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Antimicrobial Activity of Aroma Compounds against *Saccharomyces cerevisiae* and Improvement of Microbiological Stability of Soft Drinks as Assessed by Logistic Regression▽

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The combined effects of a mild heat treatment (55°C) and the presence of three aroma compounds [citron essential oil, citral, and (*E*)-2-hexenal] on the spoilage of noncarbonated beverages inoculated with different amounts of a *Saccharomyces cerevisiae* strain were evaluated. The results, expressed as growth/no growth, were elaborated using a logistic regression in order to assess the probability of beverage spoilage as a function of thermal treatment length, concentration of flavoring agents, and yeast inoculum. The logit models obtained for the three substances were extremely precise. The thermal treatment alone, even if prolonged for 20 min, was not able to prevent yeast growth. However, the presence of increasing concentrations of aroma compounds improved the stability of the products. The inhibiting effect of the compounds was enhanced by a prolonged thermal treatment. In fact, it influenced the vapor pressure of the molecules, which can easily interact within microbial membranes when they are in gaseous form. (*E*)-2-Hexenal showed a threshold level, related to initial inoculum and thermal treatment length, over which yeast growth was rapidly inhibited. Concentrations over 100 ppm of citral and thermal treatment longer than 16 min allowed a 90% probability of stability for bottles inoculated with 10^5 CFU/bottle. Citron gave the most interesting responses: beverages with 500 ppm of essential oil needed only 3 min of treatment to prevent yeast growth. In this framework, the logistic regression proved to be an important tool to study alternative hurdle strategies for the stabilization of noncarbonated beverages.

The chemicophysical and composite characteristics of soft drinks make these products susceptible to microbial spoilage. They are usually characterized by high C/N ratios and low pHs (<3.5), which allow the growth of specific microbial groups, such as acetic and lactic acid bacteria, molds, and yeasts (5). The addition of CO_2 in beverages further reduces growth possibilities, mainly favoring yeasts (35). The stability of these beverages often depends on thermal treatments to which ingredients and intermediate and final products can be subjected. However, products packaged in polyethylene terephthalate (PET) bottles are often not thermally treated because of the susceptibility of plastic material to heat. For this reason, their stability relies upon the addition of preservatives, generally belonging to the weak acid group, such as sorbic and benzoic acids. The effectiveness of these antimicrobials depends on several factors, among which the most important are pH, microbial cell concentration, and the intrinsic resistance to weak acids of the microorganisms present after bottling (45, 47).

The pressure from consumers for minimally processed products free from traditional preservatives has induced manufacturers to find new strategies for the stabilization of food (2, 7, 22). In fact, consumers are inclined to consider these preservatives as extraneous and unsafe because they have no connection with the food matrix. This framework has recently been complicated by the information that in beverages a chemical reaction can induce the transformation of part of benzoic acid in benzene, as reported by the FDA (36a) and some European agencies, such as the United Kingdom Food Standards Agency (46a) and the Federal Institute for Risk Assessment in Germany (15a). Even though this reaction has been known about for many years (19), many beverage companies did not replace benzoic acid with sorbic acid due to the higher costs of sorbic acid, its inclination to oxidation and degradation, and also its reduced yeast inhibition compared with that by benzoic acid (5).

In this scenario, the search for new strategies and new antimicrobials for stabilization of beverages (and other products) has become a central goal for producers. Aroma compounds and essential oils can be an interesting alternative. Their antimicrobial potential is well known (6, 9, 10, 13, 15, 21), and a key, even if indirect, role of an orange essential oil on the stability of an orangeade has been described by Ndagijimana et al. (38). The principal limitations to an industrial use of these substances as preservatives are their organoleptic impact and the variable composition of the essential oils (which can be reflected in their antimicrobial activity) (9, 28).

Up to now, the mechanisms of action and the interactions between aroma compounds have not been completely under-

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stood (9, 43). Even though the antimicrobial activity of single constituents of essential oils has been extensively studied (9, 23) (mainly in model systems under laboratory conditions), the literature on their use in soft drinks is scarce. In particular, Fitzgerald et al. (16) demonstrated that vanillin has the potential to preserve some fruit juices and soft drinks. The combination of essential oils with other stabilizing mild treatments (i.e., thermal treatment) can reduce the impact of the latter on the final quality and costs of the products (1). In fact, the resulting antimicrobial effect is not always the sum of the single effects, as antagonistic and synergic interactions may be present.

