calcoaceticus*. The model presented here was developed using parameters (pH, °Brix, and preservative concentration) that are directly applicable to product formulation. This model will assist product developers in the creation of more stable cold-filled ready to drink products in a timely and efficient manner.

MATERIALS AND METHODS

Organisms and cocktail preparation

Cultures of *Acinetobacter calcoaceticus* and *Gluconobacter oxydans* were obtained from Kraft Foods, Inc., Microbiology Department, Tarrytown, NY. These organisms were grown in malt extract broth solutions (Difco Laboratories, Detroit, MI) for 2 d at 30°C, while being swirled at 140 rev min^{-1} on a Laboratory-Line® Orbit Environ-Shaker (Melrose Park, IL). This medium was chosen not only because it is commonly used in the beverage industry for spoilage micro-organism enumeration (Dworetsky 1998), but also because it is a low-nutrient medium that selects for *Acinetobacter* and *Gluconobacter*. 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 d. Decimal dilutions of the cultures were made using phosphate buffer solution (Butterfield's buffer, Nutramax Products, Inc., Gloucester, MA). The solutions were refrigerated (5°C) until enumerated. No significant change of viable counts was observed after 5 d of refrigeration. Once quantified, the solutions were diluted with buffer to obtain a concentration of $5{\cdot}0 \times 10^5$ cfu ml^{-1}. The individual solutions were then blended together in equal amounts and mixed thoroughly to form the bacterial cocktail.

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), and potassium sorbate (100, 225 and 350 ppm).

Preparation of beverages

The beverages were prepared with bottled water (Poland Springs®, Poland, ME), high fructose corn syrup (HFCS 42) (Cargill, Edyville, IA), granular citric acid (Cargill), potassium sorbate (Sorbistat®-K, Cultor Food Science Inc., Ardsley, NY) 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 disposable filter units (Nalgene Company, Rochester, NY), and cold-filled into sterile, 50-ml centrifuge tubes (Corning Inc., Corning, NY). 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 samples were inoculated with the bacterial cocktail (100 μl/50 ml) and immediately plated on MEA medium. The inoculated samples were also stored at 25°C without shaking and sampled after one, two, four, six and eight weeks, using the same tube each time. Decimal dilutions were made using phosphate buffer solution. The total number of bacterial colonies was enumerated after incubation for 5 d at 25°C.

Model development

Growth rates were calculated using Equation 1.

$$\text{Rate} = [\log(\text{cfu ml}^{-1})_a - \log(\text{cfu ml}^{-1})_b]/(\text{time}_a - \text{time}_b) \quad (1)$$

where a and b are defined as points that yielded the most conservative rates for each sample, as described below. For samples that supported bacterial growth, the fastest growth rates were calculated by obtaining the steepest slope ($\Delta\log_{10}$(cfu ml^{-1})/Δtime) between points. For samples that did not support bacterial growth, the slowest inactivation rates were calculated by using data until the first consecutive plate count with no growth (< 1 cfu ml^{-1}) was observed. Where a decline in bacterial population preceded an increase, the fastest growth rate was used. Growth and inactivation rates were used in the same analysis to produce a single, unified model.

Regression analysis was conducted on the data using JMP® software. Standard least squares regression was

performed utilizing a second-order model for five input variables: pH, titratable acidity, °Brix and concentrations of potassium sorbate and sodium benzoate. A simplified model was generated using backward stepwise regression ($P = \leq 0{\cdot}10$). The parameter estimates and the corresponding prediction equation for growth rate were obtained.

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. Additionally, four beverages were tested with constant levels of sugar (12 °Brix), sodium benzoate (100 ppm), and potassium sorbate (100 ppm) to better define the relationship between pH (2·9 or 3·5) and TA (0·2 or 0·6%).

RESULTS

The bacterial growth rates and binomial growth responses for each of the 42 duplicated experiments are shown in Table 1. About half of the samples supported bacterial growth. There were six instances where the duplicate samples did not exhibit the same response.

