ORIGINAL ARTICLE

Combined effect of crude herbal extracts, pH and sucrose on the survival of *Candida parapsilosis* and *Zygosaccharomyces fermentati* in orange juice

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Abstract

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The purpose of this study was to evaluate the antimicrobial activity of crude extracts of cinnamon and clove compared with potassium sorbate against food spoilage yeasts isolated from orange juice (*Candida parapsilosis* and *Zygosaccharomyces fermentati*). The ethanolic extract of both cinnamon and clove showed activity against *C. parapsilosis* and *Z. fermentati* with minimum inhibitory concentrations (MIC) of 1.0 and 0.5 mg/ml by agar dilution method, respectively. While potassium sorbate showed activity against both yeasts with MIC 0.4 mg/ml. The combined effect of pH, concentration of sucrose and cinnamon extract or clove extract or potassium sorbate in orange juice on the survival of the isolated yeasts were studied using Response Surface Methodology. The pH of orange juice and the concentration of cinnamon or clove extract had the most significant ($P<0.05$) effect on the survival of *C. parapsilosis* and *Z. fermentati* while concentration of sucrose had the least effect. Survival of *C. parapsilosis* and *Z. fermentati* was mostly affected by the concentration of potassium sorbate but pH and the concentration of sucrose did not have significant effect on the survival of *Z. fermentati*.

Key words : orange juice, sucrose, herbal exacts, cinnamon, clove, potassium sorbate

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บทคัดย่อ

ดาวริน สุขเกษม ทิพรัตน์ หงภัทรดีรี และ อรัญ หันพงศ์กิตติกูล

ผลร่วมระหว่างสารสกัดพืชสมุนไพร พีเอชและความเข้มข้นของน้ำตาลซูโครสต่อการเจริญของ *Candida parapsilosis* และ *Zygosaccharomyces fermentati* ในน้ำส้มคั้น

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การศึกษาประสิทธิภาพของสารสกัดอบเชยและกานพลูที่สกัดด้วยเอทานอล 95% เปรียบเทียบกับโพแตสเซียมซอร์เบทต่อการยับยั้งยีสต์ *Candida parapsilosis* และ *Zygosaccharomyces fermentati* ที่แยกได้จากน้ำส้มคั้น (ชนิดน้ำส้มเกล็ดหิมะ) พบว่า ค่าความเข้มข้นต่ำสุด (MIC) ของสารสกัดอบเชย กานพลู และโพแตสเซียมซอร์เบทต่อการยับยั้งยีสต์ทั้ง 2 ชนิด โดยใช้วิธี agar dilution เป็น 1.0 0.5 และ 0.4 มก/มล ตามลำดับ เมื่อศึกษาผลร่วมของพีเอช ความเข้มข้นของซูโครส และสารสกัดอบเชย หรือกานพลู หรือโพแตสเซียมซอร์เบท ในน้ำส้มคั้นต่อการยับยั้ง *C. parapsilosis* และ *Z. fermentati* โดยใช้วิธี Response Surface Methodology พบว่า พีเอชและความเข้มข้นของสารสกัดอบเชยหรือกานพลูในน้ำส้มคั้นมีผลต่อการยับยั้ง *C. parapsilosis* และ *Z. fermentati* อย่างมีนัยสำคัญ ($P<0.05$) ในขณะที่ความเข้มข้นซูโครสมีผลต่อการยับยั้งน้อยมาก ความเข้มข้นของโพแตสเซียมซอร์เบทในน้ำส้มคั้น มีผลต่อการยับยั้ง *C. parapsilosis*, *Z. fermentati* อย่างมีนัยสำคัญ ($P<0.05$) แต่พีเอชและความเข้มข้นของซูโครสมีผล ต่อการยับยั้ง *Z. fermentati* น้อย

