

Survival of *Salmonella eastbourne* and *Salmonella typhimurium* in chocolate

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SUMMARY

Experiments were carried out to assess the reduction rate of two salmonella strains (*S. eastbourne* and *S. typhimurium*) in chocolate bars. After artificial contamination of chocolate, after 'conching', with about 10^6 *S. eastbourne*/g. this organism was still recovered after 9 months storage. The strain of *S. typhimurium* was less resistant. Both serotypes died off more rapidly in bitter chocolate than in milk chocolate.

After contamination with a smaller dose (about 10^3 /g.) with these two serotypes, similar differences were observed.

INTRODUCTION

During the period July 1973 to June 1974 more than 200 cases of food poisoning were observed in Canada and the U.S.A. caused by the otherwise relatively rare *Salmonella eastbourne*. The major group affected were children aged 1-4 years. An epidemiological inquiry showed that in about 50 % of these cases consumption of chocolate items, produced for the Christmas season in one specific chocolate factory, could be ascertained.

Upon investigation a number of chocolate novelties made in this factory, the code numbers of which corresponded to those of the products eaten by some of the infected persons, were shown to be contaminated with *S. eastbourne*. A survey of the plant revealed the presence of the serotype both on the premises and in some lots of roasted cocoa beans and one end-product (Handzel, 1974; Craven *et al.* 1975).

Reports of the occurrence of *Salmonella* in chocolate products had been published before (Lennington, 1967; Depew, 1968). The above-mentioned outbreak, however, was the first one in which a clear connexion could be demonstrated between chocolate and illness.

The contaminated samples had been produced during the period May-October 1973. In view of the fact that most cases occurred long afterwards, the strain concerned is likely to live long in chocolate. Indications that salmonellas can

actually survive in chocolate for quite a long time, in spite of the presumably low water activity (a_w) values, have been found before by Foster (1968) and Rieschel & Schenkel (1971). In the latter case salmonellas could be detected even after 15 months. These investigations, however, did not yield figures regarding the numbers of salmonellas or the speed of their reduction. Barrile, Cone & Keeney (1970) did determine some quantitative data, but these were restricted to milk chocolate, and the initial numbers were very low. Even with these low initial numbers, however, after 15 months storage at room temperature salmonellas were still present.

Upon inquiry the chocolate incriminated in the *S. eastbourne* outbreaks appeared to be of a composition different from that normally manufactured in The Netherlands. The question arose whether, in chocolate with a composition as usual in The Netherlands, long survival of salmonellas and especially of the strain concerned is also possible, and if so, what the reduction rate would be. It appeared advisable to study, besides the *S. eastbourne* strain, a strain of another serotype; for this purpose *S. typhimurium*, phage-fermentative type II 505 was chosen, because the majority of human strains in The Netherlands belong to this type.

MATERIALS AND METHODS

Two series of experiments were carried out. In the first, *S. eastbourne* isolated from chocolate associated with the food-poisoning cases in the U.S.A. was used; the strain was obtained from Dr W. Barker, Communicable Disease Centre, Atlanta, Ga, U.S.A. In the second series *S. typhimurium* II 505 was used as the inoculum.

In both series of experiments weighed portions of 3–4 kg. of melted chocolate held at a temperature of *ca.* 40° C. were inoculated with 2–3 ml. of a fully turbid 24 hr. broth culture or a suitable dilution of the *Salmonella* strain. Two types of chocolate as ordinarily used for the preparation of chocolate bars were used:

(a) 'bitter' – of the following composition: sucrose 49.4 %; cocoa butter 10.5 %; cocoa mass 39.5 %; lecithin 0.6 %;

(b) 'milk' – of the following composition: sucrose 40.6 %; cocoa butter 25.0 %; cocoa mass 9.1 %; lecithin 0.5 %; skim milk powder (1 % fat) 9.9 %; whole milk powder (25 % fat) 14.9 %.

The cocoa mass used consisted of fat 54.5 %, water 1.5 %, non-fat cocoa solids 44 %.

After inoculation the chocolate was thoroughly mixed for about 15 min. in a Hobart mixer.

Starting from both types of chocolate (bitter and milk) two portions were prepared with approximate concentrations of 10^6 and 10^3 salmonellas/g. respectively. Thus in each series of experiments four portions of material were available.

From each mixture thus prepared about 30 chocolate bars were cast in moulds as ordinarily used in a chocolate factory. As far as possible the procedure was similar to that used in a chocolate plant. The bars were kept at 4° C. for about $\frac{1}{2}$ hr., after which they were wrapped in the same kind of wrapping as used in practice,

the staff wearing sterile gloves to protect themselves, as well as the bars, against contamination.