The most important factor appeared to be pH. *Acinetobacter calcoaceticus* and *G. oxydans* did not grow in any samples with a pH 2·8. At pH 3·8, bacteria were able to grow in most of the samples. The effect of titratable acidity (TA) on bacteria growth was equivocal. Whether the TA was high or low, bacteria were able to grow only when other factors (i.e. low total preservative levels, lowest °Brix) favoured bacterial growth. Of the eight conditions that included the highest level of °Brix, in two conditions one of the duplicates exhibited bacterial growth, while the other did not.

All of the samples with 8 °Brix exhibited bacterial growth except for those with either a low pH (2·8) or a high potassium sorbate level (350 ppm). Conversely, bacteria were not able to grow in all samples with a high sugar (16 °Brix), except for one of the two duplicates with low potassium sorbate (100 ppm) or high pH (3·8).

The ability of bacteria to grow in the beverages used in this study was dependent on the potassium sorbate level. At a high potassium sorbate level (350 ppm), bacteria were not able to grow except for those that had either a low sodium benzoate (100 ppm) or high pH (3·8). Conversely, bacteria were able to grow in all samples at low potassium sorbate (100 ppm), except for when the pH was low (2·8). Samples with low potassium sorbate (100 ppm) that had either high sodium benzoate (350 ppm) or high sugar (16 °Brix) exhibited bacterial growth in one of the duplicate samples.

Bacterial growth was also influenced by the sodium benzoate level, though not to the degree that it was influenced by the potassium sorbate level. Bacteria were able to grow in all samples with low sodium benzoate (100 ppm) except for those samples that had either low pH (2·8) or high sugar (16 °Brix). Samples with high sodium benzoate levels (350 ppm) showed bacterial growth at low sugar (8 °Brix) and high pH (3·8). Bacteria were not able to grow in those samples with both high sodium benzoate (350 ppm) and high sugar (16 °Brix), high potassium sorbate (350 ppm), or low pH (2·8). The bacterial growth responses in samples with high sodium benzoate (350 ppm) and either low potassium sorbate (100 ppm), high TA (0·6%) or low TA (0·2%) were inconsistent. In these samples, bacteria were able to grow in one of the duplicates, indicating the inability of high levels of sodium benzoate alone to guarantee inhibition of bacterial growth.

Growth rate model

A full second-order model utilizing all of the linear, quadratic and interaction terms was generated. This 21-term model had a coefficient of multiple determination (r^2) of 0·822. The prediction profile for each of the five variables is shown in Fig. 1. These profiles indicate the extent of the effect that each variable has on bacteria growth. The curvature of the lines gives a visual indication of the significance of the quadratic terms in the model. As Fig. 1 shows, the pH of the beverage has a significant effect on the growth rate. As pH increased, the growth rate also increased. As the °Brix, potassium sorbate or sodium benzoate levels increased, the growth rate decreased. The curvature of the pH, potassium sorbate and sodium benzoate lines indicates the importance of the quadratic terms in predicting the bacterial growth rate. The line for TA has a slope close to zero and therefore does not appear to have any effect on the growth rate.

Of the 10 possible interactions in the full second-order model, four were found to be significant: pH and TA, °Brix and potassium sorbate, potassium sorbate and pH, and sodium benzoate and potassium sorbate (Fig. 2).

There is a strong interaction between pH and TA (Fig. 2a). At a low TA level (0·20%), decreasing the pH causes the bacterial growth rate to decrease. The antimicrobial effect of pH, however, was reduced as the TA level increased. At a high TA level (0·60%), the pH has little influence over the bacterial growth rate.

The effect of potassium sorbate was increased, as the pH decreased (Fig. 2b). At a high pH, increasing the level of potassium sorbate did not affect the growth rate. At low pH, however, increasing the potassium sorbate decreased the growth rate.