In this perspective, predictive microbiology is a useful tool to determine shelf life and stability of food products treated with combined stabilizing techniques. As observed by Battey et al. (5), predictive microbiology has been mainly focused on the creation of pathogen models. In literature, spoilage models are less prevalent (39, 48) but include models for yeasts (5, 26, 34, 40), molds (4, 20, 31, 37), and bacteria (8, 32, 41, 46). Logistic regression models are gaining importance in predictive food microbiology (52). In fact, in many cases the observations to be modeled are not continuous but express the probability of an event (for example, growth/no growth and toxin produced/toxin not produced). Based on empirical data, logistic regression calculates the probability of a binary outcome as a linear function of a combination of predictor variables (24). The application of logistic models can be found in food microbiology papers concerning bacterial (25, 27, 29, 42) and fungal (12, 33, 34) growth. Recently this approach has been used to estimate the stability against fungal spoilage of cold-filled ready-to-drink beverages (4, 5).

The aim of this work was to assess the growth probability of a *Saccharomyces cerevisiae* strain in beverages in relation to the length of a mild thermal treatment (55°C), yeast inoculum, and the presence of flavoring agents. The *S. cerevisiae* strain SPA used in this work was isolated from spoiled orangeade, in which it grew independently on the maximum benzoic acid concentration allowed by Italian legislation (160 mg/liter) and on its initial inoculum (38). The aroma compounds tested were two specific molecules, namely, (*E*)-2-hexenal and citral, and a citrus essential oil obtained from citron (*Citrus medica*). (*E*)-2-Hexenal is one of the metabolites of the lipoxygenase pathway. These metabolites act as substrates metabolizable to compounds having important roles in plant defense with a protective action against microbial proliferation in wounded areas (11). Many of the natural aromas of fruits and vegetables (18) responsible for their "green notes" are six-carbon aliphatic compounds (aldehydes or alcohols) formed through this pathway. Among these compounds, (*E*)-2-hexenal showed strong antimicrobial properties at low concentrations (13, 17). Citral is an acyclic α,β-unsaturated monoterpene aldehyde naturally occurring in many essential oils of citrus fruits or other herbs or spices (51). The name citral indicates a mixture of two isomers, geranial and neral, known for their remarkable antimicrobial activity. This property is due to its carbonyl group adjacent to α- and β-carbons, which makes it very reactive and available for nucleophilic attack (3) and acts as a direct alkylating agent (49) capable of modifying cellular processes (51). The citron essential oil used in this work was an industrial product used for flavoring citrus-based beverages at concentrations usually up to 800 ppm. Its relevant antimicrobial effect has been evaluated in in vitro trials (6) and has also been tested in fruit-based salads (N. Belletti, R. Lanciotti, F. Patrignani, and F. Gardini, submitted for publication).

The growth/no growth data observed in beverages after 60 days of storage were analyzed with logistic regression, and through the logit transformation the probability of growth of the yeast and, consequently, its ability to spoil the beverages were obtained.

MATERIALS AND METHODS

Strain. *S. cerevisiae* SPA, the strain used in this work, belongs to the strain collection of the Department of Food Science of the University of Bologna (38). The culture was maintained on slants of Sabouraud dextrose agar (SDA) (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom). The suspension was prepared by inoculating a loop of the culture in Sabouraud dextrose broth and incubating it for 2 days at 30°C. After quantification of the culture with a Bürker chamber, serial dilutions were performed in order to obtain the different concentrations required by the experimental plan. However, the data reported in Results are the outcome of a plate count on SDA incubated at 28°C for 72 h.

Experimental design. An experimental design with three variables (inoculum level, aroma concentration, and length of thermal treatment at 55°C) at five levels was used. The variables and levels used in this work are reported in Table 1.

