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The reliability of the examination of foods, processed for safety, for enteric pathogens and Enterobacteriaceae: a mathematical and ecological study

By E. F. DRION*
Institute for Mathematics and Statistics, Central Organization TNO, The Hague, The Netherlands
and D. A. A. MOSSEL
Chair of Food Microbiology, University of Utrecht, The Netherlands

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SUMMARY

Because of the paucity of quantitative data on numbers of other enteric pathogens in food, the reliability of the examination of processed foods for Enterobacteriaceae was estimated taking *Salmonella* as a model. For this purpose an assessment was carried out of the risk of accepting *Salmonella* contaminated consignments of foods, despite a negative outcome of (i) examination of 1·5 kg samples for *Salmonella*; (ii) examination of one or two 1 g samples for Enterobacteriaceae; (iii) simultaneous application of both tests. The computations were based on the results of the examination of 6830 samples of dried foods, processed for safety, out of a total of 18170 samples.

Only 69 samples permitted the exact calculation of the ϵ-factor, defined as c.f.u./g of Enterobacteriaceae/c.f.u./g of *Salmonella*; 4642 were positive for the former group but 'free' from *Salmonella*, and the rest were negative in both tests. Numbers of c.f.u./g for both groups, and hence the ϵ-factors, varied widely between commodities and also between different consignments of the same food product. The average for ϵ amounted to $5{\cdot}8 \times 10^3$, far from the base-line value of $0{\cdot}75 \times 10^3$ assessed earlier. In only 0·1 % of samples did the Enterobacteriaceae test fail to achieve the required consumer protection.

This investigation therefore substantiates that testing foods processed for safety by examining accurately chosen quantities for ecologically well selected and taxonomically thoroughly defined index organisms is a most effective procedure in terms both of consumer protection and simplicity of examination without compelling the food industry to achieve hardly attainable microbiological quality standards.

INTRODUCTION

The history of the use of Enterobacteriaceae as index organisms

The examination of potable water for the group of micro-organisms that would later come to be known as 'coli-aerogenes bacteria of faecal origin' was introduced independently by Schardinger in 1892 and Smith in 1895. Such a test aimed at

* Retired.

replacing testing for specific pathogenic Enterobacteriaceae, particularly *S. typhi*, which at that time presented almost insurmountable difficulties (Koch, 1890). From about 1920 onwards, the entire coli-aerogenes group has been used as indicator or index organisms in the examination of bottled milk, ice-cream and shellfish (Mossel, 1967) and, more recently, of animal feeds (Willems & Thomas, 1959; Van Schothorst, Mossel, Kampelmacher & Drion, 1966; Reusse, Haffke & Meyer, 1975).

At the present day, effective testing for enteric pathogens is possible. *Salmonella* can be detected with a high degree of reliability by methods now available (Edel & Kampelmacher, 1973) and techniques in current use for detecting *Shigella* (Taylor & Schelhart, 1971), enteropathogenic serotypes of *Escherichia coli* (Brent & Vosti, 1973; Mehlman *et al.* 1975) and *Vibrio parahaemolyticus* (Fishbein & Wentz, 1973), although less refined, are also sufficiently accurate for practical purposes. Nevertheless there is still a place in modern analytical food microbiology for suitably designed tests for indicator organisms in addition to the direct search for pathogens. The justification for this is as follows: (1) failure to detect pathogenic Enterobacteriaceae is of limited significance owing to the highly heterogeneous distribution of these bacteria in many foods (Kallings, Laurell & Zetterberg, 1959; Semple, Graham & Dutton, 1961; Silverstolpe, Plasikowski, Kjellander & Vahlne, 1961; Zipplies, 1964; Ray, Jezeski & Busta, 1971*a*) and feeds (Jacobs, Guinée, Kampelmacher & Keulen, 1963; van Schothorst *et al.* 1966); (2) the detection of certain enteric pathogens e.g. hepatitis A virus (Holmes *et al.* 1973; Dienstag, Flinstone, Kapikian & Purcell, 1975) cannot be entrusted to a non-specialist laboratory; (3) if enteric pathogens are in fact absent from a given sample drawn from a given consignment of food, this result has significance only to the particular consignment sampled; however, if the absence of indicator organisms can be repeatedly established in a series of samples, drawn in succession from a given processing line then the probability that such a commodity would be dangerously contaminated is virtually nil. The latter information is of particular value to Public Health authorities, manufacturers and consumers alike.

A considerable advance on the use of the coli-aerogenes bacteria as indicator organisms was made by the substitution of the entire group of the Enterobacteriaceae for this purpose. This was suggested independently by Seeliger (1952) for the assessment of the microbiological quality of products such as milk, processed by heat for safety, and by Henriksen (1955) for monitoring commodities treated with chemical disinfectants, chlorinated water in particular. In such products all members of the Enterobacteriaceae are of equal significance. Since all types of this group are eliminated by the treatments mentioned, their presence in significant numbers is an indication of either inadequate processing, recontamination or microbial growth after processing; all these features hazarding the safety of the product. The customary practice of confining tests to only the lactose-positive ('coli-aerogenes') members of the Enterobacteriaceae is unwarranted for at least three reasons: (1) the coli-aerogenes organisms are, taxonomically, a very ill-defined group of bacteria (Mossel, 1967; Brown & Seidler, 1973) which leads to the inclusion of quite dissimilar organisms (Koburger, 1964; Rosen & Levin, 1970;

Table 1. *Enterobacteriaceae: significance and probability of detection of genera by 'Enterobacteriaceae' and 'coliform' tests*

Genus	Pathogenicity	Of enteric origin	Detected by 'Enterobacteriaceae' tests	Detected by 'Coliform' tests
Arizona	D	+	+	D
Citrobacter	−	−	+	+
Edwardsiella	D	+	+	−
Enterobacter	−	−	+	+
Erwinia	−	−	+	D
Escherichia	D	+	+	+
Hafnia	−	D	+	−
Klebsiella	D	D	+	+
Levinea	−	−	+	+
Proteus	D	D	+	−
Providencia	D	−	+	−
Salmonella	+	+	+	−
Serratia	−	−	+	−
Shigella	+	+	+	−
Yersinia	+	+	+	−

+ = virtually all strains.
D = variable.
− = virtually no strains.

Alichanidis & Tzanetakis, 1974); (2) a test for lactose-positive organisms can lead to falsely reassuring results in situations where lactose-negative organisms predominate (Sutton & McFarlane, 1947; Hobbs, 1955; Canale-Parola & Ordal, 1957; Emmenegger, 1959; Wundt & Voss, 1963; Messer *et al.* 1971); (3) the sensitivity of the test is sensibly reduced by its limitation to the lactose-positive bacteria (Table 1). The proportion between counts of the coli-aerogenes group and Enterobacteriaceae obviously varies greatly with the microbial ecology of a food, being roughly one in the case of milk and dairy products (Busse, 1968; Harrewijn, Mossel & de Groote, 1972) and as low as 10^{-2} in certain raw meats, poultry and egg products (Table 2).

Reliability of methods in current use

Highly reliable methods for the detection of Enterobacteriaceae in foods are now available, both for their direct enumeration in solid media (Mossel, 1957; Hunyady, Leistner & Luike, 1973) and for presence or absence tests using liquid enrichment media (Mossel, Harrewijn & Nesselrooy-van Zadelhoff, 1974). Both these techniques provide for the recovery of sublethally injured cells from foods with intrinsically antimicrobial properties, e.g. low a_w or low pH, or those that have been stored under conditions, e.g. deep-frozen, which are hostile to non-sporing bacteria (Mossel & Ratto, 1970).