Table 1 Experimental variables with logistic and growth rate responses for the growth of *Acinetobacter calcoaceticus* and *Gluconobacter oxydans* cocktail in model cold-filled ready to drink beverages

pH	TA (%)	°Brix	Potassium sorbate (ppm)	Sodium benzoate (ppm)	Potassium citrate (%)	Growth response	Growth rate log(cfu ml^{-1}) week^{-1}
2·8	0·2	12	225	225	0	–	−3·09691
						–	−3·19590
	0·4	8	225	225	0·03	–	−0·78893
						–	−0·50848
	0·4	12	100	225	0·03	–	−0·75854
						–	−0·72159
	0·4	12	350	225	0·03	–	−2·92942
						–	−3·07918
	0·4	12	225	100	0·04	–	−1·39620
						–	−0·68239
	0·4	12	225	350	0·03	–	−1·44041
						–	−1·44881
	0·4	16	225	225	0·03	–	−2·94939
						–	−1·42255
	0·6	12	225	225	0·075	–	−1·51871
						–	−0·38263
3·3	0·2	8	225	225	0·035	+	3·38917
						+	3·52496
	0·2	12	225	100	0·055	+	2·67083
						+	1·97945
	0·2	12	225	350	0·03	–	–1·57461
						+	1·18466
	0·2	12	100	225	0·06	+	1·56093
						+	1·45482
	0·2	12	350	225	0·03	–	−0·68400
						–	−1·41942
	0·2	16	225	225	0·0425	–	−0·44085
						–	−0·72023
	0·4	8	100	225	0·17	+	3·31784
						+	3·40438
	0·4	8	350	225	0·14	–	−1·58805
						–	−1·59376
	0·4	8	225	100	0·16	+	3·90003
						+	3·81116
	0·4	8	225	350	0·14	+	1·81639
						+	1·77254
	0·4	12	100	100	0·15	+	0·98687
						+	0·98040
	0·4	12	100	350	0·14	+	1·06058
						–	−0·79495
	0·4	12	350	100	0·14	+	2·83040
						+	1·78862
	0·4	12	350	350	0·13	–	−1·45424
						–	−1·53777
	0·4	12	225	225	0·14	+	1·69291
	0·4	12	225	225	0·14	+	1·83148
						+	1·45837
						–	−0·66943
	0·4	16	100	225	0·17	+	1·32820
						–	−1·53409

Table 1 Continued

pH	TA (%)	°Brix	Potassium sorbate (ppm)	Sodium benzoate (ppm)	Potassium citrate (%)	Growth response	Growth rate log(cfu ml^{-1}) week^{-1}
	0·4	16	350	225	0·14	–	−1·48886
						–	−1·47712
	0·4	16	225	100	0·16	–	−0·30386
						–	−0·30918
	0·4	16	225	350	0·14	–	−1·27203
						–	−1·29553
	0·6	8	225	225	0·26	+	3·08036
						+	3·12494
	0·6	12	225	100	0·27	+	3·70644
						+	3·00586
	0·6	12	225	350	0·26	–	−1·45154
						+	0·78002
	0·6	12	100	225	0·26	+	1·37920
						+	1·24439
	0·6	12	350	225	0·26	–	−1·24568
						–	−1·30103
	0·6	16	225	225	0·26	–	−1·41304
						–	−1·47712
3·8	0·2	12	225	225	0·12	+	2·86981
						+	2·67329
	0·4	8	225	225	0·29	+	3·41307
						+	3·32740
	0·4	12	100	225	0·3	+	1·50000
						+	1·83245
	0·4	12	350	225	0·29	+	1·59751
						+	1·83477
	0·4	12	225	100	0·31	+	2·87231
						+	3·02031
	0·4	12	225	350	0·29	+	1·99138
						+	2·85511
	0·4	16	225	225	0·29	–	−0·75678
						+	1·39499
	0·6	12	225	225	0·5	–	−0·76730
						–	−0·79402

The effect of °Brix was shown to have a significant impact on bacterial growth rate (Fig. 1), but that effect was reduced as the level of potassium sorbate increased (Fig. 2c). At a low potassium sorbate level, a high °Brix prevented bacterial growth. At a high potassium sorbate level, however, increasing the °Brix of a beverage did not dramatically decrease the growth rate.

There was also an interaction between potassium sorbate and sodium benzoate (Fig. 2d). Increasing the sodium benzoate concentration decreased the growth rate in beverages with a high level of potassium sorbate. At low potassium sorbate levels, however, increasing the sodium benzoate concentration did not affect the growth rate. Similarly, at low sodium benzoate levels, increasing the potassium sorbate level did not affect the growth rate.

