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Variation in the numbers of Shiga toxin-producing *Escherichia coli* O157 in minced beef

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Abstract

Increasing evidence indicates that unheated minced beef products are major sources of STEC O157 infection. The distribution of STEC O157 in minced beef was studied as part of a risk assessment study. This is of importance for modelling the exposure of consumers to this organism. The aim of this study was to determine which mathematical distributions describe the variation of STEC O157 counts in minced beef and how grinding and mixing influence this variation. Additionally, the question was raised as to how a surface contamination could be translated to a contamination on the basis of weight. Much attention was paid to the methodological aspects of the determination of variations of STEC O157 in meat.

Even a contamination that occurs very locally on the starting material will be spread in the batch after grinding, and usually approximates the Poisson distribution after grinding (at least two times). The Poisson distribution with a Gamma-distributed parameter λ was helpful in fitting the experimental data, testing the fit of the Poisson distribution to the empirical distribution function and exploring variations beyond randomness.

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Samenvatting

Onvoldoende verhit rundergehakt en aanverwante producten zijn een belangrijke bron voor shigatoxinen producerende *Escherichia coli* serotype O157 (STEC O157). Als onderdeel van een *risk assessment* studie werd de verdeling van STEC O157 in rundergehakt onderzocht. Dit is van belang voor het inschatten van de blootstelling van consumenten aan deze ziekteverwekker.

Literatuuronderzoek laat zien dat ongeveer 1% van het rundvlees in de winkel is besmet met STEC O157. Wanneer een karkas is besmet, kan STEC O157 vaak overal op het karkas worden aangetoond, zowel tijdens de slacht- als in de uitbeenfase. Er is een gebrek aan Nederlandse gegevens over de aanwezigheid van STEC O157 op karkassen en in de verdere verwerking.

Doel van dit onderzoek was om wiskundige vergelijkingen te vinden die de variatie van STEC O157 in rundergehakt kunnen beschrijven, en hoe deze variatie wordt beïnvloed door het malen en mengen. Veel aandacht werd besteed aan methodologische aspecten van het bepalen van variaties in aantallen STEC O157 in rundergehakt. Enkele bronnen voor variaties in aantallen, toe te schrijven aan praktijkomstandigheden, werden onderzocht.

De eerste bron, die werd onderzocht, was de fout die kan ontstaan als gevolg van het gekozen telmedium. Voor het tellen van ongestresste cellen van een nalidixinezuur-resistente, niet-toxinogene *E. coli* O157-stam in gehakt bleken er geen verschillen te zijn tussen de onderzochte media (eosine methylene blue agar met nalidixinezuur (EMB), CHROMagar O157 met of zonder nalidixinezuur (respectievelijk CAN en CA), CHROMagar O157 met cefsoludine, cefixime en telluriet (CACCT) of Sorbitol McConkey Agar met nalidixinezuur (SMAC)), hoewel op CA en SMAC wel wat stoorflora werd gevonden. EMB was het meest geschikt om zuur/zout-gestresste cellen te tellen. Bij 5°C bleek dat *E. coli* O157 zich onder milde omstandigheden (trypton soya broth TSB, pH 7,2, 0,5% NaCl) minder goed wist te handhaven dan onder stressvolle omstandigheden (TSB, pH 4,9, 1% melkzuur, 14% NaCl). Door deze waarneming kunnen vraagtekens worden gezet bij de effectiviteit van milde conserveringsmethoden binnen het *hurdle* concept, om de aanwezigheid van STEC O157 in producten zoals droge gefermenteerde worst te kunnen controleren.

De tweede bron, die werd onderzocht, was de fout die kan ontstaan bij het homogeniseren van een monster. Zowel de stomacher als de blender, vertoonden statistisch significante fouten, maar deze zijn in praktijk nauwelijks relevant.

Variaties die kunnen ontstaan tijdens het maken van decimale verdunningen van een monster, werden ook bestudeerd. Dergelijke variaties kunnen worden verminderd door verdunningen in duplo uit te platen, iets wat in de meeste gevallen al wordt gedaan. Toch is er sprake van een 'detectielimiet' voor het bepalen van spreiding in een batch, als gevolg van toevalsfouten in de verdunningsreeks. Deze detectielimiet kan worden geschat via de Poisson

verdeling, waarbij in de standaard deviatie een factor √10 moet worden ingevoerd voor iedere decimale verdunningsstap.

Tenslotte werd het effect van de gehaktmolen op de verdeling van *E. coli* O157 en op die van de van nature aanwezige flora onderzocht. Zelfs een besmetting die op de grondstof heel lokaal aanwezig is, bleek in het hele product voor te komen na één keer malen. Na minstens twee keer malen kan die verdeling worden benaderd door een Poisson verdeling. De Poisson verdeling met een volgens de Gamma verdeling gespreide parameter λ was nuttig bij het *fitten* van de Poisson verdeling aan de empirische verdelingsfunctie en het onderzoeken van extra variatie in de meetgegevens, die niet door toevalsfouten zijn te verklaren.