In comparing these methods with techniques which search specifically for pathogens, it is necessary to establish their reliability. This must obviously depend on (1) the size of the samples examined in testing for pathogens and Entero-

Table 2. *A comparison of counts of coli-aerogenes bacteria and Enterobacteriaceae in the same samples of food*

Type of food	$\log_{10}$ average of confirmed* count/g	
	'coli-aerogenes'	Entero-bacteriaceae
Ice cream	1·8	2·1
Salads	2·4	3·2
Sliced sausages	0·8	1·4

* 'Coli-aerogenes' group and Enterobacteriaceae both oxidase negative and fermentative attack on glucose in 'gram-negative diagnostic tubes' (Mossel, Eelderink & Sutherland, 1977); 'coli-aerogenes' in addition fermentative attack on lactose in lactose deep stabs (Mossel *et al.* 1977).

bacteriaceae respectively; and (2) the relative frequency at which given pathogens occur in the total population of Enterobacteriaceae. An attempt to determine this reliability was made in an earlier paper (Drion & Mossel, 1972). It was based on the reliability patterns for various modes of *Salmonella* detection suggested by the United States National Academy of Sciences (Foster, 1971). This second study takes a more general approach to the problem, although the base-line data must again be provided by examination for *Salmonella*, as there is almost no published quantitative information on the occurrence of other food-borne pathogens.

The principle governing our own approach to the use of the Enterobacteriaceae as indicator bacteria has always been to apply such methods in parallel with the usual tests for pathogenic enteric organisms (Mossel, 1958). This is similar to the current practice of examining, e.g. cured meat products for *Staph. aureus* and *Cl. perfringens* (Cragg & Andrews, 1973). Although the tests for Enterobacteriaceae and *Salmonella* are considered separate criteria, they are not unrelated. Salmonellas are members of the Enterobacteriaceae and the methods used for the detection and enumeration of the latter group have been devised to allow for the quantitative recovery of *Salmonella* (Mossel, 1957; Mossel *et al.* 1974). However, there can be considerable variation in the relative proportions of *Salmonella* to Enterobacteriaceae in various processed foods, as a result of ecological differences in their origin and production (Silliker & Gabis, 1973). Food products of animal origin are prone to recontamination from foci of recent faecal origin, with resultant predominance of the enteric flora, *E. coli* in particular and, to a much lesser and more variable extent, enteric pathogens such as *Salmonella*. Other processed foods are, as a rule, recontaminated with material of vegetable origin in which *Enterobacter*, *Serratia* and similar species are the principal Enterobacteriaceae encountered, enteric pathogens being rarely found (Splittstoesser & Wettergreen, 1964; Spicher, 1971, 1972; Sheneman, 1973).

Using our own method of assessment, foodstuffs are rejected, *ipso facto*, if enteric pathogens are detected in the samples examined. If no enteric pathogens are found, but the sample is positive for Enterobacteriaceae, the verdict depends on the usual criteria, namely (1) the count of Enterobacteriaceae found; (2) the Enterobacteriaceae types predominating in the flora (Mossel & Vincentie, 1968);

(3) the degree of risk that can be accepted by the manufacturer and by the consumer (Mossel & Drion, 1958).

However, it has been suggested by others that properly designed tests for Enterobacteriaceae could be applied as a sole criterion for this purpose, similar to the long established use of the coli-aerogenes group, instead of the more elaborate detection methods for pathogens (Van Schothorst *et al.* 1966; van der Meijs, 1970; Hechelmann, Rossmanith, Peric & Leistner, 1973; Hunyady *et al.* 1973; Nouws & Heymans, 1975). From the viewpoint of the relation between effort made and benefit accrued, it cannot be denied that this approach may offer considerable advantages. In addition, it may often be the only practicable form of monitoring that laboratories in the developing areas of the world can allow themselves. No harm would result from this approach for, as discussed above, the use of Enterobacteriaceae as index organisms will, in nearly every instance, be a more sensitive method for the bacteriological monitoring of processed foods than an examination for coli-aerogenes bacteria. It remains essential, however, when thus replacing direct examination for pathogenic types by testing for all *Enterobacteriaceae* to assess the risk of accepting consignments containing unacceptable levels of pathogenic organisms (Hobbs & Gilbert, 1975) – although the degree of protection afforded by the less-sensitive coli-aerogenes test alone does not appear to have been determined or even questioned. To facilitate an objective evaluation of this topic, we have, therefore, in the present study, tried to evaluate also the reliability of a system of testing for Enterobacteriaceae alone.

THEORETICAL CONSIDERATIONS

It has been demonstrated repeatedly that bacteria contaminating processed solid foods are rather heterogeneously distributed (Rishbeth, 1947; Kallings *et al.* 1959; Semple *et al.* 1961; Silverstolpe *et al.* 1961; Turner & Campbell, 1962; Zipplies, 1964; Ray *et al.* 1971*a*). However, theoretical deductions can only be based on a postulated homogeneous distribution of such organisms, keeping in mind, however, that in instances where the distribution of organisms which have to be detected is extremely heterogeneous, no sample can ever be even slightly representative for the consignment. Because the bacteriological examination of foods and feeds bears the character of a destructive analysis, i.e. after testing no material remains, the only way to overcome the problem of heterogeneous distribution of microorganisms is to draw the highest possible number of relatively small samples, mix these thoroughly and draw a representative aliquot from the pooled sample for examination (Harrewijn *et al.* 1972).

Let us suppose that the number of colony forming units per 1 g of food, etc., amounts to b. In that instance the probability that in a sample of g grammes no viable organisms are present equals e^{-bg}, where e is the base of the natural logarithm. Hence the probability that the examination of the sample will yield a positive result is $1-e^{-bg}$.

Often more than one sample is drawn from the same consignment and not infrequently samples are examined for more than one group of bacteria. In Fig. 1