The above procedure was preceded by preparation of *ca.* 15 bars of each type of chocolate without added salmonellas. These bars served as controls.

The number of salmonella colony forming units (cfu) per 1 ml. of the culture inoculated was determined by counting in plate count agar. The pour-plate method was used; the plates were incubated for 48 hr. at 37° C.

Artificial contamination, preparation of the bars and plate counts were carried out in the laboratory of a Dutch chocolate factory. For reasons of safety, a room was used lying at some distance from the plant where the actual preparation of the commercial products takes place.

Each portion of contaminated and wrapped chocolate was then divided in two equal parts, which were kept at 20° C. in two different laboratories.

After different periods of storage from 1 day to 9 months surviving salmonellas in each portion of chocolate were counted in both laboratories by the most probable number (MPN) method. The procedure of Edel & Kampelmacher (1969, 1973) was used. Between 40 and 60 g. of chocolate was homogenized to make a 1/10 dilution in buffered peptone water at 37° C., and further tenfold dilutions in peptone water were made from this. Five 100 ml. volumes and five 10 ml. volumes of the 1/10 dilution, and five 10 ml. volumes of any higher dilutions, were incubated for 16–24 hr. at 37° C.; all tubes were then subcultured by transferring 1 or 2 ml. to 10 or 20 ml. respectively of Muller–Kauffmann tetrathionate broth. After 24 hr. incubation all tetrathionate tubes were subcultured on brilliant green phenol red agar plates. All tubes with negative plate cultures after 24 hr. at 37° C. were subcultured again. Suspected salmonella colonies were examined serologically and biochemically.

The a_w of the contaminated chocolate was determined by the method of Northolt (1972, 1973).

RESULTS

Table 1 gives the results of the microbiological examination of the contaminated chocolate bars. The initial numbers ('0 days') have been calculated from the results of the colony counts of the inoculated cultures, the other results, as already mentioned, with the aid of MPN tables.

Although *S. eastbourne*, when inoculated at a high concentration into milk chocolate, shows a rapid decrease in numbers at first, any further decrease during 9 months is no more than about 4 logs. The reduction in numbers in bitter chocolate is greater, but after 9 months the organisms are still detectable. The difference between the results in milk and bitter chocolate is less pronounced when the original concentration of organisms is lower ('low inoculation').

S. typhimurium survives less well, and starting with a high inoculation there is a drop of about 6 logs after 6 months in milk chocolate and of nearly 7 logs in bitter chocolate. With a low inoculation *S. typhimurium* was not detectable after 34 days in bitter chocolate; in milk chocolate they were found in very small

Table 1. *Counts of S. eastbourne and S. typhimurium in milk and bitter chocolate stored at 20° C. for up to 9 months*

Storage time	Bitter (log MPN/100 g.)				Milk (log MPN/100 g.)			
	High inoculation		Low inoculation		High inoculation		Low inoculation	
	S.e.	S.t.	S.e.	S.t.	S.e.	S.t.	S.e.	S.t.
0 d*	7.81	7.94	5.20	4.86	8.30	7.93	5.20	5.04
1 d†	6.18	4.63	4.64	1.88	7.64	5.97	4.64	2.63
	—	3.73	—	1.69	—	5.45	—	2.34
6 d	4.18	2.97	1.43	0.86	5.97	4.97	3.18	1.32
	3.96	3.04	2.32	0.30	6.20	5.38	2.73	1.52
13 d	3.18	2.18	1.30	0.56	6.38	4.97	3.18	1.18
	3.45	2.34	1.90	0.30	5.73	4.69	2.54	1.36
20 d	2.97	2.34	1.18	neg	5.97	4.85	2.97	1.11
	2.96	1.79	1.56	0.30	5.54	4.85	2.54	0.89
34 d	—	1.15	—	neg	—	3.23	—	0.89
	—	1.56	—	neg	—	3.90	—	neg
41 d	2.66	—	1.18	—	5.38	—	2.38	—
	3.20	—	0.65	—	5.73	—	2.23	—
48 d	—	1.11	—	—	—	2.96	—	neg
	—	1.36	—	neg	—	4.11	—	0.89
76 d	2.38	—	neg	—	5.38	—	1.63	—
	2.32	—	1.46	—	5.32	—	1.69	—
83 d	—	0.30	—	neg	—	2.96	—	neg
	—	0.36	—	neg	—	3.54	—	0.30
6 m	2.04	1.11	neg	neg	5.54	2.96	neg	neg
	1.49	neg	neg	neg	4.73	1.32	1.23	neg
9 m	1.11	—	neg	—	5.04	—	1.11	—
	0.70	—	—	—	4.38	—	0.89	—

—, Not done.

neg, All MPN tubes negative.