The backward stepwise regression eliminated eight insignificant terms from the full second-order model. This simplified model consists of 13 terms (intercept, five linear, three quadratic and four interaction terms), as detailed in Table 2. The r^2 for this simplified model was 0·812. A statistical comparison of the both the full second-order and simplified models is detailed elsewhere (Battey 1999).

Utilizing this simplified model, the antimicrobial effectiveness of each variable can be compared. Figure 3 shows the effect of pH and potassium sorbate at constant levels of TA, sodium benzoate and sugar content. To ensure microbially stable beverages, preservative combinations can be selected that have predicted bacterial growth rates less than zero log(cfu ml^{-1}) week^{-1}.

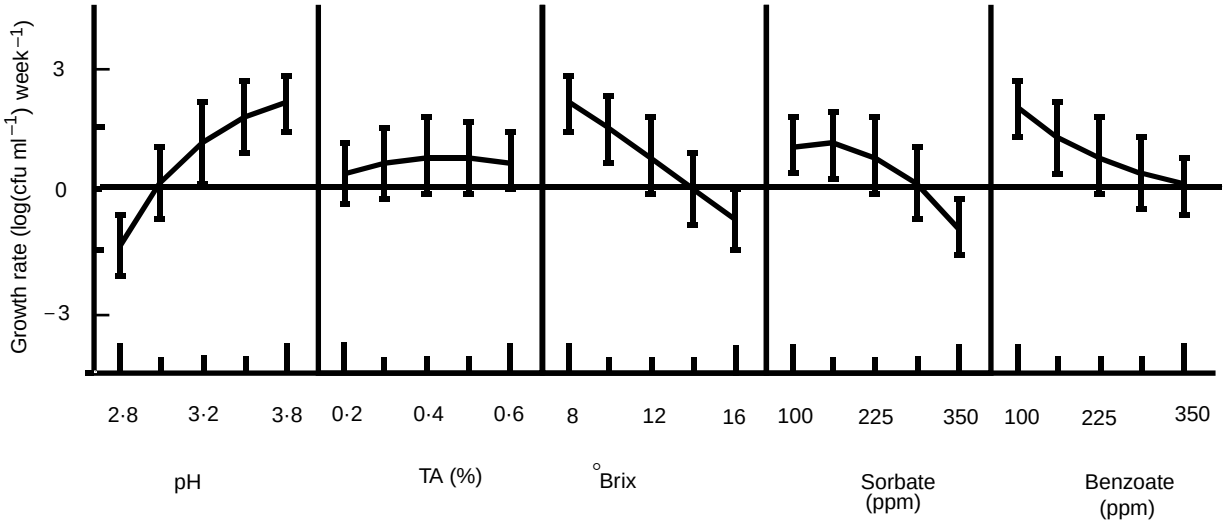

Fig. 1 Linear and quadratic effects predicting the growth of *A. calcoaceticus* and *G. oxydans* in cold-filled ready to drink beverages

The predicted and observed growth rates for the 14 beverages used to validate the simplified model are detailed in Table 3. The model predicted the outcomes successfully in 10 of the 14 beverages.

DISCUSSION

Explanation of parameters and interactions in simplified model

The microbial stability of foods is determined by a combination of several factors (hurdles) which, when acting together, inhibit the growth of micro-organisms. This 'hurdle concept' can be applied to ready to drink beverages when the simplified model (Table 2) is employed. The terms in this model and their degree of statistical significance can be applied to understand the possible mechanisms affecting bacterial spoilage of food products.

The pH of the beverage was found to have the greatest effect on bacterial growth rate. The range of pH values studied in these experiments was highly acidic (2·8–3·8). Most bacteria are not able to survive at such low pH, but some *Gluconobacter* strains can remain viable in a pH as low as 2·4 (Sand 1975). The pH of the beverage also has a significant quadratic effect on the bacterial growth rate ($P = 0{\cdot}0034$).

The TA was not a significant term on its own ($P = 0{\cdot}4289$). It was present in the model because there was a significant interaction between pH and TA ($P < 0{\cdot}0001$). This was not surprising since the growth and survival of micro-organisms can be influenced by both the pH (particularly low pH), and concentration of the acids (Young and Foegeding 1993).