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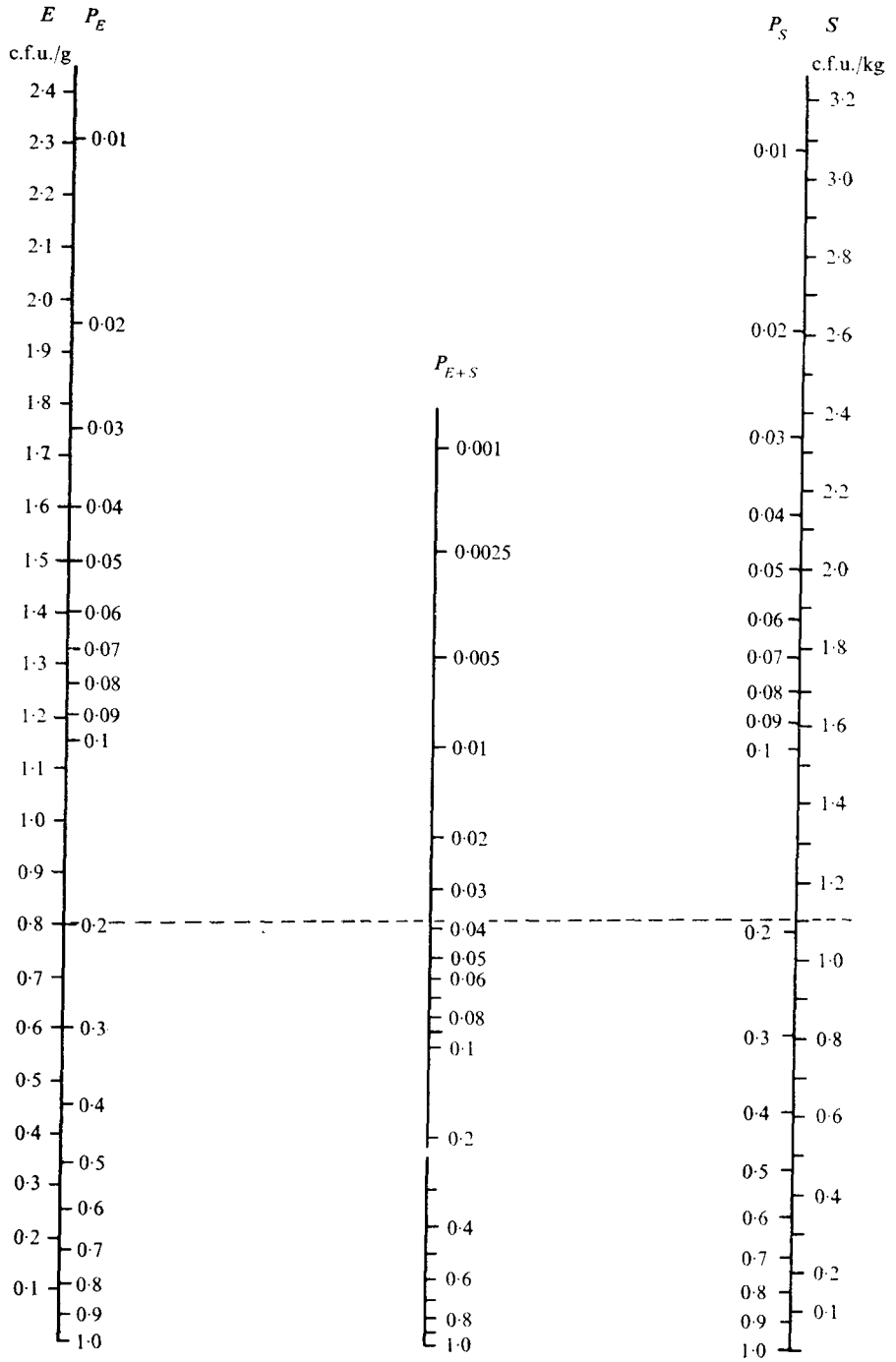

Fig. 1

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the results of computations have been brought together, allowing the assessment of the probability of obtaining a negative result for a Presence–Absence (P–A) test for Enterobacteriaceae, upon examination of two 1 g samples (Drion & Mossel, 1972) as well as a similar test for *Salmonella*, in this instance when examining sixty 25 g samples (Foster, 1971). This figure is to be used as follows:

(1) To read the probability of getting a negative result in the Enterobacteriaceae test (P_E) use the left side of the first bar: choose the appropriate level of c.f.u./g, e.g. 0·1 and read the corresponding probability of finding a negative result, hence probability of acceptance, in this instance approximately 0·81.

(2) To assess the probability of acceptance based on the outcome of an examination for *Salmonella*, use the third bar in a similar way as described under 1, starting in this instance from the c.f.u./10^3 g on the right side of the bar. Hence, when c.f.u./10^3 g = 1, then $P_S = 0{\cdot}22$.

(3) To determine the probability of getting negative results in both tests, draw a straight line joining the chosen c.f.u. values and determine the intersection with the central bar which equals the P_{E+S} value. As an example, let the *E*–c.f.u. be 0·8/g and the *S*–c.f.u. be 1·1/10^3 g then the intersection P_{E+S} is just below 0·04, with $P_E = 0{\cdot}2$ and $P_S = 0{\cdot}19$.

We have earlier defined the so-called ϵ-factor as the proportion E'/S', E' being the Enterobacteriaceae c.f.u./g and S' the Salmonella c.f.u./g (Mossel, 1975). Figs. 2 and 3 provide the means of assessing the probability of obtaining negative results in both types of tests. Fig. 2 uses E' as the parameter, together with ϵ, Fig. 3, S' and ϵ. The use of both figures is the same as explained for Fig. 1. To present two examples: when $E' = 0{\cdot}1$/g and $\epsilon = 10^2$, the probability of acceptance $P_a = 0{\cdot}18$; when $S' = 0{\cdot}5$/kg and $\epsilon = 10^3$, then P_a also equals 0·18.

EXPERIMENTAL PART

Materials

The aim of the present study was to determine ϵ-values for the most important enteric pathogens that play an aetiological role in food-borne diseases. As explained in the Introduction we had to restrict this investigation to *Salmonella*, at least for the time being, because much information is available, or relatively easy to collect, on MPN values of *Salmonella* in certain foods, whereas no comparable quantitative figures have so far been published on, for example, *Shigella*, enteropathogenic *E. coli*, *Yersinia enterocolitica* or hepatitis A virus, one of the most dangerous food-borne pathogens, isolation of which has only recently become possible (Holmes *et al.* 1973).

Two sets of results have been used for the calculations: (i) data made available by the Netherlands National Institute of Public Health (Kampelmacher and Van Schothorst, personal communication, 1973); and (ii) results collected by the authors in the course of surveys and routine monitoring. The data concern, in descending order of numbers of samples examined: (1) dried foods and special feeds that have been processed for safety during manufacture (Mossel & Krugers Dagneaux, 1959, 1963; Harrewijn *et al.* 1972; Mossel, Harrewijn & van Sprang,

