احتمال رشد Listeria spp. در برات مدل متاثر از فاکتورهای تنوع گونه، pH، حرارت، کلرورسدیم، سوربات پتاسیم و زمان نگهداری

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خلاصه:

تاثیر فاکتورهای کلرورسدیم (NaCL، ۰/۵، ۴، ۰ و ۷/۵ درصد)، سوربات پتاسیم (KS، ۰، ۱۵٪، ۳۰٪ درصد)، pH (۵/۳ و ۵/۹)، حرارت (T، ۴، ۸، ۲۰ و ۳۰ درجه سانتیگراد)، زمان نگهداری (D، تا ۵۸ -۶۸ روز) و دوز آلودگی لیستریا ($۱۰^{-۲}$ تا $۱۰^{۷}$) روی لگاریتم درصد احتمال (P) یک سلول لیستریا (لیستریامونوسایتوجنز، لیستریااینوکووا و لیستریاایوانووی) جهت رشد در آبگوشت Brain Heart Infusion، بصورت طرح چند فاکتوری مورد ارزیابی قرار گرفت. مقدار P لیستریامونوسایتوجنز بطور معنی‌داری تحت تاثیر pH، NaCL، KS، D و عمل متقابل فاکتورهای T×pH، T×NaCL، T×KS، pH×KS، NaCL×KS و مقدار P لیستریااینوکووا و لیستریاایوانووی بطور معنی‌داری تحت تاثیر pH، T، NaCL، KS، D و عمل متقابل فاکتورهای T×pH، T×KS، pH×KS، NaCL×KS، T×pH×KS، T×NaCL×KS و pH×NaCL×KS قرار گرفت ($۰/۰۲ > p > ۰/۰۰۰۱$). مقدار P با افزایش غلظت‌های KS و NaCL و با کاهش pH و T و افزایش طول مدت نگهداری (D) کاهش پیدا نمود. با کاهش میزان T، اثر باکتریواستاتیک pH، KS و NaCL تشدید و اثر باکتریوسیدی تضعیف گردید. لیستریاایوانووی حساسترین و لیستریامونوسایتوجنز مقاومترین گونه‌ها در برابر اثر جلوگیری کنندهٔ فاکتورهای مورد آزمایش شناخته شدند. یک اثر آنتاگونیسمی مربوط به غلظت توسط NaCL (۴ درصد) روی عمل ضد لیستریائی KS مشاهده گردید. بنابراین غذاهائیکه با pH بالا و مقدار متوسطی از نمک با اضافه کردن سوربات محافظت می‌شوند این موضوع باید مورد توجه قرار گیرد. معادلات ریگرسیون با ارتباط دادن P به T، pH، KS، NaCL و D محاسبه گردید. با استفاده از این معادلات تعداد سلول‌های مورد نیاز هر یک از گونه‌های لیستریا جهت رشد می‌تواند محاسبه شود.