At a higher pH (3·8), the growth rate will decrease as the TA increases (Fig. 2a). This may be due to the chelating properties of citric acid and potassium citrate (Cole *et al.* 1990; Young and Foegeding 1993). Increasing levels of these materials may potentiate the antimicrobial activity of pH by binding the heavy metals required for cell membranes and enzyme activity.

At lower pH (2·8), however, the growth rate increased at higher TA. This was consistent with Praphailong and Fleet (1997), who reported increased yeast growth at low pH in the presence of citrate. The low pH already greatly impairs the proton gradient within the organisms and was not impacted by the slight chelating effect of citrate. At higher pH, the impact of chelation due to the citrate is more appreciable. This reasoning is further supported by the differences in potassium citrate levels required at pH 2·8 vs. pH 3·8 (Table 2).

The °Brix of the beverage also has a significant effect on growth rate. As the sugar content increases, bacteria were less likely to grow. Increasing the °Brix of the beverages from 8 to 16 °Brix causes the A_w to decrease slightly, which may stress the osmoregulation mechanisms in the cell (Witter and Anderson 1987). Gram-negative bacteria (such as *Ac. calcoaceticus* and *G. oxydans*) are generally very susceptible to A_w decreases (Davidson 1997). *Acinetobacter*, for example, requires a minimum A_w of 0·99 for growth but will survive in A_w as low as 0·95 (Sperber 1983). Increased sugar levels also lower the solubility of oxygen, which is needed for the growth of these aerobic bacteria (Lueck 1980).

Potassium sorbate has a significant impact on growth rate ($P < 0{\cdot}0001$). Sorbic acid inhibits catalase-positive bacteria such as *Gluconobacter* and *Acinetobacter* (Sand 1975; Davidson 1997) by inhibiting amino acid uptake and the function of sulfhydryl enzymes (Davidson 1997).