Preparation of beverages. The beverages were prepared by aseptically diluting industrial concentrates used for soft drink preparation (Flavourint, Madone, Italy). An apple-based concentrate was used for testing the antimicrobial activity of (*E*)-2-hexenal (Sigma, St. Louis, MO) while a citrus-based concentrate was used for testing the inhibitory activity of citral (Sigma). Citron essential oil (Flavourint) was added to a nonflavored concentrate. Concentrates were mixed thoroughly with sterilized mineral water (San Pellegrino, Milan, Italy) (dilution factor, 1:6; final, 8.5 degrees Brix), and 500-ml PET bottles (San Pellegrino) were filled with the beverages. PET bottles were previously sanitized with a diluted hydrogen peroxide (3%) solution (Carlo Erba Reagents, Milan, Italy). Ten repetitions of each condition of the experimental plan were prepared. Before closing, the filled bottles were preheated at 55°C in a water bath for about 15 min, and then the aroma compound dissolved in ethanol (Merck, Rome, Italy) was added. Independently of the amount of flavoring agent added, the final concentration of ethanol was 0.5% (vol/vol) also in the samples not supplemented with flavoring agents. Afterward, the bottles were inoculated with different concentrations of yeast according to the experimental plan. Finally, they were immediately closed with screw caps and treated at 55°C in a water bath for the time defined in Table 1. After the heat treatment, samples were rapidly cooled in a water-ice bath. The bottles were stored at room temperature (25°C) and observed twice a week over a 60-day period for the presence of cloudiness, cell sediment on the bottom, and/or an evident swelling of the bottles due to yeast growth. After 60 days, where spoilage was not observed plate count analysis were performed by plating 0.1 ml of appropriate dilutions on SDA and incubating the dilutions at 28°C for 5 days to confirm the result.

Model development. Observations were transformed into positive and negative growth responses over time. The value 0 was assigned to the bottles in which yeast growth was not observed while 1 meant that growth occurred. A logistic regression analysis was conducted on the raw data using Statistica 6.1 (StatSoft Italy srl, Vigonza, Italy) in order to assess the probability of growth during the storage period as a function of aroma compound concentration, length of thermal treatment, and inoculum level. The significance of the selected variables was evaluated firstly by the relation between each variable alone and the probability of no growth examined by a likelihood ratio test; the reduction in deviance ($-2 \times$ log likelihood) when entering the variable into a model with no other variables was tested against a χ^2 value (24). In addition, the significance of each variable was tested by removing it from a complete model with all variables included. Interactive and quadratic effects of significant variables were tested in the same way. The evaluation of the goodness of fit of the model was also performed by assessing the percentage of correct predictions compared to the observed experimental results.

Sensorial analysis. A panel test concerning the aroma profile of the three different beverages was performed by a group of 12 trained panelists. Beverages supplemented with different amounts of flavoring agents were judged, and a score ranging from 1 to 5 (1, unperceivable; 2, pleasant; 3, perceivable; 4, excessive; 5, not acceptable) was assigned to each sample. The beverage flavors were considered acceptable when at least 75% of panelists assigned a score of 3 or lower.

TABLE 1. Experimental designs adopted for the evaluation of stability of the three types of beverages[a]

Run no.	Exptl design					% of spoiled bottles[b]		
	Aroma compound (ppm)			Thermal treatment (min)	Inoculum log CFU/bottle			
	(*E*)-2-Hexenal	Citral	Citron essential oil			(*E*)-2-Hexenal	Citral	Citron essential oil
1	0	0	0	0	1	100	100	100
2	0	0	0	20	3	100	100	100
3	0	0	0	20	5	100	100	100
4	0	0	0	10	3	100	100	100
5	10	30	125	5	2	100	100	20
6	10	30	125	15	2	100	100	0
7	10	30	125	5	4	100	100	80
8	10	30	125	15	4	100	100	0
9	20	60	250	10	3	100	100	0
10	20	60	250	10	3	100	80	0
11	20	60	250	10	3	100	100	0
12	20	60	250	0	3	100	100	100
13	20	60	250	20	3	80	20	0
14	20	60	250	10	1	80	0	0
15	20	60	250	10	5	100	100	0
16	30	90	375	5	2	0	100	0
17	40	90	375	15	2	0	0	0
18	40	90	375	5	4	40	100	0
19	40	90	375	15	4	0	80	0
20	50	120	500	10	3	0	20	0

[a] While the length of thermal treatment and the inoculum were the same in each run of the design independently of the beverage, the concentration of aroma compounds varied according to their antimicrobial activity and organoleptic impact.