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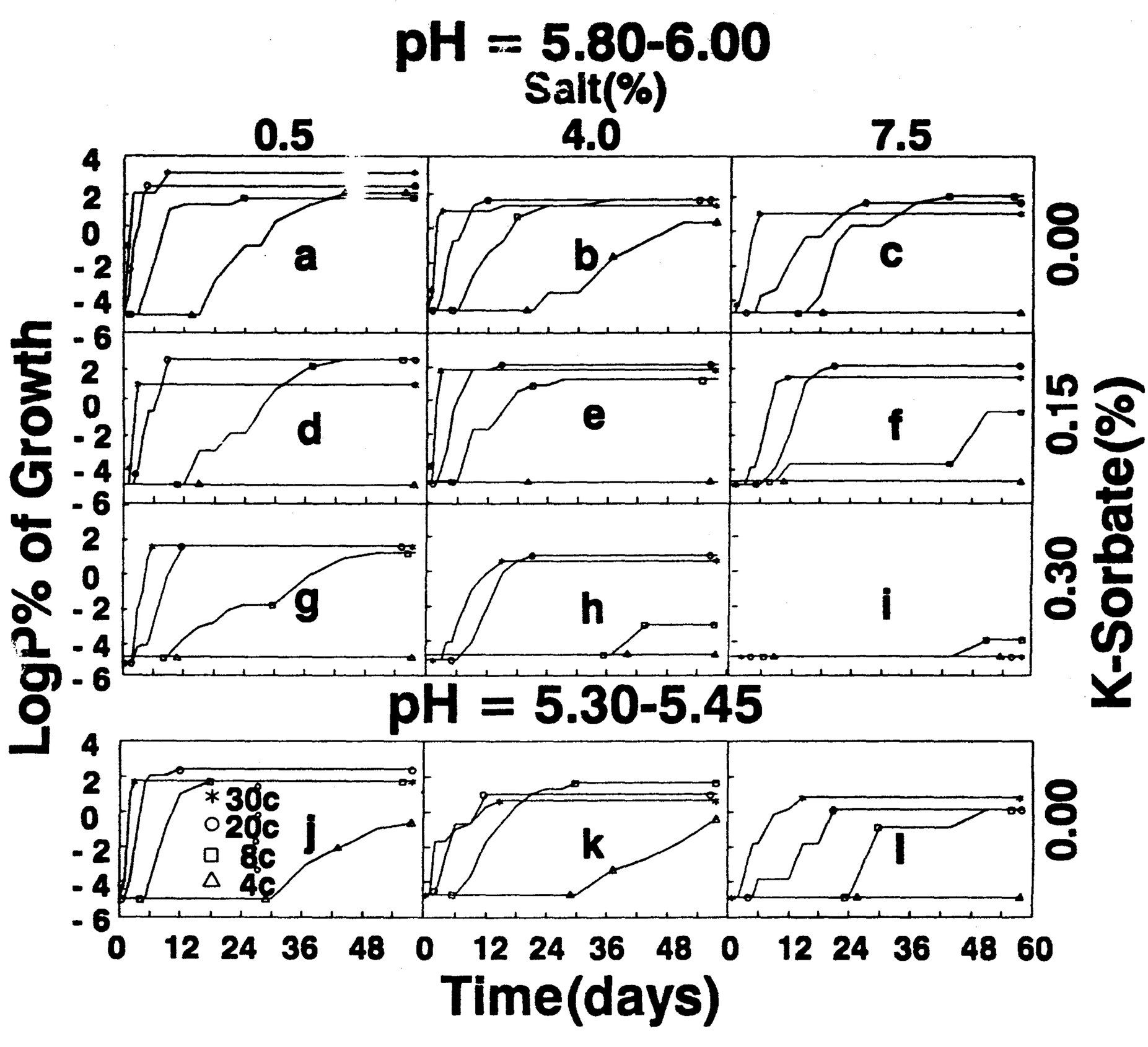
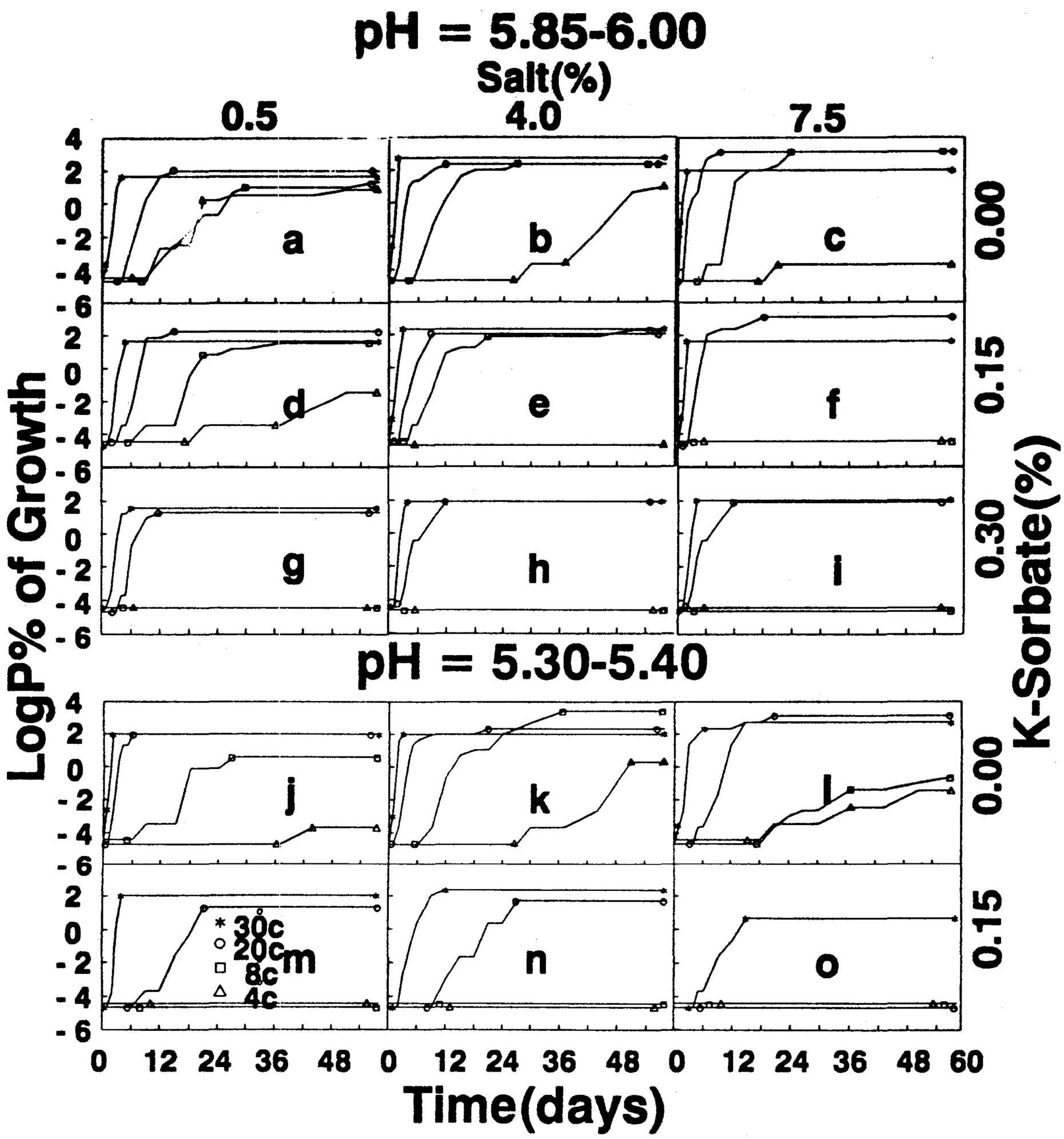
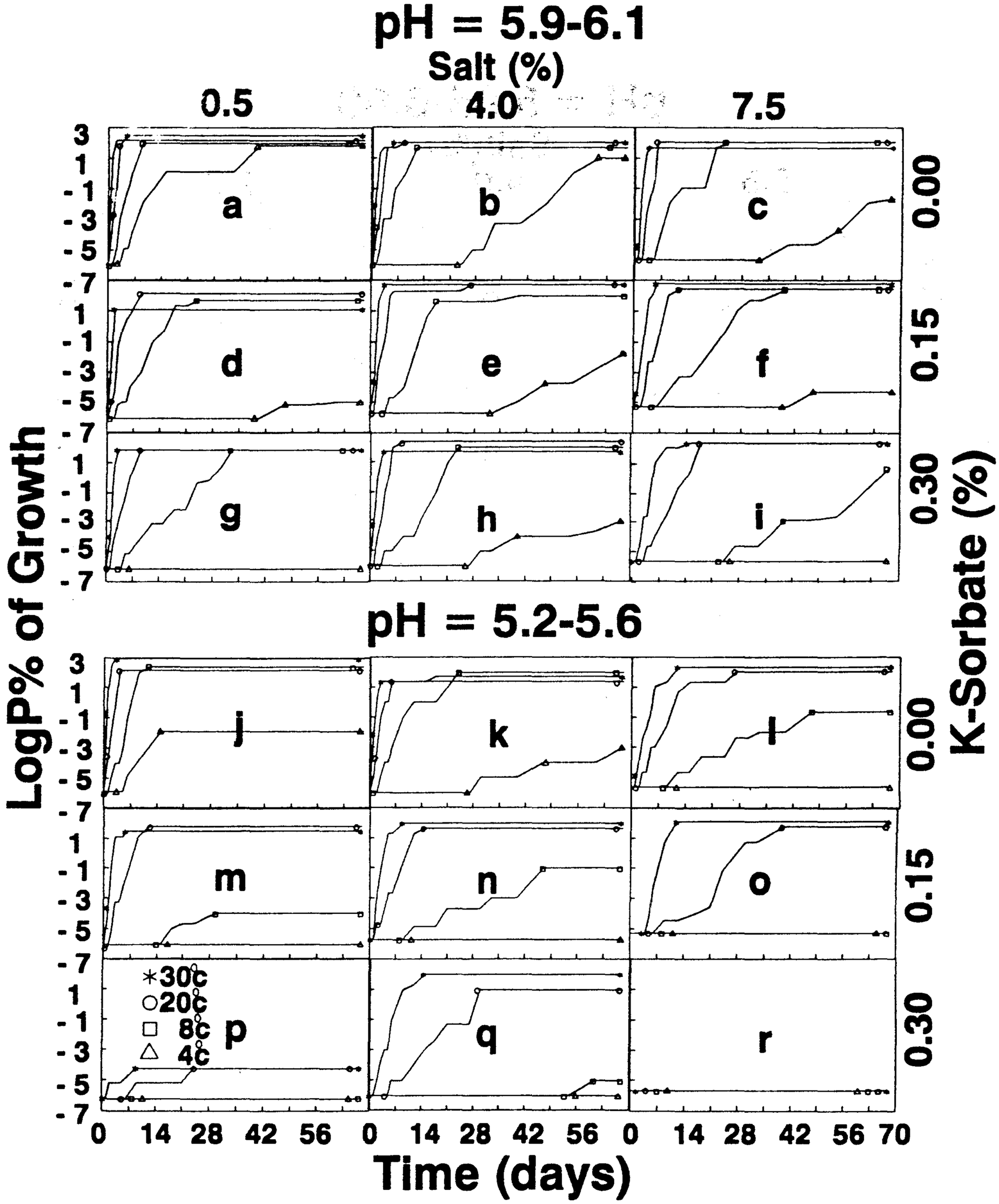


Figures 3. **Probability of growth initiation (LogP%) of L . ivanovii in BHI broth, as affected by temperature, pH, concentrations of potassium sorbate, NaCL and storage time up to 58d.**



Figures 2. **Probability of growth initiation (LogP%) of L . innocua in BHI broth, as affected by temperature, pH, concentrations of potassium sorbate, NaCL and storage time up to 58d.**



Figures 1. Probability of growth initiation (LogP%) of L . monocytogenes in BHI broth, as affected by temperature, pH, concentrations of potassium sorbate, NaCL and storage time up to 68d

Table 4. Predicted and observed values of P (log_{10}%) of growth initiation in BHI broth by one cell of three species of Listeria as affected by combinations of temperature (T), pH (range), concentration (%) of NaCL, potassium sorbate (KS) and time of storage (D), selected randomly from our data bank.