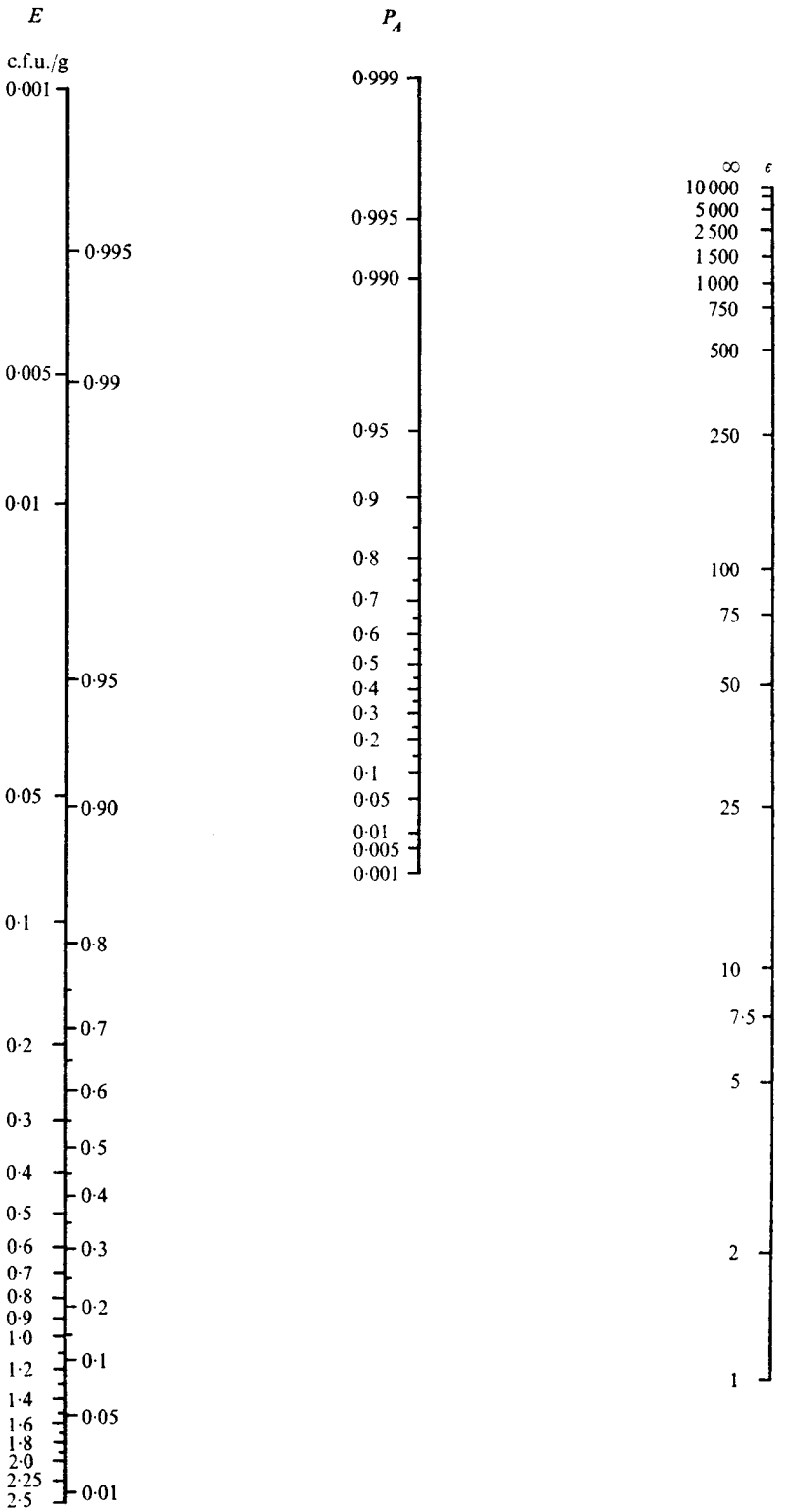

Fig. 2

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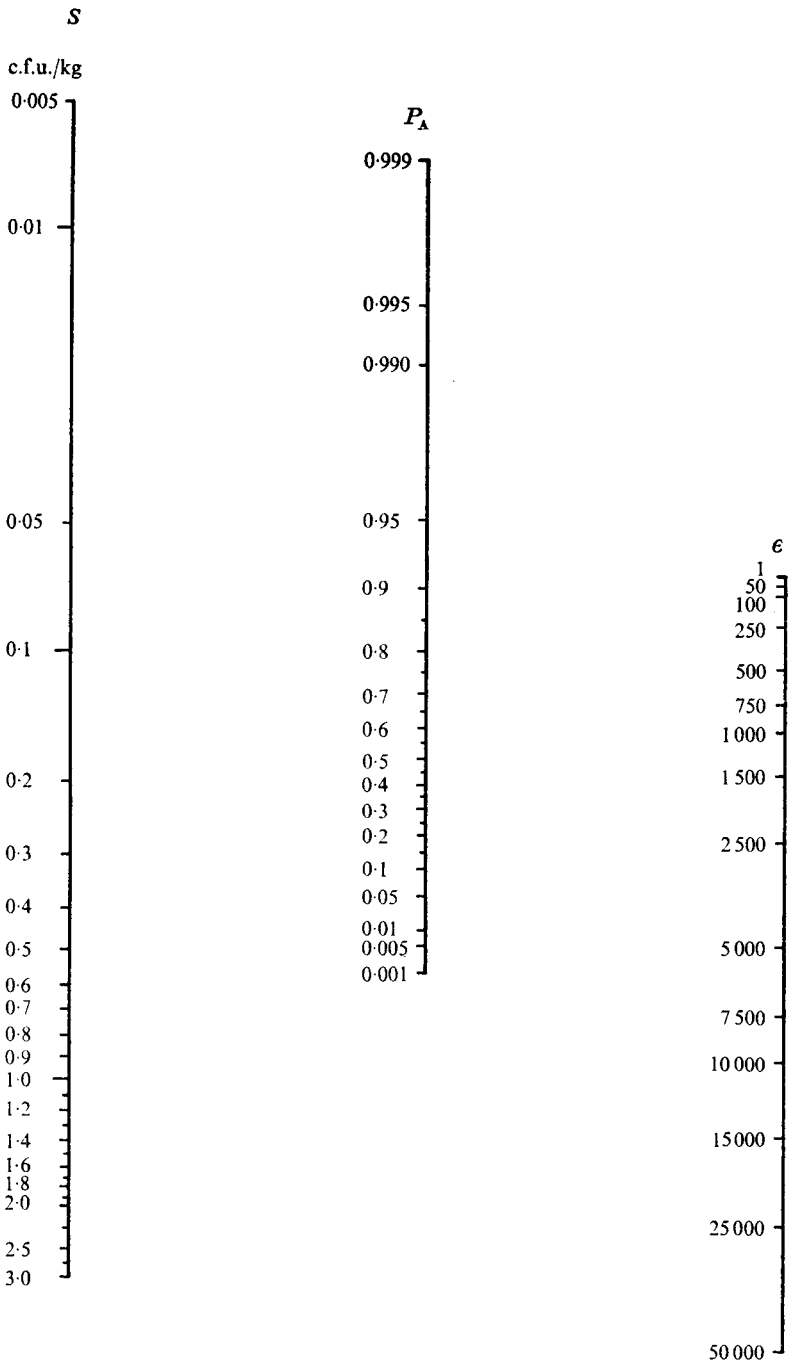

Fig. 3

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1973; Mossel, Shennan & Vega, 1973; Mossel *et al.* 1974; Mossel & Shennan, 1976); (2) margarine, ecologically related to dried foods in that the ingredients are processed for safety and the final product, if of good quality, is quite stable (Mossel, 1970); and (3) cooked meat products (Mossel, 1962).

Methods

Enterobacteriaceae

Samples were examined for Enterobacteriaceae by a selective plate count (Mossel & Ratto, 1973), an MPN procedure (Mossel *et al.* 1973) or a Presence-or-Absence test (Mossel *et al.* 1974). In all instances, culturing in selective media was preceded by adequate 'resuscitation' treatments, aiming at the recovery of sublethally impaired cells of Enterobacteriaceae (Mossel & Ratto, 1970; Ray *et al.* 1971*b*; Van Schothorst & Van Leusden, 1975). For this purpose 1/10 suspensions of the food in tryptone soya peptone broth were kept at laboratory temperature for 2 h, being resuspended by shaking every half hour. This 'deliberate' method of resuscitation, which has the reliability of a standardized procedure, was preferred to depending on the fortuitous restoration of viability which often occurs in practice when dilutions of foods are left for shorter or longer periods on the laboratory bench (Mossel & Ratto, 1970; Silliker & Gabis, 1973).

Colonies obtained on solid media, i.e. directly when plate counts were carried out, or after isolation on a solid medium following selective enrichment, were always examined for the key characteristics of Enterobacteriaceae: fermentative attack on glucose and a negative oxidase reaction. In recent years this has been done by (i) replating on lactose lauryl sulphate phenol red agar (tryptone, 20 g; lactose, 10 g; sodium chloride, 10 g; sodium dodecyl sulphate, A.R., 0·1 g; phenol red, 90 mg; agar, 15 g; water, 1 litre, pH 7·2 ± 0·1) for purification and assessment of lactose dissimilation; (ii) deep stabbing into a single 'polytropic' tube. Such tubes contain a bottom layer of about 2 cm violet red bile glucose agar, a separation layer of 2 cm water agar and a 2 cm top layer of SIM medium (Mossel, Eelderink & Sutherland, 1977). This allows in addition tentative recognition of indole positive (e.g. *E. coli*), non-motile (e.g. *Shigella*) or H_2S positive (e.g. *Salmonella*) types.