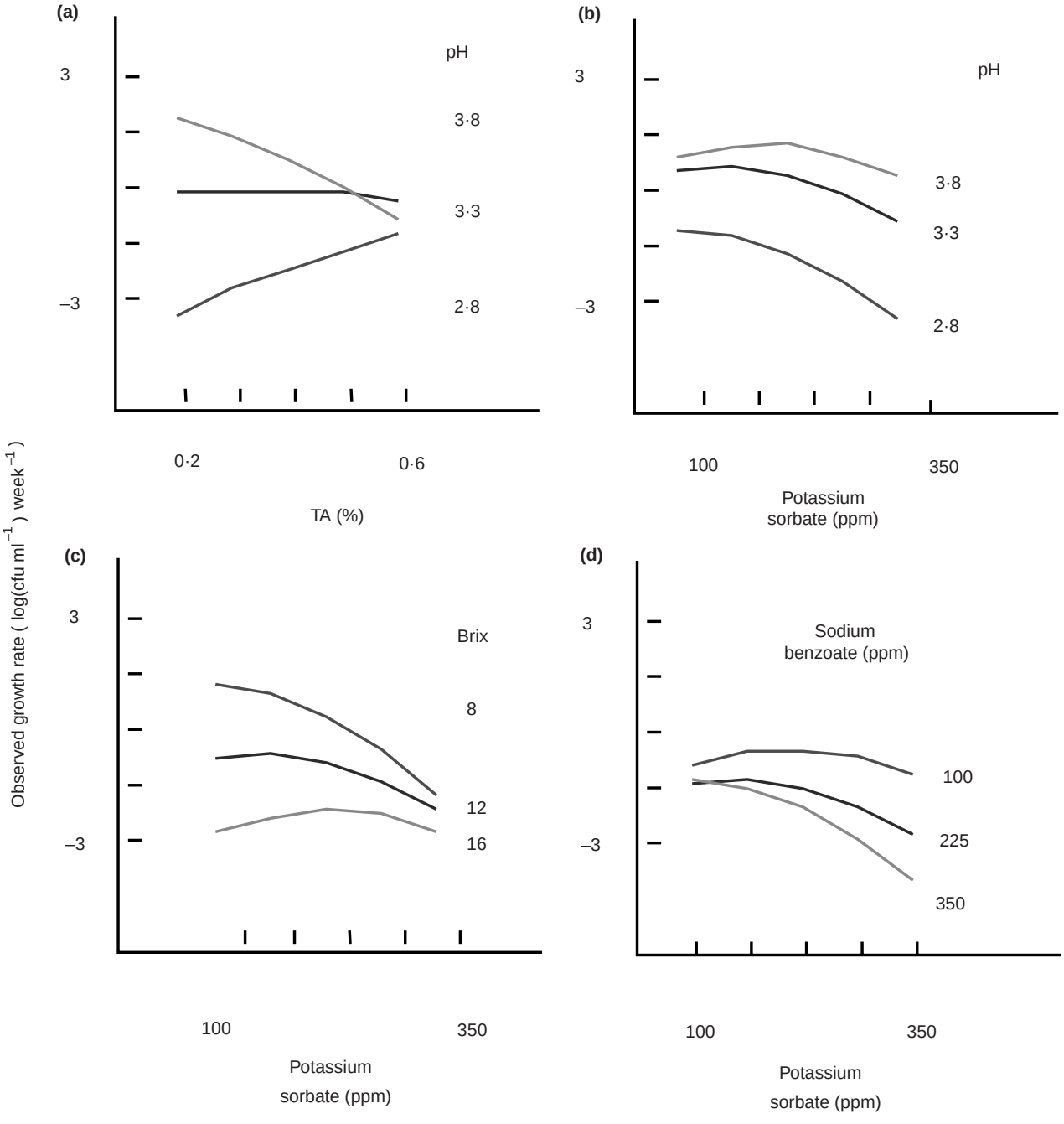

Fig. 2 Interactions between pH and titratable acidity (a), pH and potassium sorbate (b), sugar content and potassium sorbate (c), and sodium benzoate and potassium sorbate (d) at predicting the growth of *A. calcoaceticus* and *G. oxydans* in cold-filled ready to drink beverages

Sodium benzoate also had a significant impact on the bacterial growth rate ($P < 0{\cdot}0001$). Benzoic acid functions by both interrupting the electron transport system (Davidson 1997) and inhibiting enzymes involved in respiration, such as (α-ketoglutarate and succinate dehydrogenase (Bosund 1962).

In addition to the direct effects of each environmental factor on the bacteria, some of the factors acted together to inhibit cell growth. There was a slight interaction between pH and potassium sorbate ($P = 0{\cdot}0842$). The parameter estimate was positive (Table 2), indicating that there was a synergy between pH and potassium sorbate. This

Table 2 Simplified model parameters for the growth of *A. calcoaceticus* and *G. oxydans* cocktail in model cold-filled ready to drink beverages

Term	Estimate*	Prob > |t|
Intercept	−42·43177	0·0002
pH	24·97906	< 0·0001
Titratable acidity (TA)	46·76338	0·4289
°Brix	−0·77329	< 0·0001
Potassium sorbate	−0·03119	< 0·0001
Sodium benzoate	−0·00790	< 0·0001
pH^2	−2·70679	0·0034
pH* TA	−14·36986	< 0·0001
Potassium sorbate* pH	0·00926	0·0842
Potassium sorbate* °Brix	0·00179	0·0086
Potassium $sorbate^2$	−0·00004	0·0094
Sodium benzoate* potassium sorbate	−0·00005	0·0285
Sodium $benzoate^2$	0·00002	0·0877

*r^2 = 0·812.

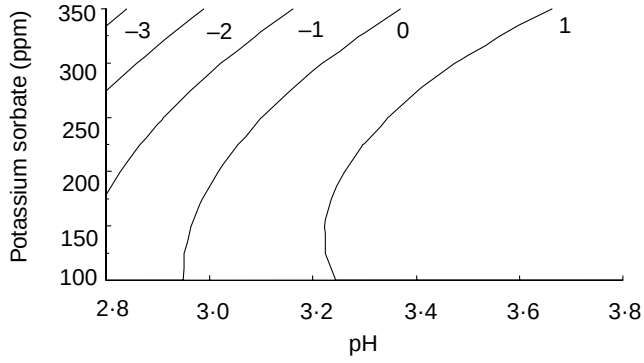

Fig. 3 The effect of pH and potassium sorbate on the bacterial growth rate at TA (0·4%), Sodium benzoate (200 ppm), and °Brix (12). Contour lines represent model predictions (log(cfu ml^{-1}) $week^{-1}$)

combination is easily understandable, as organic acids such as sorbic acid and benzoic acid are more bactericidal in their undissociated forms, which are more prevalent at lower pH