Independent variables					L.monocytogenes		L.innocua		L.ivanovii	
T°C	pH	NaCL	KS	D	Predicted	Observed	Predicted	Observed	Predicted	Observed
30	5.9-6.1	0.5	0.15	12	1.35	1.08	1.3	1.66	-1.24	1.03
30	5.3-5.5	4.0	0.00	18	1.78	1.7	1.4	2.0	-0.14	0.65
30	5.9-6.0	4.0	0.30	18	0.56	1.66	0.17	1.9	-1.86	1.66
30	5.3	7.5	0.00	5	-1.44	-0.66	-1.04	2.0	-3.07	-1.86
30	5.9-6.0	7.5	0.30	4	-1.45	-2.65	-2.37	1.25	-3.54	-4.04
20	5.3-5.6	0.5	0.15	5	-1.95	-2.26	-2.53	-4.68	-4.06	-4.97
20	5.3-5.4	0.5	0.30	30	-1.57	-4.19	-1.64	-4.44	-4.13	-4.96
20	5.3-5.4	4.0	0.15	30	0.14	1.64	-0.31	1.67	-1.92	-4.93
20	5.2-5.4	7.5	0.15	24	-1.25	-1.26	-1.06	-4.67	-3.05	-4.93
20	5.3-5.4	7.5	0.30	21	-1.92	-4.88	-2.75	-4.44	-3.42	-5.04
8	5.9-6.0	0.5	0.30	18	-2.71	-2.19	-3.42	-4.66	-3.83	-4.96
8	5.3-5.5	4.0	0.00	12	-1.96	0.00	-2.70	-0.68	-2.92	-1.69
8	5.9-6.0	4.0	0.30	5	-3.74	-5.03	-4.42	-4.66	-3.86	-4.72
8	5.3-5.3	7.5	0.00	21	-2.57	-3.66	-2.17	-3.67	-3.82	-4.86
8	5.9-6.0	7.5	0.30	5	-3.98	-4.88	-4.43	-4.44	-4.12	-4.72
4	5.9-6.0	0.5	0.00	4	-3.76	-4.87	-4.42	-4.7	-3.09	-4.97
4	5.3-6.0	0.5	0.15	6	-5.9	-5.5	-5.8	-4.44	-6.18	-4.97
4	6.0	4.0	0.15	24	-2.8	-4.94	-2.46	-4.66	-2.71	-2.72
4	5.3-5.4	4.0	0.30	3	-7.7	-5.28	-6.65	-4.66	-6.43	-4.72
4	5.3-5.3	7.5	0.00	30	-4.11	-4.90	-2.68	-4.44	-4.38	-4.86

Table 3. Summary of variables using all time values, intercepts (a), regression coefficients (b), partial correlations (r), and coefficients of determination (R^2) of regression models(1) describing the logP (%) of growth of one cell of Listeria spp (Y), as affected by a combination of temperatures (T°C), NaCl, potassium sorbate (KS), and pH levels in BHI broth stored for up to 68 days (D)

	L . monocytogenes		L . innocua		L . ivanovii	
Variables	r	b	r	b	r	b
T	0.46	0.43	0.34	0.33	0.30	0.31
pH	2.73	0.41	2.47	0.37	0.06	0.47
NaCL	0.10	0.03	0.20	0.07	0.08	0.03
KS	-10.57	-0.37	-4.85	-0.17	-4.02	-0.15
D	0.16	0.46	0.16	0.40	0.13	0.36
$NaCL^2$	-0.03	-0.10	-0.03	-0.09	-0.03	-0.11
D^2	-0.002	-0.34	-0.002	-0.29	-0.001	-0.24
T^2	-0.008	-0.28	-0.004	-0.15	-0.005	-0.17
NaCL×KS	0.81	0.15				
T×KS			-0.24	-0.16	-0.33	
a	-22.15		-20.44		-23.00	

1. Regression models for L . monocytogenes (mo), L . innocua (in) and L . ivanovii (iv):

$$Y_{mo} = -22.15 + 0.46T + 2.73pH + 0.10NaCL - 10.57KS + 0.16D - 0.03NaCL^2 - 0.002D^2 - 0.008T^2 + 0.81 NaCL\times KS.$$

$$R^2 = 0.67$$

$$Y_{in} = -20.44 + 0.34T + 2.47pH + 0.20NaCL - 4.85KS + 0.16D - 0.03\ NaCL^2 - 0.002D^2 - 0.004T^2 - 0.24T\times KS.$$

$$R^2 = 0.63$$

$$Y_{iv} = -23 + 0.3T + 3.06pH + 0.08NaCL - 4.02KS + 0.13D - 0.03NaCL^2 - 0.001D^2 - 0.005T^2 - 0.33\ T\times KS.$$

$$R^2 = 0.60$$

Table 2. Analysis of variance (P- values) of logP of Listeria spp (1) growth by one cell in BHI broth with combinations of NaCl, potassium sorbate (KS), pH and temperature (T°C), stored for up to 68d.

Source of variance	All time values included			Time to reach max. P of growth		
	L.mo	L.in	L.iv	L.mo	L.in	L.iv
Main effect						
T	0.0001	0.0001	0.0001	0.0497	0.0001	0.0004
pH	0.0001	0.0001	0.0001	0.0026	0.0001	0.0060
NaCL	0.0001	0.0001	0.0001	0.0236	0.0600	0.0400
KS	0.0001	0.0001	0.0001	0.0400	0.0001	0.0040
Time	0.0001	0.0001	0.0001	0.0925	0.0800	0.0200
Interactions						
T* pH	0.0001	0.0001	0.0001	0.0729	0.0300	0.0050
T* NaCL	0.0001	0.0001	0.1426	0.9169	0.0800	0.0300
pH* NaCL	0.1649	0.1892	0.0001	0.6292	0.0500	0.1900
T* KS	0.0001	0.0001	0.0001	0.2818	0.0060	0.1300
pH* KS	0.0001	0.0001	0.0001	0.0685	0.0020	0.0800
NaCL* KS	0.0001	0.0382	0.0001	0.3691	0.4700	0.0900
T* pH* NaCL	0.0041	0.3318	0.0234	0.4758	0.7300	0.2900
T* pH* KS	0.0001	0.0001	0.0001	0.0153	0.0002	0.2300
T* NaCL* KS	0.0001	0.0001	0.0001	0.8025	0.0300	0.0200
pH* NaCL* KS	0.0008	0.0003	0.0001	0.4737	0.0600	0.1700

(1) L.mo= L . monocytogenes, L.in= L . innocua and L.iv= L . ivanovii .

Table 1. Number of cells Listeria spp , needed (CN)[a] to imitiate growth and the day (D)[b] reach the maximum P of growth as affected by a combination of pH, temperature (T°C), concentrations (%) of NaCL and potassium sorbate (KS) in BHI broth stored for up to 68 days.