Salmonella

Many samples were also examined for *Salmonella*. Sterile wide-mouth flasks, containing an amount of tryptone soya peptone broth (Mossel & Ratto, 1970) equal to the size of the sample to be examined, were used when P–A tests were done, while an additional set of similar tubes was prepared when MPN-values had to be assessed. The material was suspended in the broth and the suspensions kept at laboratory temperature for about 2 h, the flasks and tubes being occasionally shaken to attain optimal dispersion.

This resuscitation treatment having been completed, a volume of tetrathionate brilliant green broth (Edel & Kampelmacher, 1973) equalling about nine times the volume of food plus broth was added. Incubation was for 24 h at 43 °C (Edel & Kampelmacher, 1973). Subsequently the enrichment cultures were streaked on

Table 3. *Results of all samples tested for Enterobacteriaceae during the last 25 years*

Total samples tested	18170
Tested for Enterobacteriaceae only	11340
Tested for Enterobacteriaceae and *Salmonella*	6830
Positive for *Salmonella* (50–100 g) and Enterobacteriaceae (1–2 g)	69
Negative for *Salmonella* (50–100 g) but positive for Enterobacteriaceae (1–2 g)	4642
Negative for both groups	2119

Table 4. *Distribution of Enterobacteriaceae and* Salmonella *in 18170 samples of foods and feeds, examined 1950–1976*

Class and commodity type	Commodity	Samples examined, number	Enterobacteriaceae c.f.u./g: 9th decile of distribution	Enterobacteriaceae c.f.u./g: Extremes Min.	Enterobacteriaceae c.f.u./g: Extremes Max.	*Salmonella* c.f.u./g: Pos. samples, number	*Salmonella* c.f.u./g: Maximum found
	I. *Salmonella* detected in at least one sample per group (total: 5630 samples)						
1	Cereal products, processed	3470	10	0·1	$2{\cdot}0 \times 10^4$	12	11
2	Feeds	1500	10	2	$0{\cdot}9 \times 10^3$	14	0·5
3	Gelatine, plasma etc.	300	10	8	$3{\cdot}3 \times 10^3$	15	5
4	Nuts	200	10	0·2	$1{\cdot}0 \times 10^4$	12	0·02
5	Egg products I	160	10	0·3	$1{\cdot}0 \times 10^4$	16	0·2
	II. *Salmonella* never found (total: 1200 samples)						
6	Cooked sausages	390	10	1	$1{\cdot}0 \times 10^4$	0	—
7	Milk powder	240	1	NT	NT	0	—
8	Soup	220	10^2	NT	$1{\cdot}0 \times 10^3$	0	—
9	Margarine I	210	1	NT	$0{\cdot}3 \times 10^4$	0	—
10	Cocoa products	80	1	NT	NT	0	—
11	Lactose	60	1	NT	NT	0	—
	III. No examination for *Salmonella* carried out (total: 11340 samples)						
12	Margarine	11000	1	NT	$0{\cdot}3 \times 10^4$	NT	—
13	Egg products	340	10	NT	$0{\cdot}8 \times 10^4$	NT	—

NT, Not determined. —, Not applicable.

brilliant green agar (Edel & Kampelmacher, 1973) and incubated over night at 37 °C.

Typical lactose-negative colonies were examined in gram-negative diagnostic tubes (see above), on urea agar slides and in lysine decarboxylase medium. Thus having eliminated *Pseudomonas*, *Proteus* and other non-*Salmonella* strains, the remaining cultures were identified by O and H agglutinating sera.

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Table 5. *Analysis of the results obtained on the* 69 *samples of Table* 4 *found positive for* Salmonella

Commodity group*	c.f.u./g: *Salmonella* (*S'*)	c.f.u./g: Enterobacteriaceae (*E'*)	$\epsilon = E'/S'$	Calculated verdict† for test on: *Salmonella*‡	Calculated verdict† for test on: Enterobacteriaceae§	Probability of acceptance given the c.f.u./g when tested for both *Salmonella* and Enterobacteriaceae
I. Egg products (500)	0·020	1300	7×10^4	R	R	0·000
	0·060	20	3×10^2	R	R	0·000
	0·030	8	3×10^2	R	R	0·000
	0·100	8	8×10	R	R	0·000
	0·070	2	3×10	R	R	0·000
	0·200	0·5	2·5	R	A‖	0·000
	0·200	400	2×10^3	R	R	0.000
	0·020	4000	2×10^5	R	R	0·000
	0·080	2000	3×10^4	R	R	0·000
	0·020	4000	2×10^5	R	R	0·000
	0·020	2000	10^5	R	R	0·000
	0·004	10000	3×10^6	R	R	0·000
	0·003	4000	1×10^6	R	R	0·000
	0·001	0·3	3×10^2	A	A	0·120
	0·001	10	10^4	A	R	0·000
	0·009	0·8	9×10	R	A‖	0·000
II. Processed cereal products (3470)	0·900	9000	10^4	R	R	0·000
	0·003	1	3×10^2	R	A‖	0·001
	0·003	0·1	3×10	R	A‖	0·009
	0·003	1	3×10^2	R	A‖	0·001
	0·200	20	10^2	R	R	0·000
	0·030	30	10^3	R	R	0·000
	11·000	10000	9×10^2	R	R	0·000
	0·003	0·2	7×10	R	A‖	0·001
	0·006	90	2×10^4	R	R	0·000
	0·010	10	10^3	R	R	0·000
	0·400	9	2×10	R	R	0·000
	0·200	20000	10^5	R	R	0·000
III. Gelatin and bovine plasma derivates (300)	1·300	33	3×10	R	R	0·000
	5·000	3300	7×10^2	R	R	0·000
	0·400	230	6×10^2	R	R	0·000
	5·000	17	3	R	R	0·000
	4·000	92	2×10	R	R	0·000
	2·000	49	2×10	R	R	0·000
	2·000	220	10^2	R	R	0·000
	0·200	33	2×10^2	R	R	0·000
	0·200	23	10^2	R	R	0·000
	0·300	79	3×10^2	R	R	0·000
	0·300	49	2×10^2	R	R	0·000
	1·000	8	8	R	R	0·000
	4·000	46	12	R	R	0·000
	0·300	8	3×10	R	R	0·000
	3·000	33	11	R	R	0·000

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Table 5 (*cont.*)

Commodity group*	c.f.u./g: *Salmonella* (*S'*)	c.f.u./g: Enterobacteriaceae (*E'*)	$\epsilon = E'/S'$	Calculated verdict† for test on: *Salmonella*‡	Calculated verdict† for test on: Enterobacteriaceae§	Probability of acceptance given the c.f.u./g when tested for both *Salmonella* and Enterobacteriaceae
IV. Nuts (200)	0·005	4000	8×10^5	R	R	0·000
	0·009	2000	2×10^5	R	R	0·000
	0·020	10000	5×10^5	R	R	0·000
	0·004	4000	10^6	R	R	0·000
	0·004	20	5×10^3	R	R	0·000
	0·004	100	2×10^4	R	R	0·000
	0·006	5	8×10^2	R	R	0·000
	0·001	0·3	3×10^2	A	A	0·120
	0·001	10	10^4	A	R	0·000
	0·001	0·2	2×10^2	A	A	0·150
	0·001	1	10^3	A	A	0·030
	0·001	9	9×10^3	A	R	0·000
V. Special feeds (1500)	0·001	50	5×10^4	A	R	0·000
	0·001	100	10^5	A	R	0·000
	0·001	4	4×10^3	A	R	0·000
	0·001	40	4×10^4	A	R	0·000
	0·001	2	2×10^3	A	R	0·004
	0·010	200	2×10^4	R	R	0·000
	0·006	10	2×10^3	R	R	0·000
	0·001	400	4×10^5	A	R	0·000
	0·200	40	2×10^2	R	R	0·000
	0·009	900	10^5	R	R	0·000
	0·200	90	5×10^2	R	R	0·000
	0·500	50	10^2	R	R	0·000
	0·001	5	5×10^3	A	R	0·000
	0·001	3	3×10^3	A	R	0·001

* In the remaining classes, namely milk powder, lactose, cocoa powder, dried soups and margarine, no samples containing Salmonella were found.