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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 are used to prevent the growth of most microorganisms in ready-to-drink beverages. Spoilage yeasts such as *Saccharomyces cerevisiae, Candida lipolytica* and *Zygosaccharomyces bailii*, occasionally overcome these hurdles. These microorganisms tolerate acidic environments and are resistant to chemical preservatives, such as potassium sorbate and sodium benzoate (Battey *et al*., 2002). In recent years, because of negative perception of synthetic additives, consumers are demanding natural and fresh-life food products with guaranteed safety and long shelf-life. Therefore, the interest in applying a multiple barrier approach or hurdle technology to control the growth of food borne microorganisms has increased. The main ways of enabling long storage stability (3-8 months without refrigeration) were the combination of mild heat treatment (steam blanching for 1-2 min), slight reduction in water activity (a_w, 0.98-0.92 adjusted by the addition of glucose or sucrose), lowering of pH (4.1-3.0, adjusted by the addition of citric or phosphoric acid) and the addition of preservatives (potassium sorbate, sodium benzoate or sodium bisulfite). There is a renewed interest in the use of spices, condiments and plant extracts as alternative food process method. Although the main role of these substances of plant origin is for flavoring and seasoning, they have strong medicinal, preservative and antioxidant properties, and thus contribute to overall safety and preservation of foods and beverages (Lopez-Malo *et al*., 1997).

The essential oils from cinnamon (*Cinnamomum zeylanicum*) and clove (*Eugenia aromatica*) showed activity against 13 food spoilage yeasts (Conner and Beuchat,1984). The major component of the essential oil from cinnamon that exhibit antibacterial properties is cinnnamaldehyde while from clove are eugenol and eugenyl acetate (Burt, 2004). The extracts of corni fructus, cinnamon and Chinese chive were combined and showed the antimicrobial effect in dumplings, guava juice and green and black tea. Therefore, in addition to be used as seasoning, the combined extract is suitable for incorporating in various food products where

a natural antimicrobial additive is desired (Chuan *et al*., 2001). The addition of cinnamon essential oil in broth in combination with refrigeration temperatures of $\leq 8^{\circ}$C could inhibit the growth of *Bacillus cereus* for 60 days in a model refrigerated minimally processed food product, made with carrots and tyndallized. Furthermore, a study of the sensory characteristics of the final product suggests the use of cinnamon essential oil can be considered as an alternative to traditional food preservative (Valero and Salmeron, 2003).

However, few data have been reported on the effect of herbal extracts in combination with other factors on the growth of yeasts. The objective of this work was to study the combined effect of pH, sucrose and herbal extracts from clove and cinnamon in comparison with potassium sorbate on the survival of the isolated yeasts from orange juice.

Materials and Methods

1. Yeasts strains and preparation of inocula

Candida parapsilosis and *Zygosaccharomyces fermentati* were isolated from orange juice samples obtained in Surat Thani Province. The inoculum preparation was done by transferring 1 loopful of a stock culture maintained on Yeast extract Peptone Dextrose Agar (YPDA) slant to 10 ml of Yeast Peptone Dextrose Broth (YPDB) and incubated at 30°C for 24 h. This culture was transferred 1 ml into new YPDB 9 ml followed by incubation at 30°C for 9-12 h (log phase culture) and the final concentration was adjusted to approximately 10^5 CFU/ml before use.

2. Preparation of crude herbal extracts

The crude ethanolic extracts of cinnamon (*Cinnamomum zeylanicum*) and clove (*Eugenia aromatica*) were prepared by adding 100 g. of dried and ground herbs to 500 ml of 95% ethanol and stored at 4.0°C for 5 days. The extracts were filtered and dried by rotary vacuum evaporator. The crude extracts were resuspended in 95% ethanol to give a concentration of 200 mg/ml and were sterilized by passing through a 0.45 µm Millipore membrane. The stock solutions of crude extracts were stored at 4°C until use. Minimum inhibitory concentration (MIC) of each crude extract was determined by the agar dilution method.

3. Experimental design

General Response Surface Methodology (GRSM) was used to design the experiments with three independent variable (concentrations of cinnamon extract or clove extract or potassium sorbate, pH and sucrose concentration) at three levels. The dependent variable was the survival of *C. parapsilosis* and *Z. fermentati* in the orange juice. GRSM is given in terms of coded variable, x_i (Tompson, 1982). Selection of level for independent variables was based on the result from preliminary studies. The levels of input variables in coded (x_i) and uncoded (concentration of the variables) forms are shown in Table 1. The concentrations of cinnamon and clove extracts were 0.5, 1.0 and 1.5 mg/ml. The pH was 2.0, 3.0 or 4.0 and the concentration of sucrose was 10, 15 and 20 $^{\circ}$Brix. While the concentration of potassium sorbate was 0.4, 2.0 and 3.6 mg/ml and 0.4, 1.2 and 2.0 mg/ml for the effect on *C. parapsilosis* and *Z. fermentati*, respectively. The complete design consisted of 15 experimental points, which included three replications of the center point. The orange juice was prepared in random order. Each dependent Y variable (response) was assumed to be affected by the three independent variables. Responses under observation were survival of *C. parapsilosis* and *Z. fermentati*. Each value represented the mean of three determinations. The product thus obtained was analysed and experimental values were compared with model predictions.