Variables			L.monocytogenes (pool)				L.innocua				L.ivanovii			
			5.9.6.1		5.2.5.6		5.8.5.6		5.3.5.4		5.8.6.0		5.3.5.45	
T°C	NaCL	KS	D	CN	D	CN	D	CN	D	CN	D	CN	D	CN
30	0.5	0.0	2	1.5	3	1	4	2.1	2	1	2	1	3	1.9
		0.15	2	10	5	3.6	5	2.1	4	1	3	9.3	58	NS
		0.3	3	1.5	15	8×10^{6}	6	2.7	58	NS [d]	6	2.2	58	NS
	4.0	0.0	9	1	21	1	2	1	3	1	15	4.9	15	220
		0.15	4	1	12	1	3	1	9	1	3	1.4	58	8.5×10^{6}
		0.3	4	2	15	1	4	1.2	58	NS	15	22	58	NS
	7.5	0.0	5	2	12	1	2	1	5	1	6	15	15	15
		0.15	18	1	12	1	2	2.2	15	21	12	3.9	58	NS
		0.3	15	1	68	4.6×10^{7} [c]	4	1	58	NS	58	NS	58	NS
20	0.5	0.0	4	1	4	1	9	1	6	1	4	1	6	1
		0.15	9	1	12	1.8	15	1	21	4.8	6	1	58	9.3×10^{6}
		0.3	9	1.5	24	8×10^{6}	12	5	58	4.6	12	1.2	58	9×10^{6}
	4.0	0.0	9	1	15	2	9	1	9	1	12	2.2	12	10
		0.15	27	1	15	2.3	9	1	27	2.1	12	1	58	8.5×10^{6}
		0.3	9	1	30	10	12	1.2	58	4.7×10^{6}	21	11	58	1.1×10^{7}
	7.5	0.0	6	1	27	1	5	1	15	1	27	3.3	21	72
		0.15	12	1	40	1.8	6	1	58	4.7×10^{6}	21	1	58	8.5×10^{6}
		0.3	18	1	68	2.7×10^{6}	18	4.7	58	2.7×10^{6}	58	9×10^{6}	58	1.1×10^{6}
8	0.5	0.0	9	1	12	1	58	4.7	27	27	24	1.9	18	1.9
		0.15	27	2	33	10^{6}	37	2.7	58	4.6×10^{6}	37	1	58	NS
		0.3	33	1.5	68	1.9×10^{7}	58	3×10^{6}	58	5×10^{6}	51	5.2	58	9×10^{6}
	4.0	0.0	12	1	27	1	18	1	24	1	37	2.2	30	2.2
		0.15	40	1	47	1×10^{3}	44	1	58	2.7×10^{6}	27	5.2	58	5.2×10^{6}
		0.3	21	1	61	1×10^{7}	58	5×10^{6}	58	5×10^{6}	44	1×10^{3}	58	5.2×10^{6}
	7.5	0.0	21	1	47	457	15	1	58	470	44	1.4	51	72
		0.15	40	1	68	1.8×10^{7}	58	3×10^{6}	58	2.7×10^{6}	51	520	58	5.2×10^{6}
		0.3	68	20	68	4.6×10^{7}	58	5×10^{6}	58	3×10^{6}	51	9×10^{3}	58	9×10^{6}
4	0.5	0.0	40	1.5	15	7.4×10^{3}	51	12	44	5×10^{3}	44	1	58	430
		0.15	61	1×10^{7}	68	1.9×10^{8}	51	3×10^{3}	58	3×10^{6}	58	9×10^{6}	58	9×10^{6}
		0.3	68	7×10^{7}	68	1.5×10^{8}	58	3×10^{6}	58	3×10^{6}	58	5×10^{6}	58	9×10^{6}
	4.0	0.0	61	10	68	1×10^{3}	58	6.7	51	48	51	49	58	220
		0.15	68	5×10^{3}	68	5×10^{7}	58	5×10^{6}	58	5×10^{6}	51	5.2	58	5×10^{6}
		0.3	68	1×10^{3}	68	1×10^{8}	58	5×10^{6}	58	5×10^{6}	58	5×10^{6}	58	5×10^{6}
	7.5	0.0	68	5×10^{3}	68	5×10^{7}	21	5×10^{3}	51	2.7×10^{3}	58	7×10^{6}	58	7×10^{6}
		0.15	47	2×10^{6}	68	2×10^{7}	58	3×10^{6}	58	3×10^{6}	58	5×10^{6}	58	5×10^{6}
		0.3	68	5×10^{7}	68	5×10^{7}	58	3×10^{6}	58	3×10^{6}	58	9×10^{6}	58	5×10^{6}

(a) Per 3 and 2.4 ml of BHI broth for L . monocytogenes and L . innocua , L . ivanovii respectively. (b) No further change in CN with additional incubation up to 58-68 days (58 for L . innocua and L .ivanovii and 68 for L . monocytogenes). (c) Underlined data indicate that the inoculated cells did not grow but all or a fraction of them survived. (d) NS indicates that the inoculated cells did not grow and survive to detectable levels (detection limit 100 cells/ml).

also from the comparisons of predicted and observed log P's of Table 4. Inadequacy of the models may be due to lack of a proper synergistic or additive effect between salt and sorbate variables (the main preservatives in this study). Cole et al (9) suggested the use of H - concentration rather than pH for better fitting of models for L.monocytogenes. Other reasons have been mentioned earlier.

Irrespective of the draw backs of the developed models the study has provided interesting information on the effects of important variables and their interaction on the growth of three Listeria spp. Such information can be utilized in the establishment of appropriate Listeria growth barriers in high risk foods like soft cheeses (24).

of the number of cells needed to initiate and show visible growth by a certain storage time, it did not facilitate the estimation of the true lag phase or the number of cells after D time of storage (Table 3) as have done before for the fate of S.aureus during dry salami fermentation (38).

Approaches other than log P to quantify and model the effect of a number of product formulation and environmental variables on L.monocytogenes growth have been reported recently (7,9, 43, 57). In most of these studies (7,8,43,57) data on growth curves generated in broths of different composition (pH, nitrite, salt, antioxidant) and storage conditions (temperature, atmosphere) were fitted by the use of the " Gompertz function " model. Based on linear regression analysis models were derived which related important Gompertz function parameters to the effect of the above mentioned intrinsic and extrinsic variables. From the prediction of these parameters, length of lag phase (LP) and generation times (GT) of L.monocytogenes could be calculated.

Predicted values for LP and GT compared very favorably with values published by workers from different countries after L.monocytogenes growth in various food (8). Differences between the predicted and reported values tended to be greater for LP than GT. The authors suggested that this may be due partially to the greater inherent variability of the physiological processes occurring during the LP and differences in the methods used to estimate LP. The models also tended to indicate that L.monocytogenes was more actively capable of growing under adverse conditions than was actually the case. This was probabley due to the existence of additional food factors affecting growth of L.monocytogenes but not incorporated in the models (8). Years ago, we found that models developed for the prediction of the log P of growth of S.aureus (20,25) B.cereus (42) and Salmonella (21) in broth did not predict well the growth of these pathogens in foods probably for the above mentioned reasons.

The R^2 values presented in table 3, showed that our models did not correlate very well the effect of the combinations of pH-KS-NaCL-T time to the log P of growth in the broth system. This becomes obvious

compared to absence of NaCL.