Table 3 Comparison of predicted and observed outcomes to validate the simplified model of the bacterial growth of *A. calcoaceticus* and *G. oxydans* in cold-filled ready to drink beverages

pH	TA (%)	°Brix	Potassium sorbate (ppm)	Sodium benzoate (ppm)	Observed rate (log(cfu ml^{-1}) $week^{-1}$)	Growth observed	Predicted rate (log(cfu ml^{-1}) $week^{-1}$)	Growth predicted
2·8	0·2	8	100	225	2·47049	+	0·33756	+
					2·28129	+	0·33756	+
	0·3	12	350	225	−2·73239	−	−4·29485	−
					−2·81291	−	−4·29485	−
	0·4	12	350	225	−2·65321	−	−3·64207	−
					−2·68124	−	−3·64207	−
2·9	0·2	12	100	225	1·76643	+	−1·28086	−
					0·87240	+	−1·28086	−
	0·6	12	100	225	−0·47900	−	0·75545	+
					−0·47575	−	0·75545	+
3·0	0·3	16	100	350	−2·80618	−	−2·39088	−
					−2·78533	−	−2·39088	−
3·1	0·5	8	350	100	2·84143	+	1·17565	+
					2·82217	+	1·17565	+
3·2	0·4	12	100	100	1·40409	+	1·40591	+
					1·62921	+	1·40591	+
	0·5	8	225	225	2·80543	+	1·99938	+
					2·97956	+	1·99938	+
3·3	0·4	16	350	225	−0·19131	−	−1·25497	−
					−2·77085	−	−1·25497	−
	0·4	16	225	100	−0·13700	−	0·63617	+
					−0·36316	−	0·63617	+
3·4	0·4	8	100	100	3·76740	+	4·24293	+
					3·76972	+	4·24293	+
3·5	0·2	12	100	225	1·88438	+	2·14350	+
					1·92255	+	2·14350	+
	0·6	12	100	225	1·19816	+	0·73105	+
					1·72734	+	0·73105	+

values (Eklund 1983; Chipley 1993; Davidson 1997), and this interaction is consistent with the literature (Gooding *et al*. 1955; Bell *et al*. 1959; Restaino *et al*. 1981). It has also been suggested that this synergy could also be due to cells having an increased susceptibility to organic acids when stressed at low pH conditions (Eklund 1983). Thus, decreasing the pH of the beverage means less potassium sorbate can be used to achieve the same growth rate (Fig. 3). Conversely, increasing preservative levels provides microbial stability at increased pH levels. This is consistent with Eyles and Warth (1989) who reported a dependence of pH on the minimal inhibitory concentrations of sorbic and benzoic acids for *Gluconobacter oxydans*.

There was a significant interaction between potassium sorbate and sodium benzoate in the simplified model ($P < 0{\cdot}0285$). The parameter estimate was negative (Table 2), indicating that there was an antagonism between potassium sorbate and sodium benzoate. Studies on spoilage yeasts have found an antagonistic effect between sodium benzoate and potassium sorbate (Osman and El-Mariah 1960), but this result has not been observed previously for bacteria.

There was a synergistic effect between potassium sorbate and °Brix in the model ($P < 0{\cdot}0094$). Sugars can act synergistically with potassium sorbate to inhibit microbial growth (Gooding *et al*. 1955) and solutes generally increase the inhibitory action of potassium sorbate by increasing the concentration of undissociated sorbic acid (Sofos and Busta 1993).

