SURVIVAL OF PATHOGENIC BACTERIAL ORGANISMS IN CHALLENGE STUDIES OF FINE GRANULATED SUGAR

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SUMMARY

Pathogenic organisms such as *Salmonella typhimurium*, *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* are not capable of surviving sugar refining process temperatures. Therefore, these organisms can be introduced into granulated sugar only after production if good manufacturing and warehousing practices are not employed. Hence, a series of challenge studies using different concentrations of the above organisms were carried out at American Crystal Sugar Company to determine the duration of survival of these organisms when inoculated into fine granulated sugar.

The results obtained showed that the duration of survival of pathogens evaluated was dependent on innoculum concentration – the higher the concentration, the longer the survival and vice versa. *E. coli* was found to give the lowest duration of survival while *S. typhimurium* gave the longest survival at 10^7 concentration. However, *Staph. aureus* gave varying results in two different sets of studies with differing series of concentration (10^0, 10^2, 10^4, 10^6, and 10^1, 10^3, 10^5, and 10^7). The studies with 10^0, 10^2, 10^4, and 10^6 concentration for *Staph. aureus* gave a maximum average survival of 81.25 days at 10^6 concentration, while the two studies with 10^1, 10^3, 10^5, and 10^7 concentration gave a maximum average survival of 47.5 days at 10^7 concentration. For the most part low concentration levels of organism of 10^0-10^4 in the case of *E. coli* and 10^0 concentration for *S. typhimurium* and *P. aeruginosa* did not survive at all or gave a low survival rate of less than one day on average. However, in contrast *S. aureus* at a low concentration of 10^0 cfu/ml gave a long average duration of survival of 15.75 days. Also it was disturbing to find out that at higher concentrations of 10^6-10^7 cfu/ml *S. typhimurium, S. aureus, P. aeruginosa* were capable of surviving for a long period of time in granulated sugar with an average duration of survival of 10-81.25 days. Pathogenic organisms such as these may be introduced after production by humans, animals (e.g. rodents), birds, and insects. Therefore, good warehousing practices become very important to prevent risk of pathogen contamination even in a low risk product such as sugar, which has not been associated with an outbreak of food poisoning due to its low water activity.

INTRODUCTION

Four pathogen challenge studies of fine granulated sugar with *Salmonella typhimurium, Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa* were carried out at American Crystal Sugar Company during 1996, 1997, and 1998. Each of these studies lasted for a period of 2-3 months, and two series of concentrations of microbes 10^0, 10^2, 10^4, 10^6 cfu/ml and 10^1, 10^3, 10^5, and 10^7 cfu/ml were evaluated.

MATERIALS AND METHODS

A) Test organisms – 18-24 hr cultures of *Salmonella typhimurium, Escherichia coli, Staphylococcus aureus,* and *Pseudomonas aeruginosa* grown on standard plate count agar slants were used for inoculation.

B) Substrate – Sugar was used as the substrate for the challenge studies. Ten-pound packages of sugar were obtained from the Moorhead factory on 3/21/96. Eight hundred grams of sugar were then aseptically weighed into each of 42 – 1L sterile plastic bottles for Study I. However, the numbers of bottles used in the next studies were reduced as a feel for the duration of survival of the organism was obtained after Study I.

C) Preparation of Inoculum
   1) Concentrated Bacterial Suspension – The 18-24 hour bacterial cultures were washed down with 0.85% saline solution.
   2) MacFarland's Standard #1 – This standard was made with 0.1 ml 1% barium chloride and 9.9 ml 1% sulfuric acid resulting in a bacterial concentration of 300 x 10^6 cfu/ml.
   3) Turbidimeter (Biolog, Inc.) – The MacFarland's Standard #1 was set to a specific transmittance. The washed down slant, bacterial suspensions were then added to 0.85% saline to obtain the same transmittance as MacFarland's Standard #1. Therefore cultures of concentration equal to 300 x 10^6 cfu/ml were obtained.
   4) Plate Counts – The bacterial suspensions equal to the MacFarland's Standard #1 were plated and gave counts of 10^8 cfu/ml in one series of studies and 10^7 cfu/ml in the next series of studies.
   5) Serial Dilutions – These were prepared using bacterial cultures of *E. coli, Staph. aureus, S. typhimurium*, and *P. aeruginosa* standardized with MacFarland's Standard #1 which were found to have 10^8 cells/ml on plating. This tube (#1) was then diluted in series into six other tubes containing 0.9 ml of saline with 0.1 ml aliquots of sample being used for transfer between tubes. This gave a series of dilutions from 10^8 to 10^2. Next 0.1 ml of sample from 10^8, 10^6, 10^4, and 10^2 concentrations now containing 10^7, 10^5, 10^3, and 10^1 cells respectively were added to 10 ml of saline (for easier dispersion in sugar) and this complete volume was added to each of 800 g of sugar used in the experiment. In another series of trials, dilutions of 10^6, 10^4, 10^2, 10^0 concentrations were inoculated into sugar and prepared similar to above starting with 10^7 cells/ml on plating.