In this study it is interesting that in most cases, combination of 4% salt with sorbate decreased the inhibitory action of sorbate (Figures 1-3). Several researchers (18,50,51) reported the synergistic relation between NaCL and sorbate against other microorganisms. However, in one study (50) NaCL (1.25 and 2.5%) in nutrient broth decreased the effect of sorbate against C.botulinum and elimination of NaCL increased the inhibitory effect of sorbate. Dallmier and Martin (12) reported that, the catalase and supperoxide dismutase activity of L.monocytogenes was enhanced by culturing in media with NaCL ranging from 1.5 to 4.6% and 1.5 to 3.5%, respectively. Our findings have shown that the antimicrobial activities of the combination of salt and sorbate on L.monocytogenes are concentration - dependent and that at a moderate salt cocentration (4%) they have an antagonistic effect on the growth of the organism. On the other hand there is ividence that virulence of L.monocytogenes may be enhanced by incubation at 4C (53). These findings along with ours indicate that, a particular caution may be needed in high pH foods with a moderate salt concentration, preserved with sorbate and stored at cold temperatures.

Twenty years ago Genigeorgis and co - workers (20,25) utilized the concept of probability of growth initiation by one baterial cell (dependent variable) to quantify the effect of intrinsic (food, media) and extrinsic (environmental) independent variables on the growth of Staphylococcus auresus. Using regression methods, they developed mathematical models to predict the behavior (log P) of the pathogen as affected by the independent variables. These studies were later extended to include Bacillus cereus (42) Salmonella (21) and toxigenesis by C.botulinum (6,34). In the latter studies, both linear and logistic regressions were used to predict the length of the lag phase as well as the log P of toxigenesis. In the present study, we used again log P as the dependent variable and quantified and later modeled the effect to KS,T,NaCL and storage time on log P for each one of the three Listeria spp studied. This approach while it allowed the estimation

of KS all Listeria spp in our study were able to grow very well in broths with pH 5.2 to 5.6 at 4 to 30°C.

The ability of L.monocytogenes to grow at 0 to 1°C and L.innocua at 1.6C has been reported (28,32). In this study all temperature levels supported the growth of the organisms with a very simillar log P but an extended LP at lower temper atures. The bacteriostatic effect of pH, KS adn NaCL was enhanced and the bactericidal effect was diminished with the decreasing of temperature. As Table 1 shows, at 30°C, observed 9 cases of death to undetectable levels and only one at all other temperatures. These observations are in agreement with previous reports (9,49).

Of all the variables studied, NaCL, within the concentration range need, had the lowest values, indicating the lowest antimicrobial activity against all three Listeria spp. Only at concentration of 7.5%, pH 5.9 and 5.3 at 4C, NaCL showed a significant effect on the growth of the organisms. A case of human septicemia due to the consumption of salted mushrooms with 7.5% NaCL and pH 5.9 has been reported recently (31). Scott (47) cited unpublished work by Sorrells indicating growth of L.monocytogenes at 25°C in 7d in liquid media with 12% NaCL. Sozzi et al (52) reported growth of L.monocytogenes in broth with a_W 0.92 (adjusted with NaCL) and pH 6.5 and Petran and Zottola (41) in broth with a_W 0.92 (adjusted with 39.4% sucrose) pH 6.8 at 30°C. McClure, et al (36) studied the effect of interactions of pH and NaCL on L.monocytogenes. At 10% NaCL (highest concentration studied), the minimal pH supporting growth at 25°C was 5.0. The highest NaCL concentrations supporting growth of L.monocytogenes at 5,10 and 30°C at optimal pH (7.0) was 8, 10 and 12%, respectively.

The ability of Listeria spp. to survive for up to 58 to 68 in inhibitory broths is not surprising. Published data for L.monocytogenes have shown its ability to survive in broths with 25.5% NaCL for 24 to 32d at 22°C and longer times in media and foods with lower concentrations of NaCL stored at lower temperatures (39,48). Cole, et al (9) demonstrated the protective effect of low concentrations of NaCL (4 to 8%) to low pH injured L.monocytogenes cells as

the maximum log P of growth of L.monocytogenes, L.innocua and L.ivanovii. L.ivanovii was the most and L.monocytogenes was the least sensitive species to the inhibitory effect of the variables used in this study.

Increasing levels of KS decreased the probability of growth of the test organisms. The inhibitory action of sorbate was enhanced by decreasing the broth pH and storage temperature. These observations are in agreement with the report of El-Shenawy and Marth (15), who studied the growth of L.monocytogenes in broth with 0 to 0.3% KS, pH 5 to 5.6 at 4 to 35C. With a pKa=4.78 for sorbic acid, the enhanced antimicrobial activity of KS with decreasing pH from 5.9 - 6.1 to 5.2 - 5.6 is understandable since an increasing level of undissociated acid becomes available (15,46). Complete inhibition of Listeria spp growth in broths with 0.5% NaCL was observed in our study only when the pH was≤6.1 in the presence of at least 0.3% KS and storage at 4C. Inhibition at higher temperatures required lower pH and increased levels of NaCL. When growth was not observed, inoculated cells died off with time and faster at 30°C than lower temperatures. This was most dramatic with L.ivanovii (Table 1) at 30°C. The increased death at higher temperatures may be due to a serious damage of important enzymes at optimum temperature (30°C) which is considered as an important mechanism in the inhibitory action of sorbate (33). El Shenawy and Marth (15) observed complete inhibition of L.monocytogenes growth at≥13C when the pH was 5.0 and the KS concen- tration 0.2% .

The minimum pH supporting L.monocytogenes growth has been studied by a number of researchers. The minimum pH at optimum temperature varied from 4.3 to 5.6 (16,26,40) depending on the acid and strain used. Below these pH's the rate of cell death has been found to accelerate and the lag period before cell death begings to be prolonged with increasing storage temperature (40,49). Because of this, Parish, et al (40) noted that low pH products would be of concern in Listeria outbreaks under conditions of gross contamination, followed by rapid consumption of the food product. In the presence of 0.5% NaCL and absence

study, the lowest values of r were 0.03, 0.07, 0.03 exhibited by NaCL for all three models for L.monocytogenes, L.innocua and L.ivanovii, respectively. The highest r's of 0.46 and 0.40 for L.monocytogenes and L.innocua, respectively were exhibited by storage time. In the case of L.ivanovii the highest r (0.47) was contributed by the pH variable. The values of b for KS and NaCL in the models were negative for KS and positive for NaCL. There were significant interaction effects on L.monocytogenes by KS×NaCL and on L.innocua and L.ivanovii by T×KS. From these models the number of cells needed (CN) to initiate growth can be calculated using the formula CN= 100/P(%).

Table 4 compares 20 randomly selected cases of calculated and observed logP of one cell of Listeria spp initiating growth as affected by T,pH,NaCL,KS and storage time levels. The residual values (predicted - observed) for these cases showed 0 to 4 log difference out of a range of log P of -5.5 to ≥ 2. These differences for L.monocytogenes were in 45% of the comparisons 0-1 log, in 35% 1 - 2 logs, and in 20% 2 - 3 logs. For L.innocua in 30% of the comparisons the differences were 0-1 log, in 40% 1-2 logs, in 20% 2-3 logs and in 10% 3-4 logs. For L.ivanovii, the differences were in 40% of the comparisons 0-1 log, in 45% 1-2 logs, in 10% 2-3 logs and in 5% 3-4 logs.