† For each sample the verdict of a test (specified in note ‡ and §) is indicated by either R or A. R means that except for a probability of at most 5% the sample will be rejected. A means that the probability of acceptance is more than 5%.

‡ Examination of sixty 25 g samples (Foster, 1971) or of suitable pools of these. (Silliker & Gabies, 1973.)

§ Examination of two 1 g samples (Drion & Mossel, 1972).

|| See page 314, line 8.

RESULTS AND DISCUSSION

In Table 3 a survey is given of all samples examined for Enterobacteriaceae during the last 25 years. A complete record of the data is presented in Table 4. Among the 6830 samples examined for Enterobacteriaceae and *Salmonella* only 69 were found positive for *Salmonella*, whereas 4711 contained Enterobacteriaceae above the specified limit of detection, expressed in its ninth decile.

Table 5 presents the analysis of the results obtained for the materials in which *Salmonella* spp. were encountered. In columns 5 and 6 of this table the samples have been indicated with an R when they would be rejected with a probability

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of at least 95 % when being examined for Enterobacteriaceae in two 1 g samples, or for *Salmonella* using 60 samples of 25 g. Correspondingly, the samples with a probability of acceptance > 5 % have been indicated with an A sign. These verdicts have been derived from Fig. 1, as have those in column 7, applicable to combined testing.

If only the data in Table 5 were considered, a wholly false impression of the value of testing for Enterobacteriaceae for consumer protection would be obtained. At first sight in no less than 6 out of 69 cases, marked with parallel lines, a P–A test for *Salmonella* would have led to rejection at the level $P = 0{\cdot}99$, whereas the Enterobacteriaceae test would have resulted in a probability of rejection between 19 and 86 %. However, according to Table 3 these 69 samples were only about 1 % of the total material tested for both groups of bacteria. Hence, only in 6 out of 6830 cases, i.e. less than 0·1 %, did the Enterobacteriaceae test really fail to achieve its purpose.

On the other hand, 4711 samples showed a positive result when tested for Enterobacteriaceae at the specified level; amongst these only 69, i.e. 1·5%, were found to contain *Salmonella*. It is far from improbable that more than this number of samples really contained *Salmonella*, but that these were not detected, because the size of the samples examined was usually only 50–100 g; for the computations we used the average value of 75 g.

In addition it is most reassuring that no ϵ-values of approximately or even below 1 were obtained. Hence the method used for the enumeration of Enterobacteriaceae would recover *Salmonella* quantitatively as has always been claimed by our group (Mossel, 1957; Mossel, Mengerink & Scholts, 1962; Mossel, Visser & Cornelissen, 1963; Van Schothorst *et al.* 1966; Mossel & Vincentie, 1968; Mossel *et al.* 1974) but often disputed (Hobbs & Gilbert, 1975) – although without any experimental and certainly without any taxonomic support!

Because the majority of P–A tests for Enterobacteriaceae, namely those done since 1969, were carried out with two 1 g samples, a rough estimate of the average of the number of c.f.u./g of Enterobacteriaceae in the material under review can be obtained. By the method of calculation recorded in the Appendix, the average of the number of Enterobacteriaceae in all 6830 samples amounted to 0·78 c.f.u./g, with conservative 95 % confidence limits 0·723 and 0·841. For *Salmonella* the corresponding figures were 0·135 with conservative 95 % confidence limits 0·081 and 0·189 c.f.u./10^3 g, respectively. Hence the average ϵ-value for the total material analysed may be assessed at $(0{\cdot}78 \times 10^3)/0{\cdot}135 = 5{\cdot}8 \times 10^3$. If one uses the higher confidence limit for S' and the lower for E', the ϵ-value thus obtained is about $0{\cdot}723 \times 10^3/0{\cdot}189 = 3{\cdot}8 \times 10^3$. This figure is still more than five times the base line for ϵ, established in an earlier study as safe with regard to consumer protection (Drion & Mossel 1972).

From the point of view of epidemiology the data in Table 5 demonstrate that the criteria c.f.u./g of *Salmonella* and ϵ-factor vary widely between different classes of processed foods. This has been recognized in a more qualitative sense

for many years: in contrast with dried egg products, dried milk is only very rarely found to be contaminated with *Salmonella* and is even conspicuously 'free' from Enterobacteriaceae. In addition, the data in Table 5 illustrate the considerable variation that can exist between the ϵ-factor assessed for different consignments of the same product. This fact is also well known, particularly to practitioners of microbiological monitoring of processed foods.

CONCLUSIONS

It thus appears that demonstration of the presence of Enterobacteriaceae in two samples of 1 g of foods processed for safety will in the great majority of cases lead to their justified rejection, based on testing for *Salmonella* in sixty 25 g samples, because the prevailing ϵ-factors generally surpass the safe base line of 750. The very few exceptions (0·1 %) generally barely justify additional testing for *Salmonella* when the differences in required skill, facilities, effort and time are taken into account. Two reservations remain valid, though.

Firstly, testing for Enterobacteriaceae is obviously of no avail in epidemiological follow-up of outbreaks of food-borne salmonellosis. In addition justification for thus relaxing the severity of the microbial monitoring of foods for safety by omitting direct examination for other pathogens can be derived only from bacteriological surveys followed by mathematical interpretation of the results obtained as developed in this paper. However, the perspectives for testing for Enterobacteriaceae seem good, because other enteric pathogens seem to occur much less frequently than *Salmonella* spp. and are also almost invariably accompanied by other Enterobacteriaceae (Mossel, 1975).

It may also be useful to stress that although the computations made in this study were based on the strictest U.S. testing scheme for *Salmonella* the Enterobacteriaceae test has general validity. When for example, a much more relaxed, *Salmonella* requirement is used – as a rule absence in 50 g is required – then the Enterobacteriaceae testing scheme can be similarly relaxed. In this particular instance examining two samples of $50/1500 \times 1\text{ g} = 0{\cdot}03\text{ g}$ will suffice. Hence consumer protection can be equalized or even further increased by testing, for example, two 0·1 g samples, or by allowing no more than a total of 2 colonies per 2 poured plates of violet red bile glucose agar inoculated with 1 ml of a 10^{-1} dilution of the food.

Finally, the high frequency of rejection of samples based on a positive outcome of testing for Enterobacteriaceae while *Salmonella* were not isolated (Table 3) has often prompted the question whether examination for the former group is at all justified. We hope to have demonstrated that the answer is in the affirmative for the following reasons: (1) failure to demonstrate *Salmonella* in samples does not indicate that such bacteria were not present; (2) absence of *Salmonella* from samples has only a very limited significance for the absence of *Salmonella* in the consignment; (3) absence of *Salmonella* in a consignment of food processed for safety that – as the presence of Enterobacteriaceae shows – has been overtly recontaminated after processing, may still involve occurrence of other enteric pathogens.

This clearly requires that the limits set for Enterobacteriaceae are attainable, i.e. determined as a result of an experimental survey of a sufficient number of samples taken from production lines of factories applying good manufacturing and distribution practices (Mossel, 1975).