S.e., *Salmonella eastbourne*.

S.t., *Salmonella typhimurium*.

* Average of two determinations in duplicate.

† Numbers determined during the storage period in the two laboratories are recorded separately.

Table 2. *Water activity (a_w) values of the chocolate bars*

Type ...	Bitter			Milk		
	None	High	Low	None	High	Low
Inoculation ...						
Series I (<i>S. eastbourne</i>)	0.30	0.51	0.44	0.32	0.41	0.38
Series II (<i>S. typhimurium</i>)	0.42	0.48	0.42	0.32	0.40	0.37

numbers up to 83 days, but occasional cultures were negative. The control bars gave negative results.

As far as the a_w is concerned, somewhat higher figures were found in the contaminated bars owing to the fact that water was added with the inoculation (Table 2).

DISCUSSION

As was to be expected from the investigations mentioned in the Introduction, in the present experiments a number of the contaminating salmonellas survived in chocolate for a considerable time. Indeed, a rapid initial decline took place directly after the inoculation; this may have been caused by osmotic shock (Mossel & Koopman, 1965).

A striking feature is that, notwithstanding the longer intervals between the determinations of the MPN's, in the long run the differences between the numbers became continually smaller. In other words, upon graphical plotting the survival curves would approximate more and more to a horizontal line.

From the results it is clear that the *S. eastbourne* strain tested lives longer in chocolate than the strain of *S. typhimurium*. This is also true if the more rapid die-off of *S. typhimurium* immediately after inoculation is taken into account. For comparison, attention may be drawn to the differences in value of the high-inoculated samples after 6 days and after 76 and 83 days. In both series of experiments the chocolate had a similar composition. Consequently, one may assume that the two strains differ in susceptibility to storage at low a_w , the *S. eastbourne* strain having a higher resistance than other *Salmonella* serotypes. This could offer an explanation for the fact that, amongst all serotypes, the relatively rare *S. eastbourne* caused the food poisoning cases brought about by chocolate, a commodity of low a_w . Whether the difference between the two strains examined is also a property of the two serotypes in general should be further investigated.

Some insight into the other intrinsic factors, together with a_w , at work during storage of the chocolate may be obtained by examination of the differences between the death-rate of salmonellas in bitter chocolate and milk chocolate. Obviously in bitter chocolate salmonellas die off more rapidly than in milk chocolate. Ostovar (1973) observed a similar effect when studying the survival of *Staphylococcus aureus* in chocolate. When considering the compositions mentioned in the section on Materials and Methods the following differences may be noted:

- (a) the presence of milk constituents in milk chocolate,
- (b) a considerably larger amount of non-fat cocoa solids in bitter chocolate.

The difference in reduction may be most easily explained by taking into account the antimicrobial effect of cocoa constituents on salmonellas, observed by Busta & Speck (1968). In addition, a protective activity of milk constituents against the antimicrobial effects of low a_w and/or cocoa constituents may be assumed. Investigations in progress may shed more light on this matter.

In connexion with the cases of food poisoning mentioned in the Introduction, the question might be posed whether, in addition to the possible influence of a longer storage life, a greater heat resistance of the *S. eastbourne* strain might have

contributed to the harmful effects of the chocolate. During its preparation chocolate is heated at 60–70° C. ('conching') for a considerable period of time (8–24 hr.). Earlier investigations have demonstrated that several salmonella strains may show high decimal reduction times (*D*-values) in chocolate, suggesting a protective effect by the chocolate mass against the damage of heating (Goepfert & Biggie, 1968; Barrile & Cone, 1970; Barrile *et al.* 1970; Rieschel & Schenkel, 1971). Consequently, if the chocolate is infected before heat treatment, by whatever salmonella strain, part of the initial contamination will always have a considerable chance of survival. It is possible that *S. eastbourne* has still higher *D*-values than most other strains. Further investigations would be necessary to obtain certainty regarding this question.

From the experiments already completed the conclusion can be drawn at least that under the circumstances in this Dutch factory salmonellas, once they have gained access to the product, will die off slowly. In view of the fact that in the food-poisoning cases mentioned before (Craven *et al.* 1975) the average number of *S. eastbourne* organisms was 2.5/g. of chocolate (corresponding to a log number per 100 g. of 2.40), even the chocolate with the small amount of added salmonellas could have caused food poisoning if eaten shortly after manufacture, especially in susceptible persons like young children. As stated before, heating time and temperature during processing cannot guarantee complete destruction of all salmonellas which might be present in the raw materials.

There is therefore in the manufacture of chocolate every reason to exert a scrupulous control of all raw materials and to take all measures during production to prevent contamination of the chocolate mass with salmonellas.

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