Discussion:

The ability of L.monocytogenes to grow in foods and laboratory media under a variety of conditions has been studied and reviewed (1,7,9,11,14,15,16,26,40,41,45,49,52).However methods which can quantify the risk of Listeria growth (L.monocytogenes as well as other species) as affected by various intrinsic and extrinsic factors, have seldomly been utilized (7,9,43,57). In the present study, we utilized the concept of the probability of one cell to initiate growth (20,21,25,34,42) in order to quantify the effect of potassium sorbate (KS), pH, storage temperature (T) and time and NaCL on the growth iniation by one cell of L.monocytogenes, L.innocua and L.ivanovii.

Statistical analysis demonstrated the significant effect of KS,pH,T,NaCL and storage time and a number of interactions including T×pH and T×pH KS, on the log P of growth after a certain storage time and on

T, NaCL, KS, time, the two way interactions of T×pH, T×NaCL, T×KS, pH×KS and NaCL×KS and the three way interactions of T×pH×NaCL, T×pH×KS, T ×NaCL×KS and pH×NaCL×KS. The maximum logP was affected significantly ($0.0003 \leq p \leq 0.05$) only by T, pH, NaCL, KS and the interaction T×pH×KS.

For L.innocua and L.ivanovii, when all time observations were included, the logP of growth initiation was affected significantly ($0.0001 < p < 0.02$) by T, pH, NaCL, KS, time, T×pH, T×KS, pH×KS, NaCL×KS, T×pH×KS, T×NaCL×KS, and pH×NaCL×KS. The logP of L.innocua was affected also by T×NaCL and L.ivanovii by pH×NaCL and T ×pH×NaCL. For L.innocua and L.ivanovii the maximum logP was affected significantly ($0.004 < p < 0.04$) by T, pH, KS, T×pH and T× NaCL×KS. In addition for L.innocua the maximum P was affected significantly ($0.0001 < p < 0.05$) by pH×NaCL, T×KS, pH×KS, T×pH×KS and for L.ivanovii by time and T×NaCL (table 3).

Using the results of ANOVA, which identified the main effects and their interactions influencing significantly the log P of growth, an effort was made to model the behavior of the three Listeria species as affected by the important independent variables and their transformations. The general model for the best fit equation was $Y = a + b_1x_1 + b_2x_2 + b_3x_3 + \ldots\ldots b_nx_n$, where Y= the dependent variable log P(%), $x_1x_2x_3 \ldots x_n$= the independent variables, a= intercept and $b_1b_2b_3 \ldots = b_n$= regression coefficients. Using the the stepwise regression program BMDP2R the best fit equations for each of L.monocytogenes, L.innocua and L.ivanovii were derived. Pertinet information for the important variables, their regression coefficients, values of a's, partial correlations (r) and multiple correlations (R^2) of the three equations are summarized in table 3. Overall the p values for all models were strongly significant ($p < 0.001$). R^2 values for the models, were 0.67, 0.63 and 0.60, respectively. These R^2 show that a large proportion of the variation in the dependent variables (logP of growth for each Listeria)is explained by the various combinations of the independent variables, their transformations and interactions. Of the 5 independent variables used in this

3×10^6 cells at T≤8°C and any NaCL concentration, yet at 20 to 30°C, ≤5 cells could initiate growth in the presence of 0.3% KS and 7.5% NaCL. Decreasing the pH to 5.3 to 5.4 had a dramatic effect on the number of L.innocua cells needed to initiate growth in the presence of NaCL and KS. No growth was observed in 18/36 situations. No growth was observed at T≤8°C even by 2.7×10^6 cells at KS≥0.15% and any NaCL conditions. NaCL condition of 4% seemed to be more conducive to growth than 0.5%. Furthermore, KS at 0.3% was more detrimental to the L.innocua at 30°C than≤20°C at all NaCL levels. At 30°C there were no surviving cells out of≥10^6/3ml inoculated, while at≤20°C there was no growth in all but one situation and survival of all inoculated cells.

L.ivanovii required greater numbers of cells to initiate growth than did either L.monocytogenes or L.innocua. This was even more dramatic at pH 5.3 to 5.45. At this pH growth was observed in only 11 of the 36 NaCL-KS-T situations, all representing absence of KS. As was observed for L.innocua, L.ivanovii was also more sensitive to the lethal effect of KS at 30°C than at≤20°C.

Overall, of the 73/216 pH-NaCL-KS-T situations which did not support growth in the broths, in 10 there were no surviving cells out of the 10^7 inoculated after 58d of storage. Nine of these situations represented incubation of the broths at 30°C and one at 8°C (table 1).

Statistical analysis :

Two ANOVA's were included for each of the three Listeria species studied. In one analysis we included the effects of all the levels of pH,T,NaCL and KS conditions, their 2 and 3 - way interactions and the time to reach maximum log P (as covariance) on the logP of growth initiation by one cell. In the second ANOVA we also included the logP's corresponding to all observations (19 to 21)during storage for 58 to 68d (time as a covariate). Table 2 presents the results of these ANOVA's for L.monocytogenes, L.innocua and L.ivanovii.

When all time observations were included in the analysis then the logP of L.monocytogenes growth initiation was affected significantly (0.0001≤p≤0.004) by pH,

at pH 5.8 to 6. The LP was extended to≥18d by the combinations of the variables of T≤4°C, NaCL≥4% and KS≥0.0% or T≤8°C, NaCL≥ 4% and KS≥3% or T≤4C, NaCL≥4% and KS≥0.15%. At pH 5.3-5.45 and KS = 0.0% the LP was extended to≥30d by NaCL≥0.5 and T≤4C. In the presence of KS≥0.15% and NaCL≥0.5% at this pH no growth was observed after 58d at≤30C. Overall L.ivanovii seemed to be more sensitive to pH, NaCL, KS and storage temperatures than either L.monocytogenes or L.innocua.

Number of cells needed to initiate growth:

Table 1 presents the calculated number of cells needed for each pH, NaCL, KS, and T conditions to initiate growth by the day at which the maximum log P of growth was observed.

At a pH of 5.9 to 6.1, ≤20 Cells/3ml broth of L.monocytogenes were required to produce visible growth (≥1×10^7/ml broth) with NaCL≤7.5% and KS≤0.30% stored at≥8°C for 2-68d. At 4°C, ≤10 cells could initiate growth in broth with NaCL≤ 4% and no KS. In the presence of NaCL≥ 4% and KS≥0.15%, 5×10^3 to 1×10^7 were needed to show visible growth only after 68d incubation. Presence of 0.30% KS at any NaCL level did not permit growth of L.monocytogenes. Decreasing the pH to 5.2 to 5.6 required greater number of cells to initiate growth under the same conditions of NaCL, KS and T than pH of 5.9 to 6.1. Of 36 possible conditions of NaCL, KS and T in only 16 conditions, growth was initiated by≤10 cells, in one by 457, in two by 1×10^3 cells. In all other conditions≥1×10^5 cells were needed to initiate growth. Actually, in most of these cases even 3×10^6 to 1.9×10^8 did not initiate growth. In the presence of 0.3% KS,≥10^6 cells/3ml were required for growth or even no growth by such and greater inocula at all T and NaCL concentrations. The only two exceptions which led to visible growth within 30d by 1 and 10 cells respectively, were at 20 to 30°C and 4% NaCL. It seems that NaCL at this concentration protected cells from the action of KS, something not done by 0.5 or 7.5% of NaCL.