An interaction between pH and °Brix was expected but was not significant in the model. Synergies between pH and solutes have been reported for bacteria (McClure *et al*. 1993) and spoilage fungi (Cole and Keenan 1986), but did not appear to be significant under the tested conditions. The influences of °Brix and pH were accounted for by their highly significant linear terms in the simplified model (Table 2), and their effects are additive. This is consistent with Cole *et al*. (1990) who reported that there was no synergy between pH and salt on the inhibition of the growth of *Listeria monocytogenes*.

Model validation

Ideally, any model would have no differences between the predictions and observations in the validation samples, since the value of predictive microbiology is based on the premize that the responses of populations of micro-organisms to environmental factors are reproducible (Ross and McMeekin 1994). There was concordance between the observed and predicted bacterial growth responses for most of the samples used to validate the model (Table 3). Even when the outcomes agreed, however, the predicted and observed rates do not match exactly. There were two beverage formulas where the model predicted growth would occur, but no growth was observed (false positives). Another beverage formula predicted no growth but bacterial growth was observed (false negative).

Two of these discrepancies occurred in the samples specifically chosen to understand the relationship between pH and TA. When the pH of the validation samples was 3·5, growth was expected and observed (Table 3). At pH 2·9, however, the interaction between pH and TA was expected to be more critical (Fig. 2a). Higher levels of TA were expected to increase the bacterial growth rate. This was not observed in these validation points. At low TA (0·2%), growth was not expected (growth rate of –1·28 log(cfu ml^{-1}) week^{-1}) but was observed in duplicate (growth rates 1·77 and 0·87 log(cfu ml^{-1}) week^{-1}). At high TA (0·6%), growth was expected (growth rate of 0·76 log(cfu ml^{-1}) week^{-1}) but was not observed in duplicate samples (growth rates –0·48 and –0·48 log(cfu ml^{-1}) week^{-1}). These results indicate a more focused study analysing the interaction between pH and TA is merited.

Implications

The pH of the beverage was found to have the greatest effect on bacterial growth rate, but there are challenges associated with formulating acceptable beverages at very low pH levels (Gomez and Herrero 1983). Buffers such as potassium citrate can be used to provide appropriate flavour characteristics, but the consequences for microbial stability must be taken into consideration.

The sugar content of the beverage can be balanced against the titratable acidity to achieve desired Brix/acid ratios. In fruit flavoured beverages, Brix/acid ratios are chosen to provide the flavour characteristics for a particular fruit. The effect of both titratable acidity and °Brix of a beverage on microbial behaviour must be considered when formulating ready to drink beverages.

A clear benefit of using either higher proportions of potassium sorbate or sodium benzoate to prevent the growth of *Ac. calcoaceticus* and *G. oxydans* was not observed. The levels of each preservative can be adjusted to balance antimicrobial effectiveness against organoleptic characteristics. Benzoic acid imparts a burning after-taste while sorbic acid tends to be neutral (Dryden and Hills 1959). The costs of potassium sorbate, however, tend to be higher than sodium benzoate. Furthermore, the chemical structure of potassium sorbate is more prone to oxidation and degradation (Sofos 1989).

This model can help to quantify the effects of several formulation factors (Whiting 1995). This allows product developers to select a 'preservative system' (Kabara 1981) that provides a hostile environment to spoilage organisms while considering the ingredient costs, quality, and flavour implications.

Microbial models greatly reduce the need for ad hoc microbiological examination and allow for predictions concerning quality and safety to be made swiftly and with considerable financial benefit (Ross and McMeekin 1994). Increased productivity should result by reducing the need for the time-consuming microbiological testing procedures currently practiced. Predictive microbial models provide better understanding of the effects of each variable, and therefore function as a product development tool. Models, however, do not replace microbial testing or the judgement of a trained and experienced microbiologist.

CONCLUSION

A mathematical model predicting the growth of *Acinetobacter calcoaceticus* and *Gluconobacter oxydans* 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, titratable acidity, sugar content (°Brix), sodium benzoate and potassium sorbate. Product developers can utilize this model to predict microbial stability while considering the ingredient costs, quality and flavour implications of various preservative systems.

ACKNOWLEDGEMENTS

The authors would like to thank Ms. Kristin Jackson and Ms. Siobain Duffy for editorial assistance in preparation of the final version of this manuscript.

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