4. Preparation of the system

The orange juice was extracted from fresh orange, centrifuged and sterilized by passing through 0.45 µm Millipore membrane. Then sterilized sugar solution was dissolved in orange juice to obtain the desired final concentrations ($^{\circ}$Brix) and the pH was adjusted with 30% citric acid or 1N NaOH to the desired value. This solu-

Table 1. Experimental data for the three-factor, three level response surface analysis [a]

Treatment	Independent variables			Dependent variables[b]					
	°Brix	pH	Extracts /PS	Yeasts					
	X_1	X_2	X_3	Y_1	Y_2	Y_3	Y_4	Y_5	Y_6
1	1 (20)	1 (4.0)	0 (1.0)[c](2.0)[d](1.2)[e]	6.05	4.36	4.17	3.30	5.84	4.82
2	1 (20)	-1 (2.0)	0 (1.0)[c](2.0)[d](1.2)[e]	5.34	3.49	0.00	0.00	0.00	0.00
3	-1 (10)	1 (4.0)	0 (1.0)[c](2.0)[d](1.2)[e]	6.64	4.07	0.00	0.00	5.59	5.55
4	-1 (10)	-1 (2.0)	0 (1.0)[c](2.0)[d](1.2)[e]	5.38	3.65	0.00	0.00	0.00	0.00
5	1 (20)	0 (3.0)	1 (1.5)[c](3.6)[d](2.0)[e]	6.11	0.00	0.00	0.00	0.00	0.00
6	1 (20)	0 (3.0)	-1 (0.5)[c](0.4)[d](0.4)[e]	6.77	6.79	5.75	5.65	5.86	5.79
7	-1 (10)	0 (3.0)	1 (1.5)[c](3.6)[d](2.0)[e]	6.27	0.00	0.00	0.00	0.00	1.93
8	-1 (10)	0 (3.0)	-1 (0.5)[c](0.4)[d](0.4)[e]	6.81	6.74	5.57	3.49	5.71	5.94
9	0 (15)	1 (4.0)	1 (1.5)[c](3.6)[d](2.0)[e]	6.32	0.00	0.00	0.00	5.98	1.99
10	0 (15)	1 (4.0)	-1 (0.5)[c](0.4)[d](0.4)[e]	6.84	6.90	5.54	5.75	5.80	5.87
11	0 (15)	-1 (2.0)	1 (1.5)[c](3.6)[d](2.0)[e]	4.23	0.00	0.00	0.00	0.00	0.00
12	0 (15)	-1 (2.0)	-1 (0.5)[c](0.4)[d](0.4)[e]	5.39	4.57	4.30	0.00	5.49	5.17
13	0 (15)	0 (3.0)	0 (1.0)[c](2.0)[d](1.2)[e]	6.27	4.14	3.53	3.30	4.82	2.15
14	0 (15)	0 (3.0)	0 (1.0)[c](2.0)[d](1.2)[e]	6.34	4.17	3.77	3.49	4.78	2.11
15	0 (15)	0 (3.0)	0 (1.0)[c](2.0)[d](1.2)[e]	6.20	4.23	3.17	3.25	4.87	2.12

Values in parentheses are the uncoded independent variables
[a] Mean of three replication and the experimental runs were performed in a random order
[b] Survival of yeasts expressed as Log of CFU/ml (Y_1, Y_3, Y_5 = *C. parapsilosis* Y_2, Y_4, Y_6 = Z. *fermentati*)
[c] Concentration of cinnamon or clove extract expressed as mg/ml
[d, e] Concentration of potassium sorbate expressed as mg/ml ([d] for *C. parapsilosis* and [e] for *Z. fermentati*)

Table 2. Regression coefficient and analysis of variance of the second order polynomial for response variables