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APPENDIX

1. The data on contamination with Enterobacteriaceae or *Salmonella* of food processed for safety were collected from experiments performed with another aim than determining the exact, quantitative, contamination of each sample. This implies that in those cases, where the contamination remained under a certain limit no further analysis was performed and the sample was recorded as 'negative'. It is therefore not possible to calculate with a standard method the mean of the number of c.f.u./g of either Enterobacteriaceae or *Salmonella* averaged over all 6830 samples considered in this paper. Moreover, the average calculated by dividing the total number of c.f.u./g (summed over all samples considered) by 6830 (number of samples) does not seem reasonable. This can be clearly shown with the results of the salmonella tests. Of the 6830 samples 6761 gave a negative result; among the 69 positive results there were only 10 samples with a density of *Salmonella* of 1 or more c.f.u./g.

Among these there was one sample with 11 c.f.u./g. This one sample would have elevated the mean number of *Salmonella* with $11/6830 = 0{\cdot}0016$; this is more than the upper limit of the average given in this article. We consider it more reasonable to calculate an average using a method that is not so sensitive to one or a few extreme results.

There is a second reason for tackling the mathematical–statistical problems in the way to be described. We should have liked to calculate directly the mean of the ϵ-value. However, this does not lead to useful results as a possible value for ϵ is infinite. An infinite value of ϵ means that in a sample no salmonella organisms at all are present whereas there are Enterobacteriaceae. For this reason it was necessary to find another way – even another definition – for the 'average content' of

Table A. *Number of samples positive for* Salmonella

Enterobact. contaminat.	Commodity group: Egg prod.	Processed cereal prod.	Gelatin, etc.	Nuts	Spec. feeds	Total
< 10 c.f.u./g	6	5	2	5	4	22
⩾ 10 c.f.u./g	10	7	13	7	10	47
Total	16	12	15	12	14	69

Enterobacteriaceae and of *Salmonella* in the samples investigated. Before explaining this method it must be shown that it is not necessary to make separate calculations for each of the commodities.

2. Table 4 shows that positive results for *Salmonella* were found in only five commodities. If we consider as a 'heavy' contamination with Enterobacteriaceae of a sample, the presence of 10 or more c.f.u./g then the number of samples positive for *Salmonella* that were heavily contaminated with Enterobacteriaceae and the number that were not can be found per commodity from table 5. The results are given in table A.

A chi^2 test performed on this 2×5 table shows no significant deviation from proportionality ($P = 0{\cdot}43$). Therefore in this material samples contaminated with *Salmonella* have about the same fraction of samples highly contaminated with Enterobacteriaceae in each of the five commodities. To avoid any misunderstanding: no samples contaminated with *Salmonella* were found that were not contaminated with Enterobacteriaceae.

3. We propose to calculate in the first place the confidence interval for the mean of the probabilities to get a positive result when testing for Enterobacteriaceae in 1 or 2 g and when testing for *Salmonella* in 50 or 100 g. As for each sample this probability may be different we must use Poisson's Theorem (Uspensky, 1937, p. 208). From this mean probability $\bar{p}$ of finding a positive result we find the mean probability $\bar{q}$ of finding a negative result. If a material is contaminated with b c.f.u./g and a g are tested then the probability of obtaining a negative result is $q = e^{-ab}$. It is, however, not possible to conclude from $\bar{q} = e^{-x}$ that the mean value of b equals x/a. Only if $\bar{q}$ would have been a geometric mean would this have been correct. Nevertheless we propose to use $(-\ln \bar{q})/a)$ as the average contamination. This average will be smaller than the value we would have found if we could have determined the geometric mean, as the geometric mean is smaller than the arithmetical mean, and therefore $-\ln \bar{q}/a$ is smaller than minus the natural logarithm of the geometric mean. As minus the logarithm of the geometric mean equals $(a \Sigma b_i)/n$ (n being the total number of samples, i.e. 6830) it follows that the average we are using is smaller than the arithmetrical mean of the contamination expressed as c.f.u./g. However, this is not unacceptable in this case: in the first paragraph of the appendix we showed that a mean calculated directly from all observations may give a wrong impression of the average contamination if there are one or more samples with extremely high c.f.u./g.

4. To calculate the average contamination with Enterobacteriaceae $\bar{p}[E]$ in this

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material let us put $p_i[E]$ for the probability that the ith sample will give a positive result when testing 1 g for Enterobacteriaceae ($i = 1, \ldots, 6830$).

$$\bar{p}[E] = \frac{\Sigma\, p_i[E]}{6830}.$$

In the 6830 samples tested both for Enterobacteriaceae and Salmonella $m = 4711$ were positive for Enterobacteriaceae. Therefore $\bar{p}[E] = 0{\cdot}690$. If we put $\eta = 0{\cdot}05$ then the inequality $|(m/6830) - \bar{p}[E]| \leqq \delta$ holds with a probability at least $1-\eta$ ($= 95\,\%$) when $\delta = 0{\cdot}027$. This follows from Poisson's theorem as given by Uspensky (Uspensky, 1937, p. 208, 209). In the proof of the theorem Uspensky shows that the above given inequality holds, provided that $\eta \leqq B[E]/n^2\delta^2$.
In this formula $B[E]$ is the variance of the sum of the positive results of all n (in this case $n = 6830$) experiments. An upper limit for $B[E] = \frac{1}{4}n$. Therefore

$$-\delta < \bar{p}[E] - \tfrac{4711}{6830} < \delta, \quad 0{\cdot}690 - 0{\cdot}027 < \bar{p}[E] < 0{\cdot}690 + 0{\cdot}027,$$
$$0{\cdot}663 < \bar{p}[E] < 0{\cdot}717.$$

$$q = 1-p, \text{ therefore } 0{\cdot}283 < \bar{q}[E] < 0{\cdot}337.$$

In paragraph 3 we have argued that we may use for the average c.f.u./g of Enterobacteriaceae $-\ln \bar{q}[E])/a$, where a is the sample size, in these experiments 1 g or 2 g, say 1·5 g. We may therefore put

$$\frac{-\ln 0{\cdot}283}{1{\cdot}5} > E > \frac{-\ln 0{\cdot}337}{1{\cdot}5}, \quad \frac{1{\cdot}262}{1{\cdot}5} > E > \frac{1{\cdot}088}{1{\cdot}5},$$

$$0{\cdot}725 < E < 0{\cdot}841.$$

$\bar{E}$ is the average number of Enterobacteriaceae/g. From $-\ln(1-\frac{4711}{6830}) = -\ln 0{\cdot}310 = 1{\cdot}171$ we find as estimate for the average of E the value $1{\cdot}171/1{\cdot}5 = 0{\cdot}780$.

5 (*a*). For the calculation of the average of S, i.e. the average of the number of salmonella organisms per gram we may use in principle the same method. It is, however, possible to obtain a shorter confidence interval for $p[S]$, the average value of the probability of getting a positive result when testing for *Salmonella*. This can be attained here by finding a rather precise, low, estimate for $B[S]$ the sum of the variances of the 6830 tests. In the tests for Enterobacteriaceae the mean value of the p's was $\bar{p} = 0{\cdot}690$, so $\bar{p} \times \bar{q} = \bar{p} \times (1-\bar{p}) = 0{\cdot}690 \times 0{\cdot}310 = 0{\cdot}214$, which is not much lower than the maximum value 0·25, assumed in the calculation for the confidence interval. A much sharper estimate for $B[E]$ in the case of the Enterobacteriaceae seems not well possible. The situation is, however, quite different in the case of the *Salmonella* tests. Only 69 out of 6830, that is a little more than 1 % of all tests, were positive. Moreover, for each of the positive *Salmonella* tests the value of the probability p of obtaining a positive result was known, namely $p = 1 - e^{-a \times s}$, s being the density of *Salmonella* per gram, a the sample size in grammes (in the earlier tests 50 g, later 100 g; on the average 75 g) and e the base of the natural logarithms, namely 2·718.