D) Spiking of Sugar – Duplicate 10 ml aliquots of 10^1, 10^3, 10^5, and 10^7 cfu/ml concentrations or 10^0, 10^2, 10^4, and 10^6 concentrations (as the case may be) for each test organism were inoculated into each of 800 g of weighed sugar in plastic bottles and shaken up vigorously for even spread of inoculum. One bottle of sugar was also spiked with 10 ml of 0.85 % sterile saline as a negative control. The bottles were then placed in a cupboard with the cap loose until survival check were made daily or weekly as required depending on the concentration of organisms in question.

E) Survival Checks – for pathogenic test organisms were initially done weekly and then daily for those organisms showing low survival especially at lower inoculum concentrations. The test methods used for these pathogenic organisms were AOAC approved rapid methods or conventional methods as given below. All tests were carried out along side a positive and negative control.

   Escherichia coli – Coli complete disc method (Bio Control Systems) 50 g of the sugar sample was weighed into 450 ml of buffered water. One (1) ml of this 1:10 dilution was used to inoculate each of three tubes of Lauryl Sulfate Tryptose Broth (LST). One Coli complete disc was then added aseptically to each of the three tubes inoculated per sample. The inoculated tubes were incubated at 35°C and observed under UV light (366 nm) at 24 and 48 hrs for presence or absence of milky blue fluorescence and blue disc. The

presence of milky blue fluorescence was interpreted as a confirmed positive result for *E. coli* and presence of a blue disc as a confirmed positive result for total coliforms.

Salmonella typhimurium – 1-2 test (BioControl Systems Inc.) The sample (25 g) enriched in lactose broth (225 ml) per 24 hr at 35°C was added to the 1-2 test gel diffusion chamber. The 1-2 test chamber in turn had been prepared for addition of inoculum by prepping it with Reagent #1 (iodine-iodine solution) and Reagent #2 (antibody preparation). The 1-2 test unit was incubated at 35°C for 14-30 hr and observed for a U-shaped immuno band and compared to the control. The absence of an immuno band indicated a negative test.

Staphylococcus aureus – 50 g of the sugar sample were weighed into 450 ml buffered water. Additional dilutions of 1:100 and 1:1000 were made. This was inoculated into three tubes of Tryptic Soy Broth (TSB) for each of the three dilutions with 1 ml aliquots of decimal dilutions. Tubes were incubated at 35°C for 48 hrs. Positive tubes were streaked on Baird Parker Agar plates which were incubated for 48 hrs at 35°C. Suspect colonies were picked and transferred to 0.2 ml of Brain heart infusion (BHI) broth for 18-24 hr at 35°C. Next 0.5 ml of EDTA coagulase plasma was added to BHI tube and observed for coagulation.

Pseudomonas aeruginosa – 10 g of sample were added to 90 ml of Tryptic Soy Broth (TSB) and incubated for 48 hr at 35°C. Turbid TSB bottles were then streaked on cetrimide agar plates that were in turn incubated at 35°C for 48 hr. Suspect greenish blue colonies were then transferred to a cytochrome oxidase strip. A purplish smear developing in 30 sec. indicated a positive test (organism should also be gram negative). A clear TSB bottle after incubation on initial test indicated a negative *Pseudomonas* test.

F) Water Activity Measurements (a_w) were made using an Aqua lab CX-2 unit. The instrument was standardized using saturated K_2SO_4 and water. Measurements were made between 21 and 23°C. Ten (10) ml of 0.85% saline was added to 800 g of sugar similar to test samples. Four bottles (two sets) were used for evaluations (one set started at Day 1 and the other set started at Day 3). Each set had a bottle with a loose cap and a tight cap. These evaluations were carried out through a period of 52 days.