L.innocua required more cells to initate growth than L.monocytogenes under the same pH, NaCL, KS and T conditions. At pH 5.85 to 6, KS did not support growth by

remained ≤5d. No change in LP by the 68th day of incubation was observed at 4°C with 0.3% KS and any level of NaCL.

At pH 5.2-5.6 (5.3 before autoclaving the broths) LP was extended to≥21d at≤4°C with NaCL≥0.5% and KS≥0.15% or at ≤8°C with NaCL≥7.5% and KS≥0.15% or at T≤8°C with NaCL≥4% and KS≥0.3%. Under all other conditions LP remained≤9d. At≤8°C with KS≥0.15% growth initiation seemed to be better in the presence of 4% rather than 0.5% NaCL. A number of conditions did not support any visible growth after 68d. They included storage at≤30°C with NaCL≥7.5, KS≥0.30 and pH≤5.6 or at≤4°C with NaCL 0.5%, KS≥0.30% and pH≤6.1 or at≤4°C with NaCL≥7.5%, KS≥ 0.30% and pH≤6.1 or at≤4°C, NaCL≥4%, KS≥0.15% and pH≤5.6.

Figure 2 illustrates the log P of growth of L.innocua as affected by the independent variables. Like fig.1 on the response of L.monocytogenes, fig.2 indicates also that the LP and the change in log P with time were affected by pH, T, NaCL and KS concentrations. The LP was significantly extended by decreasing pH or temperature and by increasing levels of NaCL or KS. At pH 5.85-6 (5.9 before autoclaving of broths) the LP was extended to≥18d by the combination of T≤4°C with NaCL≥4% and KS≥0% or T≤4°C, NaCL≥0.5% and KS≥0.15% or T≤8°C with NaCL≥7.5% and KS≥0.15% or T≤8°C with NaCl≥7.5% and KS≥0.15% or T≤8°C with NaCL≥0.5% and KS≥0.3%. At pH 5.3 to 5.4 (5.3 before autoclaving of broths) the LP was extended to≥18d by the combinations of T≤4°C, NaCL≥0.5% and KS≥0.0% or by T≤8°C, NaCl≥7.5% and KS≥0.0% or by T≤8°C NaCL≥0.5% and KS≥0.15%. After 58d incubation at≤8°C no visible growth was observed in any of the broths with the highest inoculum and pH≤5.4, NaCL≥0.5% and KS≥0.15% or in broths with NaCL≥ 0.5% and KS≥0.3% stored at≤30°C. At pH 5.85 to 6 no growth occurred in broths with NaCL≥ 4% and KS≥0.15% stored at≤4°C or in broths with NaCL≥0.5% and KS≥0.3% stored at T≤8°C or in broths with NaCL≥ 7.5% and KS≥0.15% stored at T≤8°C. Overall L.innocua seemed to be more sensitive to T, NaCL and KS than was L.monocytogenes.

Figure 3 illustrates the response of L.ivanovii to the effect of T, NaCL, and KS

Statistical analysis:

The main and interactive effects of pH, temperature, concentration of NaCL and KS, time of incubation and time to reach maximum growth on $\log_{10}$ P(%) (to be referred hereafter as log P)were evaluated by analysis of variance using the Biomedical Computer Program BMDP2V (13). The stepwise multiple regression program BMDP2R (13) was used to select the best fitting models to predict the log P (dependent variable) of one Listeria spp cell initiating growth as affected by independent variables of pH,NaCL,KS,temperature and time of storage and their relevant transformations. The coefficient of determination (R^2) was used to express the proportion of variation of the dependent variable explained by the variation in independent variables. Partial correlation (r)was used simply to explain the correlation between the dependent variable and each individual independent variable. Regression coefficients (b) of the equations expressed the change in log P of growth of each Listeria on a unit change in independent variables. Sources of variation (independent variables) having regression coefficients with $p<0.05$ were considered as significant.

Results:

Probability of growth initiation

The effect of pH,NaCL,KS,temperature and time of incubation on log of one cell of L.monocytogenes, L.innocua and L.ivanovii to initiate growth by a certain time period was evaluated in three factorial design experiments.

Figure 1 illustrates the log P of growth of L.monocytogenes as affected by the different independent variables. Overall this figure indicates that the lag phase (LP) (time interval from the beginning of inoculation until first increase of log P is observed) and the rate of growht (described by the rate in the change of log P with time) were affected by the pH, T and concentration of NaCL and KS. The LP was extended with increasing levels of NaCL or KS and with decreasing pH and temperature.

At pH 5.9-6.1 (5.9 before autoclaving the broths) the LP was extended to over 21d only when T≤4°C with NaCL≥4% or T≤4°C with KS≥0.15% or T≤8°C with NaCL≥7.5% and KS≥0.3%. Under all other conditions LP

and L.ivanovii the contents of the last 8 tubes were dispensed in portions of 2.4 ml in triplicate into the wells of a tissue culture plate (Beckton Dickinson Labwere) making a total of 24(3×8) wells for each pH-NaCL-KS-T combination. To avoid evaporation all tissue culture plates were wrapped with a polyethylene film having an oxygen permeability of 1600-3200 cc/m^2/24h at 25°C (Stretch'n Cling Wrap, Safe Way Stores, Inc., Oakland, CA). Equal sets of inoculated tubes and tissue culture plates representing all pH-NaCL-KS-T combinations were stored at 30, 20, 8 and 4°C for 68 or 58 days, respectively. During these periods all the tubes or plate wells were observed for visible growth 19-21 times. The number of tubes/wells showing growth at a particular observation and the date were recorded.

Calculation of probability of growth:

From the number of total tubes (out of 27) or wells (out of 24) for each pH-NaCL-KS-T situation showing visible growth up to a certain observation time, and use of 3×9 or 3×8 (for tubes or wells respectively) Most Probable Number (MPN)tables (17,25,34) the fraction of the inoculum which was inhibited by each broth environment up to that time was estimated from the formula $\log_{10}$ I/G. I is the number of cells inoculated in the highest cell concentration tube or well and G is the MPN of cells in the same tube or well which managed to grow (19). The probability P (%) of any given cell initiating growth under each broth environment within a certain period of time was defined as P (%) = 100/antilog ($\log_{10}$I-$\log_{10}$ G). Based on MPN scaling (17) when no growth was observed in any of the tubes/wells then G was set at 0.17 cells. The numbers of cells needed (CN) to initiate visible growth for each pH-NaCL-KS-T-time situation were estimated from CN=100/p (%). To determine whether nonvisible growth (<5×10^6 cells/ml) or death occurred in the tube/well with the highest number of inoculated cells at any particular time, 0.01 and 0.001 ml were plated on TSBA agar using standard platinum loops. The plates were incubated at 35°C for 24 to 48h and the colonies were counted.

Preparation of inocula:

Listeria spp. inocula were prepared by transferring cells from working cultures to BHI broth. After 24 hrs incubation at 35°C a second subculture was prepared, which was incubated for 18h at 35°C. Equal volumes of the two strains of L.monocytogenes cultures were mixed in a 13×100 mm sterile cuvet (Fisher scientific, Pittsburgh, PA) and the optical density at 600 nm was adjusted with BHI broth to 0.25-0.30 using a Spectronic 20 spectrophotometer (Bausch and Lomb, Rochester, N.Y). Adjustment of OD to these levels gave a cell concentration of $\log_{10}$ 8-8.5/ml broth. Individual cell suspensions of L.innocua and L.ivanovii were prepared in a similar fashion. The numbers of cells in the suspensions were estimated by plating ten-fold serial dilutions on TSBA agar in duplicate and counting the colonies after 24h incubation at 35°C.