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Table B. *Summary of Table 5, giving also the information necessary to calculate B[E]*

Salmonella (c.f.u./1000 g) S	Observed frequency k	Probability of result $p = 1-e^{-0{\cdot}05s}$	Result $q = e^{-0{\cdot}05s}$	Maximum likelihood estimate of n	Maximum likelihood estimate of npq
1	15	0·05	0·95	308	14·25
3	5	0·14	0·86	36	4·30
4	4	0·18	0·82	22	3·28
5	1	0·22	0·78	5	0·78
6	3	0·26	0·74	12	2·22
9	3	0·36	0·64	8	1·92
10	2	0·39	0·61	5	1·22
20	5	0·63	0·37	8	1·85
30	2	0·78	0·22	2	0·44
60	1	0·95	0·05	1	0·05
70	1	0·97	0·03	1	0·03
80	1	0·98	0·02	1	0·02
100	1	0·994	0·006	1	0·01
200	8	0·999955	0·000045	8	0·00
> 200	17	1·000·000	0·000·000	17	0·00
Total	69			435	30·37

Tables 4 and 5 show that the material under consideration, namely 6830 samples tested for Enterobacteriaceae and for *Salmonella*, may be subdivided in several strata according to the degree of contamination with *Salmonella*. In strata consisting of heavily contaminated lots all samples will be positive so all these lots will be recognized as contaminated with *Salmonella*. However, among the lots contaminated with say 1 *Salmonella* per kg the probability that in testing 50 g of material a positive result will be obtained is only 5% (namely $1-e^{-0{\cdot}05}$). In order not to underestimate $B[S]$ we used for a in the following calculations the value 50 g. In all, 15 samples were found with a contamination of 1 *Salmonella*/kg, so an estimate (maximum likelihood estimate) of the number of lots with such a contamination is $15/0{\cdot}05 = 300$. In other words the stratum of lots contaminated with 1 *Salmonella* per kg has a size of 300 lots. Such calculations were made for all strata that have a contamination of 1 *Salmonella*/kg or more. The results are given in the fifth column of Table B under the heading maximum likelihood estimate of n. This table was derived from Table 5. The sum of this column given in the last line is 435 so about 435 lots had a contamination of 1 *Salmonella* or more per kg. There is left a stratum of $6830-435 = 6395$ lots. This stratum consists almost certainly of lots which had either a contamination of less than 1 *Salmonella*/kg or which were not contaminated at all with *Salmonella*. It is, however, not probable that all these lots were entirely free from *Salmonella*. On the other hand, it is most improbable that there were lots among them that were heavily contaminated with *Salmonella*.

The last column of Table B gives the contribution of the 435 lots to the total variance $B[S]$. The total contribution is shown in the last line, namely 30·37. An estimate of the contribution to $B[S]$ of the $6830-435$ samples having all (or

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almost all) a contamination of less than 1 *Salmonella* per kg can be found in the following manner.

Let q_i ($i = 1, 2, \ldots, 6395$) be the probability that the ith sample gives a negative result when tested for *Salmonella*. Then $1 > \prod_{i=1}^{i=6395} q_i > a$, where a is some small number.

This formula implies that there were positive samples, but that all *test results* were negative. As has been remarked it is improbable that for many values of i the probability q_i was much lower than 1, for, in that case, the compound probability Πq_i would be inacceptably low. We may therefore suppose that a has a value of at least 0·05.

To find the contribution of the 6395 samples to $B[S]$ we must estimate

$$\Sigma p_i q_i \ (i = 1, \ldots, 6395) = \Sigma q_i(1-q_i) = \Sigma q_i - \Sigma q_i^2.$$

We may put $6395 > \Sigma q_i$

To obtain an upper limit for $-\Sigma q^2$ we need the lemma: If the product of n positive quantities not all equal to one is one then their sum is greater than n (for proof see Korovkin, 1960).

$$\Pi q_i = a \quad (1 > a > 0{\cdot}05),$$
$$\Pi q_i^2 = a^2,$$
$$a^{-2}\Pi q_i^2 = 1,$$
$$\Pi(a^{-2/6395} \times q_i^2) = 1.$$

We may therefore apply our lemma to the quantity $a^{-2/6395} \times q^2$.
This gives: $\Sigma\,(a^{-2/6395} \times q_i^2) = a^{-2/6395}\,\Sigma q_i^2 > 6395$.

The value of $c = a^{2/6395}$ may be found in the following way:

$$\ln c = (2 \ln a)/6395.$$

As $\ln a$ is larger than $\ln 0{\cdot}05 = -3$, $(2 \ln a)/6395 > -0{\cdot}000936$. Therefore

$$c = \exp \frac{2 \ln a}{6395} = 1 + \frac{2 \ln a}{6395} + O(10^{-6}).$$

So
$$\Sigma q_i^2 \geqq 6395\left(1 + \frac{2 \ln a}{6395}\right) = 6395 + 2 \ln a.$$

$$-\Sigma q^2 \leqslant -6395 - 2 \ln a$$

$$+\ \Sigma q < \quad 6395$$

$$\Sigma(q - q^2) < \quad -2 \ln a \quad +.$$

Therefore the contribution of the 6395 negative tests for *Salmonella* to $B[S]$ is smaller than $-2 \ln a$. If we put for a the value 0·05 (namely 5 %), as is also usual for confidence intervals, $\ln a = -3$ and $-2 \ln a = 6$. So $\Sigma(q - q^2) < -2 \ln a < 6$. If we put therefore $B[S] = 37$, namely 30·37 + 6·00 rounded up to the next integer, we will probably overestimate its value.

5(*b*). It is now possible to calculate a confidence interval for the probability $p[S]$ of a positive result when testing for *Salmonella* in the same way as has been done for Enterobacteriaceae.

$$P\left(\left|\frac{m}{n} - p[S]\right| \leqq \delta\right) > 1 - \frac{B[S]}{n^2\delta^2} > 1 - \frac{37}{6830^2 \times \delta^2}.$$

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Putting $\frac{37}{6830^2 \times \delta^2} = 0{\cdot}05$ we find for δ

$$\delta^2 = \frac{37}{0{\cdot}05 \times 6830^2}$$

$$\delta = \frac{\sqrt{(20 \times 37)}}{6830} = \frac{\sqrt{(740)}}{6830} = 0{\cdot}0040.$$

The mean value of $p = \frac{69}{6830} = 0{\cdot}0101$.
Therefore $q = 0{\cdot}9899$ with the limits $0{\cdot}9899 - 0{\cdot}0040 = 0{\cdot}9859$ and $0{\cdot}9899 + 0{\cdot}0040 = 0{\cdot}9939$.

Hence the average value of S is

$$\frac{-\ln 0{\cdot}9899}{75} = 0{\cdot}000135 \text{ c.f.u./g} = 0{\cdot}135 \text{ c.f.u./1000 g}$$

with the limits

$$\frac{-\ln 0{\cdot}9859}{75} = 0{\cdot}000189 \text{ c.f.u./g} = 0{\cdot}189 \text{ c.f.u./1000 g}$$

and $$\frac{-\ln 0{\cdot}9939}{75} = 0{\cdot}000082 \text{ c.t.u./g} = 0{\cdot}082 \text{ c.f.u./kg.}$$

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