Coefficient	*C. parapsilosis*	*Z. fermentati*	*C. parapsilosis*	*Z. fermentati*	*C. parapsilosis*	*Z. fermentati*
	Y_1	Y_2	Y_3	Y_4	Y_5	Y_6
β_{ko}	2.58	0.22	-9.95	-26.43	-11.68	6.00
Linear						
β_{k1}	-0.27	-0.19	0.85	0.86	2.01	-0.20
β_{k2}	3.81	3.68	7.18	11.64	1.88	0.77
β_{k3}	-1.30	4.59	-5.45	9.94	-3.54	-6.14
Quadratic						
β_{k11}	-0.009	-0.004	-0.04	-0.03	-0.06	0.01
β_{k22}	-0.55	-0.40	-1.50	-1.68	0.01	-0.03
β_{k33}	-0.09	-3.64	1.14	-0.89	-0.27	0.15
Interaction						
β_{k12}	-0.005	0.02	0.20	0.16	0.004	-0.11
β_{k13}	0.01	0.004	-0.01	-0.21	0.88	-0.40
β_{k23}	0.32	-1.16	0.62	-2.87	-0.09	1.52
Variability Explained (R^2)	97.31	99.22	95.92	96.53	91.95	90.34

tion was distributed in 10 ml amounts into steriled bottles (three bottles for each treatment) and each herbal extract solution (diluted as needed) was added. This solution was then dispensed 1.8 ml into sterile test tubes and 200 µl of an inoculum was added. Sterile control (200 µl of sterile orange juice solution in place of the inoculum) was included. Incubation was carried out at 30°C for 48 h. At the end of the incubation period the yeast count was done by pour plate technique on YPDA plates and incubated at 30°C for 48 h. The mean number of colonies on the triplicate plates was determined and expressed as the number of colony-forming units per ml (CFU/ml).

5. Statistical analysis

Data were analysed to fit the following second-order polynomial equation to all dependent Y variable:

$$Y = \beta_{ko} + \sum_{i=1}^{3} \beta_{ki} x_i + \sum_{i=1}^{3} \beta_{kii} x_1^2 + \sum_{i=1}^{2} \sum_{j=1}^{3} \beta_{kij} x_{ij} x_i \quad (1)$$

where: β_{ko}, β_{ki}, β_{kij} are constant coefficients and x_i is the coded indepentdent variable. The SAS version 6.12 program (SAS, 1996) was used for analysis of variance and regression coefficient calculation. Control plot of response for these models was also drawn using the Statistica for Windows, Version 5.0 by plotting as a function of two variables, while keeping other variables at the constant value.

Results

Minimum inhibitory concentration (MIC) of each crude extract was determined by the agar dilution method. The ethanolic extracts of cinnamon and clove showed antimicrobial activity against *C. parapsilosis* and *Z. fermentati* with MIC 1.0 and 0.5 mg/ml, respectively. While potassium sorbate showed activity against both yeasts with MIC 0.4 mg/ml. The response surface regression (RSREG) procedure of Statistical Analysis System (Box & Draper, 1987) which was used to fit the second order polynomial Eq. (1) to the data on the survival of yeasts is shown in Table 1. The regression coefficients (β_{ki}) and the analysis of variance variables are presented in Table 2. The results (not presented) showed that the model developed for the survival of yeasts was adequate, and did not show a significant lack of fit. Further statistical analysis (not presented) revealed that concentrations of cinnamon extract, clove extract or potassium sorbate and sucrose as well as pH and °Brix had a significant overall effect on the survival of both yeasts in the orange juice. The concentrations of cinnamon extract and clove extract and pH of orange juice had the most significant effect on survival of *C. parapsilosis* and *Z. fermentati* while sugar showed the least effect. Survivals of *C. parapsilosis* and *Z. fermentati* were mostly affected by concentration of potassium sorbate but pH and contration of sucrose did not have significant effect on the survival of *Z. fermentati*.

1. Survival of *C. parapsilosis*

Contour plots of the survival of *C. parapsilosis* in the orange juice as affected by the concentrations of cinnamon extract, clove extract or potassium sorbate , pH and sucrose concentrations are shown in Figure 1. The concentrations of cinnamon extract, clove extract or potassium sorbate and pH of orange juice were the most important factors affecting on the survival of the yeasts, while sucrose concentration had lesser effect. Comparing with the same concentrations of cinnamon extract, clove extract or potassium sorbate in the orange juice, the results demonstrated that the survival of *C. parapsilosis* decreased as pH of orange juice decreased. The effect of cinnamon extract and potassium sorbate on growth were smaller than the effect of pH but clove extract showed larger effect than pH. When pH was reduced from 3.5 to 2.5 with 1.0 mg/ml of cinnamon extract, clove extract or potassium sorbate the survival of *C. parapsilosis* was reduced from 2.88×10^6 to 5.19×10^5, 1.51×10^3 to 4.16×10^2 and 1.43×10^8 to 7.37×10^6 CFU/ml, respectively. In order to obtain the same survival of *C. parapsilosis* in the orange juice with 1.0 mg/ml of cinnamon extract, clove extract or potassium sorbate at pH