RESULTS AND DISCUSSION

Summary charts of four pathogen challenge studies are given in Table 1 and Table 2. Also this data is shown in graphical form in Fig. 1 and 2. Table 3 gives water activity (a_w) information on inoculated sugar, while Table 4 gives approximate minimum levels of water activity permitting growth of microorganisms at temperatures near optimal.

The first three pathogen challenge studies were carried out for a period of 3 months. Study I was carried out from 4-17-96 to 7-30-96, Study II from 8-12-96 to 11-4-96, and Study III from 6-9-97 to 9-2-97. In addition, a follow-up study for *Salmonella typhimurium* was also carried out from 9-22-97 to 10-20-97 so as to obtain a more consistent set of data for this organism. Also Study IV for *Staph.*, *E. coli*, and *P. aeruginosa* was carried out from 9/14/98 to 11/16/98 due to different concentrations of organism being used in different studies.

Results obtained in these studies have shown that *Escherichia coli* at different concentrations of 10^0 to 10^4 cfu/ml gave a low survival rate of <1 day. However, a concentration of 10^7 cfu/ml gave a survival of 17.5 days. Also, *E. coli* was shown to give the lowest survival rate in sugar as compared to all other pathogens evaluated.

Intestinally pathogenic *E. coli* is defined as those *E. coli* strains that are capable of causing diarrheal disease in man or animals. Subdivision of the pathogenic forms is made on the basis of the mechanism underlying the illness (Levine, 1987, Doyle and Padhye, 1989). Presently, four main types of pathogenic *E. coli* have been associated with food-borne disease: enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), and enterohaemorrhagic *E. coli* (*E. coli* 0157:H7;EHEC). Therefore, these types of E. *coli* are not desired in foods or food ingredients.

Salmonella typhimurium was shown to give the longest time of survival in sugar inoculated and stored at room temperature at 10^7 concentration. The average time of survival in two studies at a concentration of 10^7 cfu/ml was 63 days while survival at 10^0 cfu/ml concentration was <1 day. Also the average time of survival at concentration of 10^1 and 10^6 cfu/ml varied between 4 and 43 days respectively. Although salmonellae do not form spores, they can survive for long periods in foods and other substrates (ICMSF 1996, Micro-organisms in Foods 5). Salmonellae have been found to survive longer than 10 weeks in butter stored at temperatures between -23° and 25°C (Sims et al., 1969) and 6 months in milk stored at room temperature or in an ice box (Berry, 1927). Their survival in dry environment of chocolate is remarkable, numbers declining only slightly over months in milk chocolate (a_w 0.32-0.41) or bitter chocolate (a_w 0.30-0.51) (Tamminga et al., 1977). Salmonellae also survive well on surfaces such as ceramic, glass, and stainless steel (McDade and Hall, 1964) and on human skin (Pether and Gilbert, 1971). The outbreak of *Salmonella* food poisoning also has been reported from cereal in the Netherlands. The cereal incriminated in food poisoning had been made from large consignments of grain on barges on canals on which pigeons and other birds have been feeding. (Struijk C.B., 1997, and D.A.A. Mossel et al, 1995, Essentials of the Microbiology of Foods).

Table 1. The Duration of Survival of Different Concentrations of Pathogenic Bacterial Organisms in Inoculated Granulated Sugar

		Challenge Study I	Challenge Study II	Challenge Study III	Challenge Study IV	Average
ORGANISM	CONCENTRATION	Approximate number of days for survival in sugar				
Salmonella *typhimurium*	10^6		61		25	43
	10^4		35		11	23
	10^2		17		1	9
	10^0		<7*		<1	0.5
Escherichia coli	10^6	8	17	5.5		10.17
	10^4	<5*	1	1		1
	10^2	<5*	<1	<1		0.5
	10^0	<5*	<1	<1		0.5
Staphylococcus aureus	10^6		80	82.5		81.25
	10^4		45	36.5		40.75
	10^2		38	4		21
	10^0		31	<1		15.75
Pseudomonas aeruginosa	10^6			17		17
	10^4			11		11
	10^2			1		1
	10^0			<1		0.5