[b] Results (expressed as percentages of spoiled bottles) observed in each run of the experimental design in relation to the type of beverage, i.e., to the aroma compound added.

RESULTS

Three types of noncarbonated beverages were prepared by using industrial concentrates. The apple-based beverage was chosen to study the effect of (*E*)-2-hexenal on *S. cerevisiae*. In fact, the six-carbon aliphatic aldehydes are typical components of apple aroma (14). The citrus-based beverage was used to support the addition of citral, a component of many citrus fruit flavors. This last beverage was also flavored with an essence (deterpenated alcoholic extract of red orange) which allowed the growth of *S. cerevisiae* SPA (38). In contrast, the citron essential oil was added to beverages not previously flavored. The ranges of (*E*)-2-hexenal and citral were chosen by considering their antimicrobial activity and their potential impact on organoleptic properties (28; Belletti et al., submitted). The amounts of citron essential oil were compatible with its industrial dosage.

After bottling, the samples were monitored for 60 days and the results were expressed as frequency of stable (not spoiled) bottles after this storage time and are reported in Table 1.

The logit model was used to find relationships among the considered variables and the probability of having stable beverages. In each model, the significance of the linear terms of the variables was first tested. Then, the effects on the models of the insertion of interactions and quadratic terms were assessed, as described in Materials and Methods.

(*E*)-2-Hexenal. In the logit model describing the microbial spoilage of the beverages supplemented with (*E*)-2-hexenal, only the linear terms of the variables were included (Table 2). In fact, the addition of any interaction and quadratic term did not significantly improve the statistics used for evaluating the goodness of fit.

This model allowed the correct classification of 192 observations out of 200 (96.0% of correct classifications), and only four spoiled samples were predicted as stable (97.3%) while four nonfermented samples were predicted as spoiled (92.0%).

Figure 1 reports the predicted probability to have yeast growth in relation to the (*E*)-2-hexenal concentration in bottles inoculated with 10^1, 10^3, and 10^5 CFU/bottle of *S. cerevisiae* SPA. The predicted values are referred to beverages not thermally treated (Fig. 1a) or treated at 55°C for 10 (Fig. 1b) or 20 (Fig. 1c) min.

In addition, the comparison among the probability plots in relation to the thermal treatment showed that the decrease of 10 min in the length of the treatment was counteracted by an

TABLE 2. Final logistic models obtained to predict the spoilage of the beverages supplemented with the three aroma compounds

Aroma compound	Logit equation	Relative χ^2 ($P < 0.0001$)	Deviance ($-2 \times$ log likelihood)
(*E*)-2-Hexenal	57.257 − 2.500[(*E*)-2-hexenal] − 1.162[time] + 5.809[inoculum]	191.46	33.55
Citral	−5.411 + 0.053[citral] + 0.321[time] + 4.094[inoculum] − 0.0147[citral][time]	146.70	53.41
Citron essential oil	1.151 − 0.010[citron] − 0.038[time] + 1.602[inoculum] − 0.0065[citron][time]	221.53	22.82