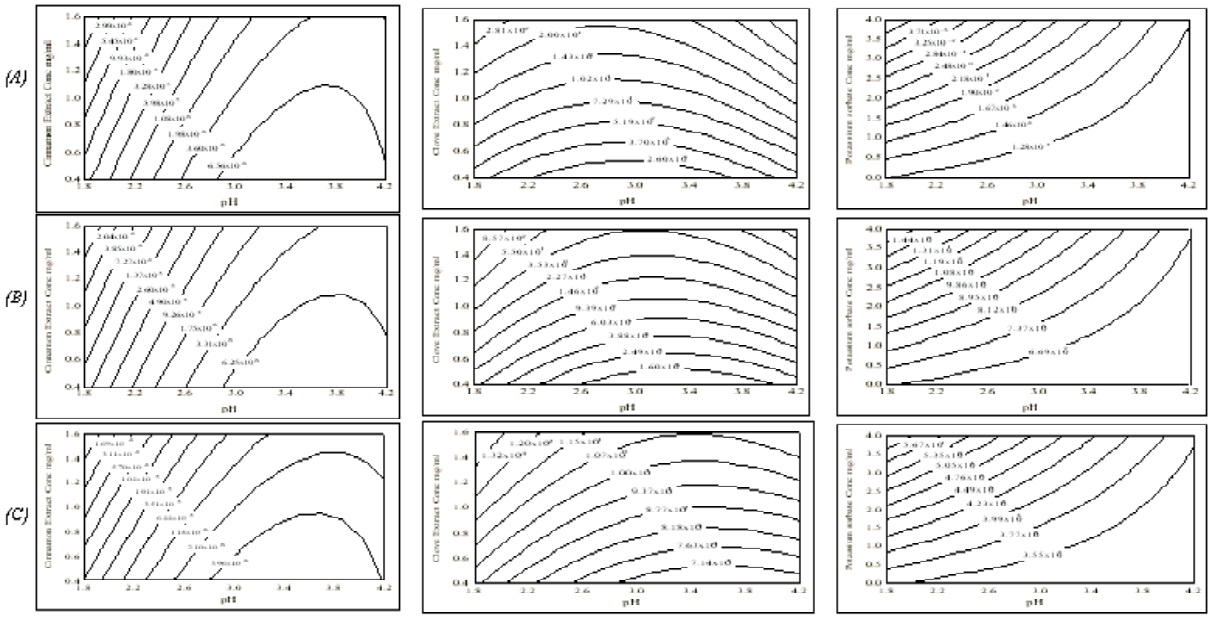

Figure 1. Contour plots showing combined effect of pH and cinnamon or clove or potassium sorbate on the growth of *C. parapsilosis* in orange juice solutions under constant sucrose (^{o}Brix) ; (A) = 10 ^{o}Brix, (B) = 15 ^{o}Brix, (C) = 20 ^{o}Brix

2.5 more than 1.6 mg/ml of cinnamon extract , about 1.1 of clove extract and 2.5 mg/ml of potassium sorbate were required in the orange juice at pH 3.5.

2. Survival of *Z. fermentati*

Contour plots of the survival of *Z. fermentati* as affected by the concentrations of cinnamon extract, clove extract and potassium sorbate are presented in Figure 2. The main factors influencing the survival of *Z. fermentati* in the orange juice were the same as those with *C. parapsilosis*. However, cinnamon extract, clove extract or potassium sorbate showed larger effect on the survival of *Z. fermentati* than pH. When the pH is reduced from 3.5 to 2.5 with 1.0 mg/ml cinnamon extract, clove extract or potassium sorbate the survival of *Z. fermentati* was reduced from 1.34 $x10^{4}$ to $5.12x10^{3}$, $1.36x10^{4}$ to $1.21x10^{3}$ and $3.30x10^{3}$ to $9.24x10^{1}$ CFU/ml, respectively. In order to obtain the same survival of *Z. fermentati* in the orange juice with 1.0 mg/ml of cinnamon, clove or potassium sorbate at pH 2.5 about 1.1 mg/ml of cinnamon extract, 1.2 mg/ml of clove extract or 1.6 mg/ml of potassium sorbate were required in the orange juice at pH 3.5.