		Challenge Study I	Challenge Study II	Challenge Study III	Challenge Study IV	Average
ORGANISM	CONCENTRATION	Approximate number of days for survival in sugar				
Salmonella typhimurium	10^7	101		25		63
	10^5	80		4		42
	10^3	24		4		14
	10^1	<7*		4		4
Escherichia coli	10^7				17.5	17.5
	10^5				8	8
	10^3				1	1
	10^1				<1	0.5
Staphylococcus aureus	10^7	45			50	47.5
	10^5	29			29	29
	10^3	10			20	15
	10^1	<7 = 3.5			15	9.25
Pseudomonas aeruginosa	10^7	17	25		25	22.3
	10^5	4.5	19		22	15.17
	10^3	2	11.5		12.5	8.67
	10^1	1	11.5		6	6.17

* Survival checked only on Day 5 or Day 7 for the first time. Therefore, these numbers were not included for calculations of average time of survival. Also <1 day was considered as 0.5 day in average calculations.

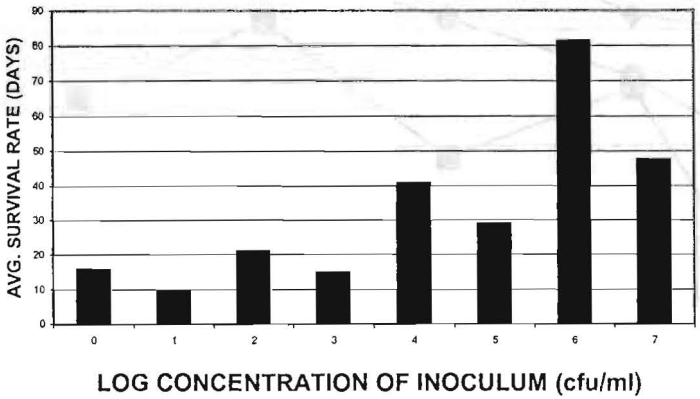
Table 2. Pathogen Survival in Sugar
A Summary of Four Studies

ORGANISM	CONCENTRATION (cfu/ml)	RANGE OF SURVIVAL (days)	AVG TIME OF SURVIVAL (days)
Salmonella typhimurium	10^7	25-101	63
	10^6	25-61	43
	10^5	4-80	42
	10^4	11-35	23
	10^3	4-24	14
	10^2	1-17	9
	10^1	<7-4	4
	10^0	<7-<1	0.5
Escherichia coli	10^7	17.5	17.5
	10^6	5.5-17	10.17
	10^5	8	8
	10^4	1-<5	1
	10^3	1	1
	10^2	<1	0.5
	10^1	<1	0.5
	10^0	<1	0.5
Staphylococcus aureus	10^7	45-50	47.5
	10^6	80-82.5	81.25
	10^5	29	29
	10^4	36.5-45	40.75
	10^3	10-20	15
	10^2	4-38	21
	10^1	<7-15	9.25
	10^0	<1-31	15.75
Pseudomonas aeruginosa	10^7	17-25	22.3
	10^6	17	17
	10^5	4.5-22	15.17
	10^4	11	11
	10^3	2-12.5	8.67
	10^2	1	1
	10^1	1-11.5	6.17
	10^0	<1	0.5

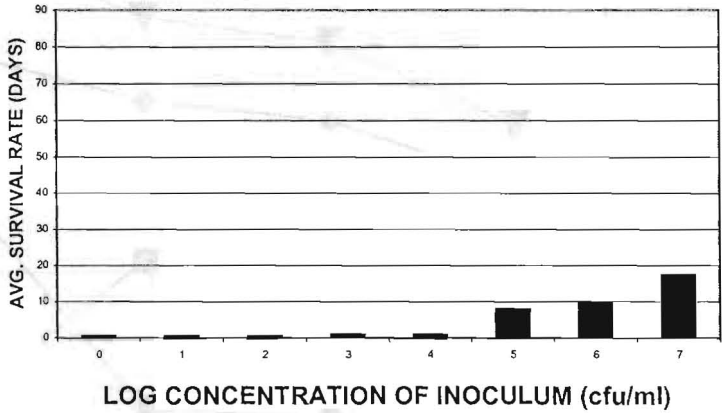
Staphylococcus aureus also showed a long duration of survival in fine granulated sugar. However, results obtained in two sets of studies at concentrations of 10^0, 10^2, 10^4, and 10^6 and two sets of studies at concentrations of 10^1, 10^3, 10^5, and 10^7 gave varying results. The first series of dilutions gave longer survival times with 10^0 dilution giving an average survival of 15.75 days and 10^6 concentration an average survival of 81.25 days. However, in the second series of dilutions the 10^7 concentration showed a lower average survival (47.5 days) as compared to that obtained at 10^6 concentration (81.25 days).