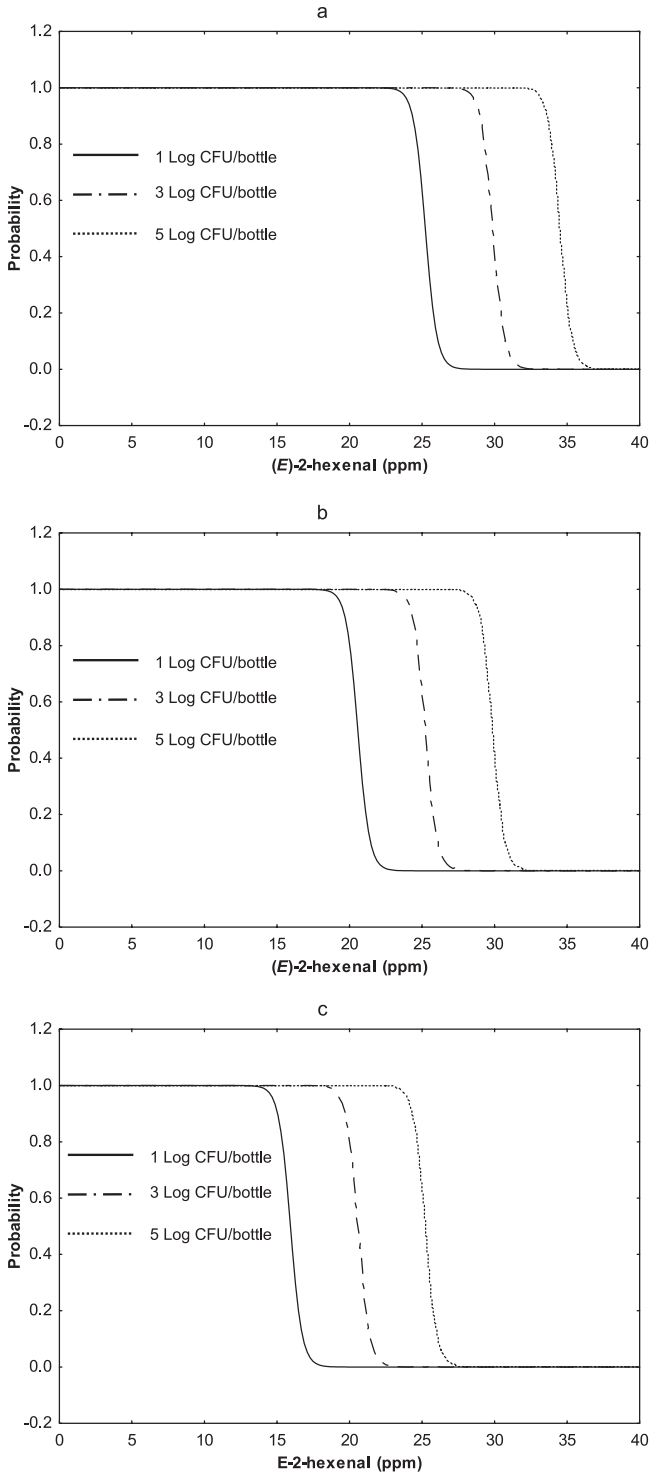

FIG. 1. Predicted spoilage probability of beverage supplemented with (*E*)-2-hexenal inoculated with *S. cerevisiae* SPA. The curves are relative to the probability in the absence of thermal treatment (a) or when a treatment of 10 min (b) or 20 min (c) was applied. For each thermal treatment, the predicted probability for an initial inoculum of 1, 3, or 5 log CFU/bottle is reported.

increase of (*E*)-2-hexenal concentration of about 5 ppm. In fact, the microbial stability of beverages was obtained at about 25 ppm of (*E*)-2-hexenal in the samples inoculated with 10^5 CFU/bottle and thermally treated for 20 min, while 35 ppm of the aldehyde was necessary to stabilize the beverages not thermally treated.

Citral. The elaboration of the data reported in Table 1 with the logit model included also the interaction (Table 2) between citral concentration and duration of thermal treatment. In fact, the inclusion of this term determined a significant improvement of the value of χ^2 and $-2 \times$ log likelihood. In contrast, no improvement was found with the inclusion of the other interactions, as well as quadratic terms.

As in the case of (*E*)-2-hexenal, 8 observations out of 200 were misclassified by the model (96.0% of correct assignments), and in particular four spoiled samples were classified as stable (97.5%) and four nonfermented bottles were classified as spoiled (90.0%) (Table 3).

Figure 2 shows the surface indicating the combination of the independent variables allowing yeast growth at $P = 0.1$, i.e., the conditions that predict 90% of nonspoiled samples ($1 - P = 0.9$). It has been obtained by posing logit (P) = ln (P/1 − P) = ln (0.1/0.9) = −2.197. With this premise, the equation of Table 2 can be rewritten as follows, with logit (P) = −2.197: [inoculum] = (3.213 − 0.053[citral] − 0.321[time] + 0.0147[citral][time])/4.094. The equation can be represented as in Fig. 2.