Discussion

The microbial stability of foods is based on the combination of several factors (hurdles) acting in concert to inhibit the growth of microorganisms (Battey *et al*., 2002). This hurdle concept can be applied to orange juice 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 orange juice. Only two of the three variables, (pH and cinnamon extract or clove extract or potassium sorbate) were found to be significant predictors for the growth of the spoilage yeasts. Sugar content (^{o}Brix) was not a significant factor affecting on the growth of these spoilage yeasts. The magnitude of each parameter can be observered by comparing the normalized parameter estimates (Table 2).

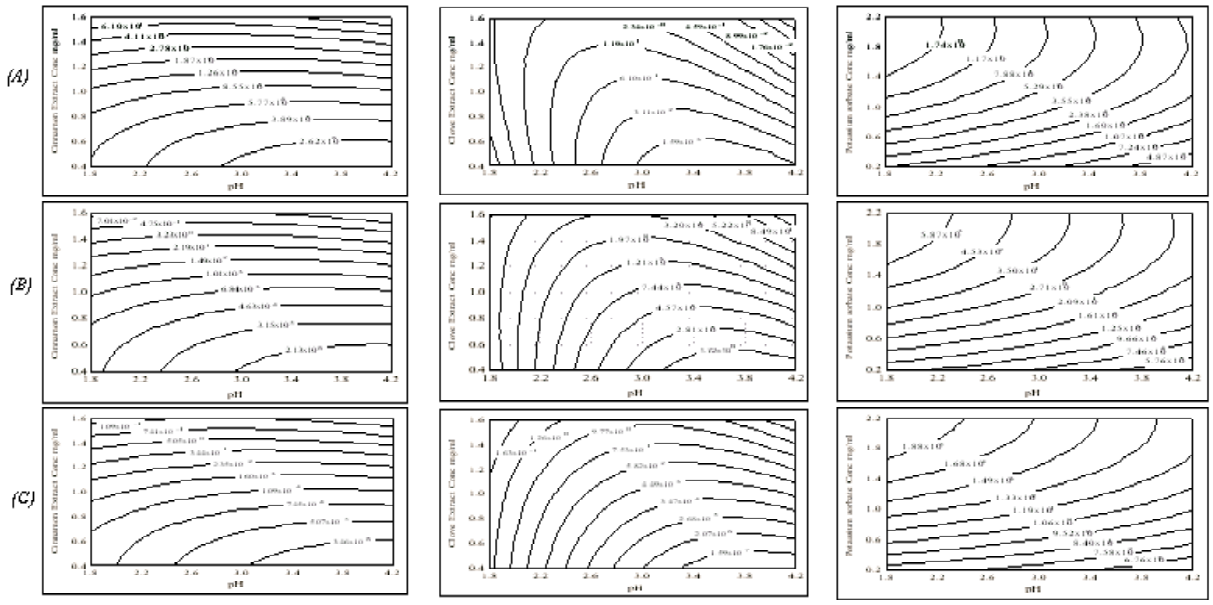

Figure 2. Contour plots showing combined effect of pH and cinnamon or clove or potassium sorbate on the growth of *Z. fermentati* in orange juice solutions under constant sucrose (^{o}Brix); (A) = 10 ^{o}Brix, (B) = 15 ^{o}Brix, (C) = 20 ^{o}Brix

In general, many yeasts are able to survive at acidic condition but do not grow at these low pH values. Although pH of the orange juice used in these experiments was highly acidic (2.0 to 4.0), the obtained result shows that *C. parapsilosis* and *Z. fermentati* could grow at pH as low as 2.0. As the pH of the beverage decreases, the amount of undissociated citric acid increases and can permeate the cell wall and alter the internal pH of the microorganism (Battey *et al*., 2002). So these two yeasts might have some mechanisms to protect cells from pH change.

The sugar content (^{o}Brix) of the orange juice in this study (10 to 20 ^{o}Brix corresponding to a_w values from 1.00 to 0.98) was not a factor in predicting growth of these spoilage yeasts. *C. parapsilosis* and *Z. fermentati* had shown the ability to grow at 50 ^{o}Brix (Praphailong and Fleet, 1997), and they contained enzyme systems that producing compatible solutes to allow them to grow at decreased a_w (Battey *et al*., 2002). 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. These results were consistent with Battey *et al*. (2002) who did not include the ^{o}Brix in their models predicting the growth of *S. cerevisae, Z. balilii* and *C. lipolytica* in the ready to drink beverages. The sugar contents of the ready to drink beverages in those studies were 8 to 16 ^{o}Brix.