Preparation of broth substrates:

As the basic substrate BHI broth was used. Broth powder (3.7 g) was dissolved with mild heating 90 ml of distilled water in flask. After cooling, NaCL and KS were added and dissolved in amounts to satisfy the experimenal design, and then the pH was adjusted with 1N HCL to 5.9 or 5.3. The final volume was brought to 100 ml with additional distilled water. pH measurements were done with a Select 2000 pH meter (Beckman Instrument Inc., Irvine, CA), having a gel-filled combination pH electrode (Orion Research Inc., Boston, MA). The content of each flask was dispensed into screwcap tubes at levels of 9 ml/tube and then the tubes were autoclaved at 121°C for 15 minutes. After cooling, the pH of each pH-NaCL-KS-T combination broth was measured and recorded as the final pH.

Inoculation and storage of broth substrates:

Serial ten-fold dilutions of each Listeria culture (OD_{600nm}=0.25-0.30) were prepared using a set of 9 tubes that contained BHI broth with a designated combination of pH-NaCL-KS-T. In the case of the L.monocytogenes pool, three portions of 3 ml each from the 9 ml content of each tube were dispensed into sterile capped poly-styrenne (12×75 mm) tubes (Beckton Dickinson Labware, oxanard. CA) resulting in a total number of 27 tubes for each pH-NaCL-KS-T combination. For L.innocua

sorbate, temperature and storge time on the probability of one L.monocytogenes, L.innocua, or L.ivanovii cell to initiate growth in a broth substrate model. Using regression methodologies an attempt was made also to derive predictive models which related the probability of growth by one cell in the broth to the effect of exposure of the cells to different levels of pH, NaCL, potassium sorbate and temperature during incubation of up to 68d. These studies were initiated in an effort to identify antimicrobial systems which could be used eventually to control the growht of L.monocytogenes in soft Hispanic cheeses mostly made without the use of starter cultures and having final pH levels above 6 (24).

Materials and methods :

Experimental design

To assess the effect of pH, NaCL and potassium sorbate (KS), temperature (T), and time of storage on the probability of one cell of each of three Listeriae spp to initiate growth three experiments were arranged in a factorial fashion. This 3(2×3×3×4) design included: three Listeriae species (a pool of two L.monocytogenes strains, L.innocua and L.inanovii), two levels of pH (5.9 and 5.3)three levels of NaCL (0.5, 4 and 7.5%), three levels of KS (0, 0.15 and 0.3%), four storage temperatures (30,20,8 and 4°C) and repeated observations (19 - 21 times) for growth in Brain Heart Infusion broth (BHI)(Difco Laboratories, Detroit MI) for up to 68 days. Previous study (22,54) from this laboratory evaluated the $\log_{10}$ P% of L.monocytogenes as affected by pH(5.3, 5.9 and 6.5), NaCL (0.5, 4 and 7.5%), KS(0,0.15 and 0.3%) and storage at 4,8,20 and 30°C for up to 68d. Data dealing with pH 5.3 and 5.9 in these earlier studies were incorporated in the data pool of this study and reevaluated.

Test organisms :

Listeria monocytogenes strains VPH-1 (a clinical isolate, serotype 4b), VPH-2 (isolated from cheese, serotype 1/2b), L.innocua and L.ivanovii (all obtained from Dr Hailu Kinde, University of California, Davis, Veterinary Diagnestic Laboratory Services), were used as test organisms. Culture stocks lyophilized on porcelain beads were kept at 2°C. Working cultures were maintained on Tryptose -Soy-Agar with 5% sheep blood (TSBA) (PMLMicrobiologicals, Sacramento CA).

cases in humans due to L.ivanovii and L. seeligeri have been reported (37,56) Listeria species, and more specificaly L.monocytogenes, are widely distributed in meat, poultry, fish, and raw agricultural products (23,27,29,34,46). Prevalence studies in both dairy and meat processing plants have demonstrated the frequent presence of L.monocytogenes in such environments (10,23,46,55,13,29). Furthermore, these studies and the repeated isolation of Listeria spp. from finished products (2,3,4,5,27,35,–41,46), lead to the belief that the problem might most often be one of contamination during process or post-process recontamination rather than one of thermal process failure (55). It has been estimated recently (44) that the annual costs of listeriosis cases in the US total $480 million or more. Lawsuits associated with the Listeriosis cheese outbreak in California have amounted to over $ 800 million the manufacturer has gone bankrupt, and officers of the company were fond to be criminally negligent and have served prison sentences (30). The regulatory agencies specify a zero tolerance for Listeriar in those foods which undergo no further processing before consumption (46). In view of these developments there has been an urgent need for information concerning the effects of various intrinsic (pH, water activity, brine, antimicrobial additives, redox potential, microbial competition) and extrinsic (temperature, atmosphere, relative humidity)food and environmental parameters on growth or inhibition of Listeria in contaminated foods.

A number of recent papers have described the effect of temperature (7,11,28,32), pH and acids (9,16,26,36,40,49), sodium chloride (11,12,39,48,47) and potassium sorbate (15) on the growth and inhibition of L.monocytogenes. The effect of these and other food and environmental variables on the other Listeria species remains unclear. A few recent attempts have also been made for the development of mathematical models which relate the growth of L.monocytogenes to a number of variables like those mentioned above (7,9,36,43,57). In this paper we report the results of a study in which we quantified the individual and interacting effects of pH, NaCL, potassium

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Probability of growth initiation of Listeria Spp in a model broth as affected by species, pH, temperature, sodium chloride, potassium sorbate and storage time

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Summery:

The effects of NaCL (0.5, 4.0, 7.5%) potassium sorbate (KS, 0.0, 0.15, 0.3%), pH (5.9,5.3), temperature (T,4,8,20,30°C), time (D, up to 58-68d) and inoculum (10^{-2}-10^{7}) on the $\log_{10}$ probability percentage (P)of one cell of L.monocytogenes, L.innocua, L.ivanovii to initiate growth in brain heart infusion broth were evaluated in a factorial design study. The P of L.monocytogenes was affected significantly ($0.0001<P<0.02$) by pH, T, NaCL, KS, D, and T×pH, T×NaCL, T×KS, pH×KS, NaCL×KS interactions and L.innocua and L.ivanovii by pH, T, NaCL, KS, D, and T×pH, T×KS, pH×KS, NaCL×KS, T×pH×KS, T×NaCL×KS and pH×NaCL×KS interactions. The P decreased by increasing concentrations of KS, NaCL, and decreasing pH and T and prolonging storage. The bacteriostatic effect of pH, KS and NaCL was enhanced and the bactericidal effect was diminished with the decreasing of T. L.ivanovii was the most and L.monocytogenes the least sensitive species to the inhibitory effect of the variables studied. There was a concentration dependent antagonism of NaCL (4%) on KS action. Therefore a particular caution may be needed in high pH foods with a moderate salt concentration preserved with sorbate. Regression equations were derived relating P to NaCL, KS, pH, T and D. From the equations, the number of cells needed to initiate growth can be calculated.

Introduction :

Of the various species of Listeria, L.monocytogenes is the most important to human and animal health (46). Rare clinical

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