It is clear that the microbial growth could not be inhibited by either thermal treatment or citral concentration considered alone. When the length of thermal treatment was 20 min and the initial yeast inoculum was 10^1 CFU/bottle, the presence of about 30 ppm of citral was needed to reach $P = 0.1$, while the same probability level was obtained in bottles with 120 ppm of citral and treatment at 55°C for about 5 min.

The effect of the interaction between thermal treatment and citral is evidenced in Fig. 2. In fact, interaction means that the effect of one factor changed nonlinearly depending on the level of the other factor. Consequently, to guarantee the same P level (0.1), there was a nonlinear proportionality between citral concentration increase and diminution of the length of the thermal treatment. This increase was remarkably higher in proportion to the shorter thermal treatment. Also, an important effect of initial inoculum is evidenced by Fig. 2, which shows the increase of citral concentration and treatment length necessary for maintaining the same level of probability. At the highest inoculum (10^5 CFU/bottle), only concentrations of citral between 100 and 120 ppm and thermal treatments between 16 and 20 min could allow the attainment of $P = 0.1$.

Citron essential oil. In addition to the linear terms of the variables, the insertion in the model of the interaction between time of treatment and essential oil concentration improved it significantly. In fact, the χ^2 value in the presence of this interaction was 221.53 (compared with 169.03 when the interaction was excluded) and the $-2 \times$ log likelihood was 22.82 (75.31). This was reflected in the number of misclassified observations. In fact, 20 observations were erroneously classified by the model without interaction. The misclassified observations were only four with the model which included the interaction. This model permitted a correct classification of 98% of the observations (Table 3).

Figure 3a shows, on the basis of the final model obtained,

TABLE 3. Comparison between observed and predicted (on the basis of the model) spoilage of the bottles of the three types of beverages

Observed condition	Value for aroma agent (% overall correct prediction)								
	(*E*)-2-Hexenal (96.0)			Citral (96.0)			Citron oil (98.0)		
	No. predicted		Correct %	No. predicted		Correct %	No. predicted		Correct %
	Spoiled	Not spoiled		Spoiled	Not spoiled		Spoiled	Not spoiled	
Spoiled	146	4	97.3	156	4	97.5	58	2	96.7
Not spoiled	4	46	92.0	4	36	90.0	2	138	98.6

the effects on the value of *P* of the time of thermal treatment and the citron essential oil concentration when the initial inoculum was kept constant at 10^3 CFU/bottle. As it is possible to observe, in the absence of thermal treatment, an increase of citron concentration could not guarantee a satisfactory stability even if added at 500 ppm ($P > 0.4$). Analogously, the absence of citron made the thermal treatment completely ineffective, even if it was prolonged for 20 min. However, the increase of citron concentration strongly increased the efficacy of the thermal treatment and the presence of 100 ppm of essential oil was sufficient to ensure the absence of yeast growth in all the beverages treated for more than 10 min. Shorter treatments required higher citron concentrations, but in the beverages with 500 ppm of essential oil a treatment of 3 min was sufficient for inhibiting yeast growth.

Figure 3b represents the combined effects of the thermal treatment length and initial inoculum with the presence of 250 ppm of citron essential oil. Under these conditions, a treatment of 8 min was needed to avoid spoilage in the presence of the highest yeast concentration tested. This time was halved (about 4 min) when the initial cell concentration was 10^1 CFU/bottle.

Analogously, when the equation was drawn keeping constant the time of treatment (10 min), the presence of about 200 ppm of citron essential oil was necessary for the beverage stability at the highest inoculum while only 100 ppm inhibited yeast growth at the lowest initial inoculum (Fig. 3c).