There are also significant interactions between concentrations of cinnamon extract or clove extract and pH on the survival of the spoilage yeasts. The effect of pH on the antimicrobial activity of natural phenolic compounds is not clearly understood. However, there was a greater effect of phenolic compounds as the pH was lowered since the increase of solubility and stability of these compounds at low pH (Lopez-Malo *et al*., 1997).

The inhibitory response is stronger at pH values less than 5.0 where a greater proportion of the acid is present in the undissociated form. It is generally reported that most yeasts initiate growth with in the pH range, 3.0-7.0 and thus a reduction in pH alone can cause a reduction in growth

(Praphailong and Fleet, 1997). However, the obtained results show that only the reduction of pH of the orange juice to pH 2.0 was not sufficient to inhibit growth of those two spoilage yeasts. In this case, the addition of cinnamon extract, clove extract or potassium sorbate was more efficient in yeasts inhibition at low pH, and could be because low pH may sensitize the microorganisms and thus they are more susceptible to other stress factors or hurdles.

In conclusion, the results obtained is useful to understand the interaction of preservation factors on the growth of the isolated yeasts from orange juice and to establish a promising application of herbal extracts. Even at low concentration of cinnamon extract or clove extract can markedly inhibit the growth of food spoilage yeasts when used in combination with low pH. The inhibitory effect of cinnamon and clove extracts on several important food spoilage yeasts was observed, which suggests the potential use of these spices to enhance food safety in the fruit juice industry. Furthermore, sensory attribute given by cinnamon and clove may appeal to the consumer. Thus, if foods and beverages are manufactured under sanitary conditions, the addition of substantial amount of herbal extracts can prolong the shelflife and safety of the products.

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References

Battey, A.S., Duffy, S. and Schaffner, D.W. 2002. Modeling yeast spoilage in cold-filled ready to drink beverages with *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, and *Candida lipolytica*. Appl. Environ. Microbiol. 68: 1901-1906.

Box, G.E.F. and Draper, N.R. 1987. A basis for the selection of response surface design. J. Amer. Stat. Assoc, 54: 622-654.

Burt, S., 2004. Essential oils: their antibacterial properties and potential application in food, a review. Int. J. Food. Microbiol, 94: 223-253.

Conner, D.E. and Beuchat, L.R., 1984. Effects of essential oils fron plants on growth of food spoilage yeasts. J. Food. Sci. 49:429-434.

Hsieh, P.C., Mau, J.L. and Huang, S.H. 2001. Antimicrobial effect of various combinations of plant extracts. Food. Microbiol. 18 : 35-43.

Juven, B.J., Kanner., Schved, F. and Weisslowicz, H. 1994. Factors that interact with the antimicrobial action of thyme essential oil and its active constituent. J. Appl. Bact., 76: 626-631.

Lopez-Malo, A., Alzamora, M. S., and Argaiz, A. 1997. Effect of vanillin concentration, pH and incubation temperature on *Aspergillus flavus, Aspergillus niger, Aspergillus ochraceus* and *Aspergillus paraciticus* growth. Food Microbiol. 14: 117-124.

Praphailong, W. and Fleet, G.H. 1997. The effect of pH, sodium chloride, sucrose, sorbate and benzoate on the growth of food spoilage yeasts. Food. Microbiol., 14: 459-468.

SAS. 1996. SAS/STAT user's guide : Statistic : version 6.12. North Cariforina : SAS Institute Inc.

Thompson, D.R. 1982. Response surface experimentation. J. Food Proces. Preserv. 6: 155-188.

Valero. M. and Salmeron, C.M. 2003. Antibacterial activity of 11 essential oils against *Bacillus cereus* in tyndallized carrot broth. Int. J. Food Microbiol. 85: 73-81.

Yuste, J. and Fung, D.Y.C. 2003. Evaluation of *Salmonella typhimurium, Yersinia enterocolitica* and *Staphylococcus aureus* count in apple juice with cinnamon, by conventional media and thin agar layer method. Food Microbiol. 20: 365-370.