SURVIVAL RATES OF 4 DIFFERENT PATHOGENS IN GRANULATED SUGAR

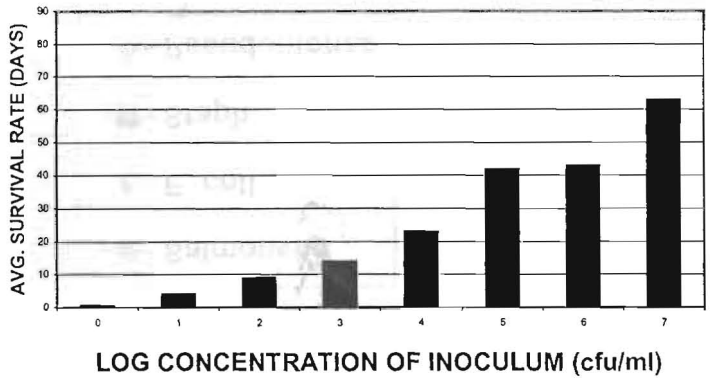
Staphylococcus aureus



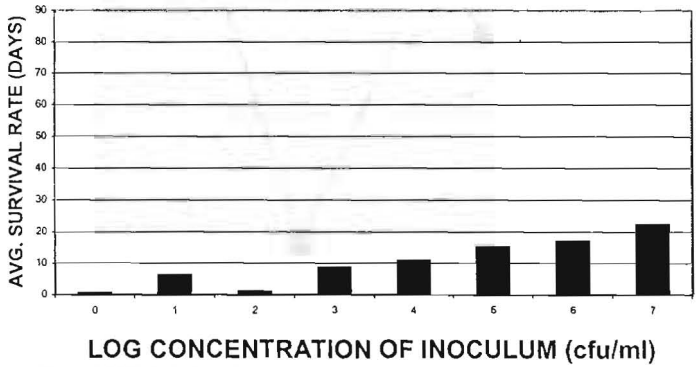
Escherichia coli

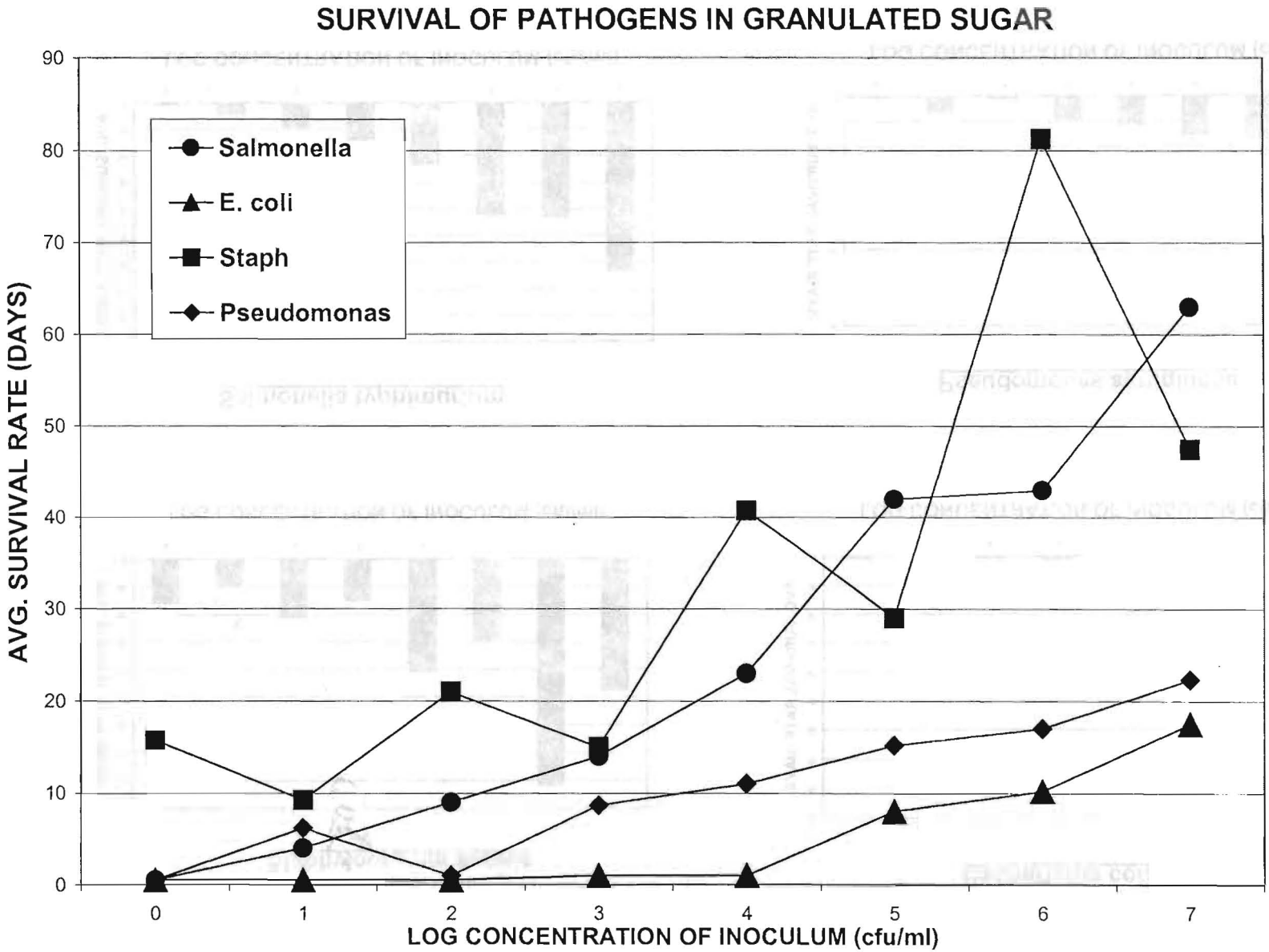


Salmonella typhimurium



Pseudomonas aeruginosa




SURVIVAL OF PATHOGENS IN GRANULATED SUGAR
AVG. SURVIVAL RATE (DAYS)
LOG CONCENTRATION OF INOCULUM (cfu/ml)
Salmonella
E. coli
Staph
Pseudomonas
0
10
20
30
40
50
60
70
80
90
0
1
2
3
4
5
6
7

Staphylococci are commensals of the body surface of warm-blooded animals. The diseases they cause include, acute infection, such as septicaemia, and acute toxemias, such as staphylococcal food poisoning, but the main source of enterotoxin-producing strains is the human carrier. Contamination of food can occur as a result of poor hygienic practices at any part of the food chain and, if combined with subsequent storage at temperatures and for periods that permit significant growth, the formation of food poisoning enterotoxins may occur (ICMSF 1996, Microorganisms in Foods 5). *S. aureus* cells are easily killed by heat, but are salt tolerant and may be selected for in salt-containing products or products with lowered water activities, e.g. sugar. They are very resistant to drying and survive well under most environmental conditions and thus can persist for some time in food-production areas and may act as a source of contamination for products that are not properly protected. Staph. enterotoxins are produced under a wide range of environmental and storage conditions. These enterotoxins are very resistant to heat and will survive cooking and some sterilization processes. (ICMSF 1996, Microorganisms in Foods 5).

Although *Pseudomonas aeruginosa* is not a major food borne pathogen, it has been incriminated in enteritis transmitted by water and foods (Thom A. R. et al., 1970; Falcos et al., 1972).

This organism was found to have an average survival time of 22.3 days in fine granulated sugar at 10^7 cfu/ml concentration. Also as in the case of *Salmonella* and *E. coli*, the 10^0 concentration was found to survive for <1 day while 10^1 to 10^6 cfu/ml concentrations were found to survive on an average for 6 to 17 days, respectively.

Microorganisms require water for survival. Therefore too much "free" water in a product can serve as a medium for microbial reproduction, travel, and contamination. But if water within a product is held with enough force (bound water), microorganisms will not be able to exert the energy required to obtain water necessary for their subsistence in this way. Therefore the term water activity (a_w) is commonly used in the evaluation of food quality and safety.

Water activity (a_w) is a water energy measurement. It is an indication of "free" water in a sample; "free" referring to the water particles in a product that are not chemically or physically bound.

Therefore products with no free water will have an a_w of 0.0 while a product such as pure water will have an a_w of 1.0.