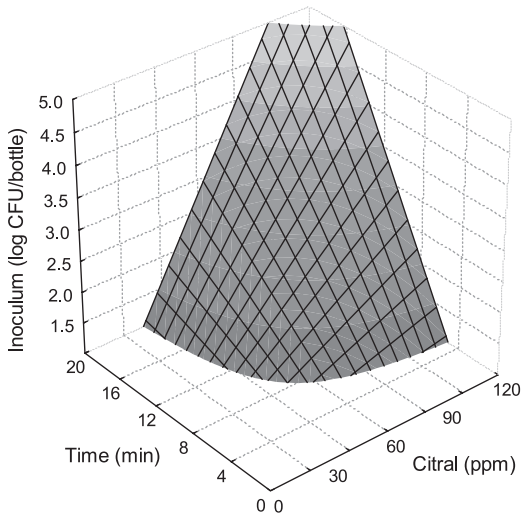

FIG. 2. Surface representing the predicted conditions which guarantee the stability of 90% of bottles ($P = 0.9$) in relation to the amount of citral added, the initial inoculum, and the thermal treatment applied.

DISCUSSION

The logit models obtained for all the substances considered were extremely precise (few samples were not correctly classified) and indicated that the presence of flavoring agents can inhibit yeast growth especially in relation to the length of thermal treatment. This is a promising alternative approach to beverage stabilization also in relation to the usual yeast contamination of industrial products immediately after bottling. In fact, under good manufacturing practices, such contamination should be lower than 1 cell/ml, i.e., below the value of 10^3 CFU/bottle, which was the central inoculum value considered in the experimental design. In this light, the stability of beverages inoculated with the higher yeast concentrations, used to comprise anomalous industrial contamination also, is of particular interest.

In any case, a stabilization strategy based only on the use of these substances can be difficult to achieve without the addition of excessive concentrations of the flavoring agent, not compatible with an acceptable flavor profile. (*E*)-2-Hexenal and citral were added to apple-vanilla- and citrus-based beverages, respectively, so that they could be compatible with the original flavor. Nevertheless, a panel (12 panelists trained in beverages) judged the aroma profiles of beverages supplemented with concentrations of (*E*)-2-hexenal and citral higher than 25 and 50 ppm, respectively (data not shown), to be unbalanced. In both cases, these concentrations are higher than those allowing a satisfactory inhibition of yeast growth in combination with a short mild thermal treatment. For this reason a hurdle strategy is more interesting and applicable to industrial products (30). In fact, the contextual application of a mild thermal treatment, not per se sufficient to prevent yeast proliferation even in the presence of the lower inocula, strongly enhanced the antimicrobial activity of the flavoring substances. This important aspect, reinforced by the presence of the interaction in the model obtained for citral and citron essential oil, could be due to the increase of vapor pressure determined by the treatment at 55°C. It is known, in fact, that the antimicrobial action of such molecules depends, in the first instance, on their presence in gaseous form (allowing their solubilization in cell membranes) which is, in turn, dependent on their vapor pressure (28). However, the mild thermal treatment applied, although unable to prevent yeast growth, probably caused damage to part of the cells, increasing their susceptibility to the antimicrobial activity of the flavor substances. Moreover, such a mild thermal treatment does not compromise the plastic bottles, reduces the thermal damage

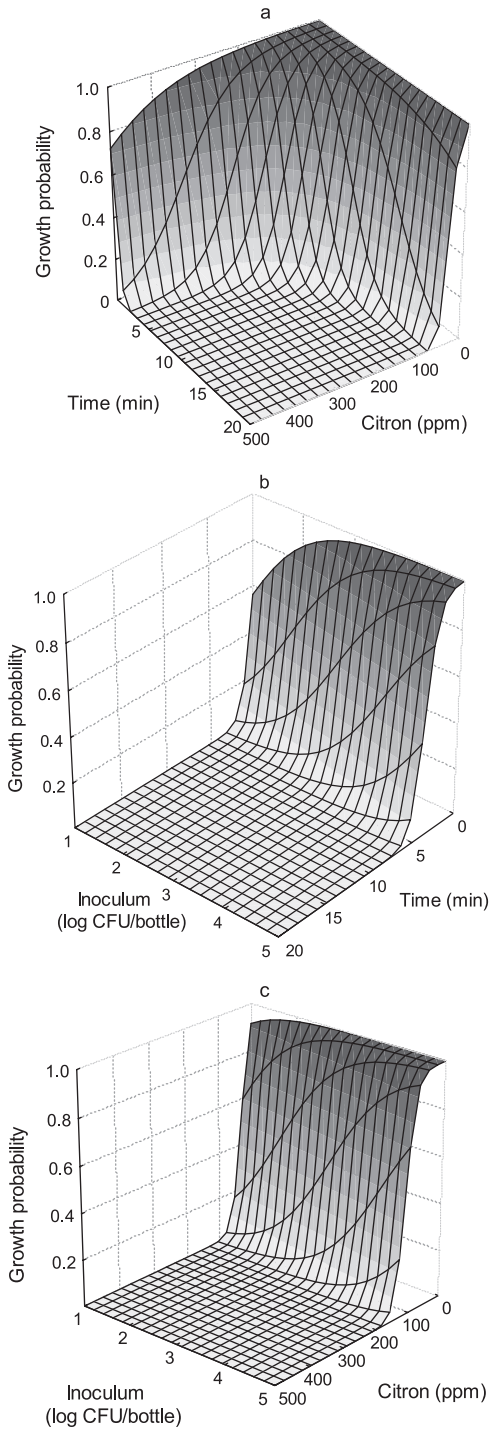

FIG. 3. Surfaces representing the predicted spoilage of beverages supplemented with citron essential oil. (a) Effects of duration of thermal treatment and citron essential oil concentration when the initial inoculum was kept constant at 3 log CFU/bottle; (b) effects of duration of thermal treatment and initial yeast inoculum when the citron essential oil concentration was kept constant at 250 ppm; (c) effects of initial yeast inoculum and citron essential oil concentration when thermal treatment was kept constant at 10 min.

to beverage components, and could represent an important reduction of the energy costs.

The model obtained for (*E*)-2-hexenal does not include the interaction between time of treatment and molecule concentration. All the curves were characterized by a sharp passage from the (*E*)-2-hexenal concentration allowing spoilage ($P = 1$) or stability ($P = 0$). This shape of the curves suggests that there was a threshold level of (*E*)-2-hexenal below which spoilage always occurred, while above the threshold yeast growth was completely inhibited. In other words, the range of values of aldehyde concentrations allowing *P* values intermediate between those of growth and those of no growth was very narrow. This phenomenon has been described by McMeekin et al. (36) as "cliff edge." Moreover, Fig. 1, independently of the intensity of thermal treatment, indicates that after the threshold value, an increase of about 2.5 ppm of (*E*)-2-hexenal could counteract an increase of 1 log unit of the initial yeast contamination.

Notably, the use of citron essential oil gave the most interesting responses in terms of microbial stability. The inhibition of yeast growth in the presence of this essential oil has been already observed in vitro and attributed to the high percentage of citral (7.1%) found in the essential oil composition. In fact, citral is considered to be among the most interesting molecules with respect to the antimicrobial activity (44, 50, 51). However, other compounds with potential antimicrobial properties were present in the essential oil, such as β-pinene (20.1%), limonene (41.1%), γ-terpinene (8.3%), *p*-cymene (5.9%), α-pinene (3.4%), and linalool (1.7%) (6). The inhibition of yeast growth in beverages was achieved at a concentration of essential oil containing amounts of citral which, if used alone, were not sufficient to allow yeast inhibition (500 ppm of citron essential oil contains about 35 ppm of citral). This was due not only to the presence of other molecules active against microorganisms but also to their synergistic action. Some studies have concluded that whole essential oils have a greater antibacterial activity than do the major components mixed, which suggests that the minor components could be critical to the bioactivity and may have a synergistic effect or a potentiating influence (9).

In conclusion, logistic regression can represent a suitable tool to set up at the industrial level preservative strategies aimed at reducing the use of traditional antimicrobials such as sorbic and benzoic acids and the severity of thermal treatments. In fact, it can allow the choice of optimal flavor concentrations able to inhibit yeast growth without detrimental effects on aroma profiles and the overall organoleptic properties of beverages. The use of mild thermal treatments enhances the antimicrobial activity of flavor agents and reduces the product damage caused by heat and the process costs. However, a more effective application of this strategy for microbial stabilization of foods needs a deeper comprehension of how these agents act and affect microbial metabolisms, also in relation to other factors such as temperature, the variability in resistance to aroma compounds within cells of the same population, and the role in this resistance of their physiological state.

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