Microorganisms generally have optimum and minimum levels of a_w for growth depending on other growth factors in their environments. Minimum levels permitting growth of a number of organisms are shown in Table 4 (Vanderzant, C. et al., 1992)

In general bacteria require higher values of a_w for growth than fungi, with gram negative bacteria having higher requirements than gram positives.

Table 3. Water Activity (a_w) Values of Sugar in Control Bottles with Sterile Saline

Set 1	Day 1	Day 2	Day 3	Day 7	Day 11	Day 21	Day 29	Day 35	Day 52
Bottle w/loose cap	0.843	0.826	0.825	0.839	0.831	0.831	0.827	0.828	0.828
Bottle w/tight cap	0.839	0.833	0.831	0.831	0.831	0.831	0.829	0.830	0.813

Set 2	Day 3	Day 7	Day 11	Day 21	Day 29	Day 35	Day 52
Bottle w/loose cap	0.838	0.834	0.830	0.833	0.831	0.827	0.829
Bottle w/tight cap	0.834	0.831	0.831	0.835	0.827	0.827	0.831

a_w value of granulated sugar = 0.622

Table 4. Approximate Minimum Levels of Water Activity Permitting Growth of Microorganisms at Temperatures Near Optimal (Vanderzant, C. et al., 1992)

Organism	Water Activity a_w
Pseudomonas spp.	0.97
Salmonella spp.	0.95
Escherichia coli	0.95
Staphyloccus aureus	0.86

In the trials carried out at American Crystal Sugar Company, 10 ml of inoculum with the desired concentration of test organism (10^7, 10^5, 10^3, or 10^1) or in the second series of trials with concentration (10^6, 10^4, 10^2, and 10^0) was added to each sterile bottle containing 800 g of sugar. This was to enable more uniform distribution of the test organism in the sugar as far as possible. However, a_w readings taken in inoculated sugar over a period of time (Table 3) show the highest a_w reading on Day 1 in bottle with a loose cap to be 0.84. This value decreased with time and at Day 52 was down to a value of 0.828. See Table 3 for detail. However, it should be noted that even at the highest a_w of inoculum of 0.84 in sugar on Day 1 of experiment this value was still below the minimum water activity levels permitting growth of test organisms. See Table 4 for detail of minimum levels of a_w permitting growth of microorganisms. The a_w value of granulated sugar was found to be 0.622. However, as mentioned previously, salmonellae have been found to survive remarkably in dry environments such as chocolate, numbers declining only slightly over months in milk chocolate (a_w 0.32 – 0.41) or bitter chocolate (a_w 0.30 – 0.51) (Tamminga et al., 1977). Therefore the survival of these test organisms in sugar is not surprising.

The food in which an organism is ingested can markedly affect the number of microbial cells required to initiate infectious disease. For example, numbers of enteric pathogens in excess of 10^4 are normally required to cause infectious bacterial enteritis (Armstrong R. W. et al., 1970).

However, if such organisms are ingested with a small volume of water or food between meals, they pass almost immediately from the stomach to the duodenum (Mossel and Oei, 1975) and infect at a markedly lower level of cfu's. Another important possibility is that enteric pathogens are protected against the marked bactericidal effect of the gastric fluid of healthy individuals when enveloped in lipids in foods (Fontaine R. E. et al., 1978).

As a consequence of such phenomena, the minimum oral infective dose of pathogens ingested with food may be as low as one to ten cells (Craven P. C. et al., 1975; Lipson and Meikle, 1977; Robinson D.A., 1981; Greenwood and Hooper, 1983; D'Aoust, 1985). This is a disturbing finding. Therefore, good manufacturing and warehousing practices are important and critical in preventing an outbreak of food poisoning even from a low risk product such as sugar.

CONCLUSION

This study has shown that generally when low concentrations of organisms are inoculated into sugar the organisms did not survive at all or gave a low average survival rate of less than one day (10^0-10^4 concentration for *Escherichia coli* and 10^0 concentration for *Salmonella typhimurium* and *Pseudomonas aeruginosa*). In contrast, *Staphylococcus aureus* at a low concentration of 10^0 cfu/ml gave a long average duration of survival of 15.75 days. Also at high concentrations of 10^6 and 10^7 cfu/ml *S. typhimurium*, *E. coli*, *S. aureus*, and *P. aeruginosa* were capable of surviving in granulated sugar for long periods of time with an average duration of survival of 10-81.25 days.

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