Dit onderzoek heeft gegevens gegenereerd, waarmee berekeningen kunnen worden uitgevoerd om variaties in de waarnemingen die het gevolg zijn van onzekerheid in de meetgegevens of werkelijke variatie van monster tot monster te kunnen onderscheiden.

Summary

Increasing evidence indicates that unheated minced beef products are major sources of STEC O157 infection. The distribution of STEC O157 in minced beef was studied as part of a risk assessment study. This is of importance for modelling the exposure of consumers to this organism.

A literature review shows that the prevalence of STEC O157 in retail meats is usually around 1%. When STEC O157 is present on carcasses, it can usually be found on most anatomical locations, and at all stages of the slaughter and deboning process. Data about the STEC O157-situation in slaughtering and deboning plants in the Netherlands are lacking.

The aim of this study was to determine which mathematical distributions describe the variation of STEC O157 counts in minced beef, and how grinding and mixing influence this variation. Much attention was paid to the methodological aspects of the determination of variations of STEC O157 in meat. Several sources for variation due to practical factors in the analysis were studied.

The first source studied, was the choice of the selective medium used for the enumeration of cells of a non-toxigenic, nalidixic acid-resistant *E. coli* O157 strain (as a simulant for STEC O157) in minced beef. It was found that for the recovery of unstressed cells, all selective media (CHROMagar O157 (CA), CA with nalidixic acid (CAN), CA with cefixime cefsolodin, and tellurite (CACCT), sorbitol MacConkey agar with nalidixic acid (SMAC) and eosin methylene blue agar with nalidixic acid (EMB)) studied showed similar productivity, but much background flora was found on media without nalidixic acid. Salt/acid stressed organisms were recovered best on EMB. At 5°C, *E. coli* O157 survived better under stressful conditions (tryptone soya broth (TSB) with pH 4.6-4.9, 1% lactic acid, 14% NaCl) than under mild conditions (TSB with pH 7.2, 0.5% NaCl). This observation raises questions about the effectivity of the hurdle concept to control STEC O157 in products such as dry fermented sausage.

The second source studied, was the error that occurs during homogenisation of a sample. The studied methods, stomacher and blender, showed in many cases statistically significant systematic errors but these appear to be of limited practical relevance.

Variations occurring during the preparation of plate counts have been studied as well. The preparation of decimal dilutions results in an increased variance of the data, but can be reduced by using multiple platings. Nevertheless, this variation always causes a ‘detection limit’ for variations in a batch, which can be estimated from the Poisson distribution with the standard deviation adjusted with a factor $\sqrt{10}$ for each level of dilution.

Finally, the effect of the meat grinder on the distribution of STEC O157 and on that of the natural contamination was studied. It was found that even a contamination that occurs very locally on the starting material will be spread throughout the whole batch after grinding,

and usually approximates the Poisson distribution after grinding at least twice. The Poisson distribution with Gamma-distributed parameter λ was helpful to fit the experimental data, and to test the fit of the Poisson distribution to the empirical distribution function and to explore variations beyond randomness.

This study has generated data for further calculations to separate of variations caused by methodological error and true differences between samples.

1. Introduction

Shiga toxin-producing *Escherichia coli*, particularly those of serotype O157 (STEC O157), can cause severe gastro-enteritis. In many cases such an infection leads to serious complications, varying from bloody diarrhoea to the haemolytic uremic syndrome (HUS). This syndrome is characterised by the damage of many organs, most importantly the kidney. Although in the Netherlands the main sources for infection remain unclear [66], observations in the US and UK point to insufficiently heated minced beef as one of the main sources [46 51]. There is no reason to assume that insufficiently heated minced beef would not be an important source for STEC O157-infection in the Netherlands, because the prevalences of STEC O157 in beef and cattle in the Netherlands and the UK are comparable (see Chapter 2). Besides, the custom in the Netherlands, to not thoroughly cook products such as steak tartare adds to the risk for infection.

Currently at the RIVM, a quantitative risk assessment (QRA) study is undertaken on the health risk related to dissemination of STEC O157 in the beef production chain.

Risk assessment consists of four steps, namely hazard identification, exposure assessment, hazard characterisation and risk characterisation [31]. The study presented here should contribute to the exposure assessment.

With respect to the production of minced beef products (Fig 1.1), three questions should be addressed:

1. What mathematical functions can describe the distribution of STEC O157-contamination in or on beef?
2. What is the effect of grinding, mixing and portioning on this distribution (functions 2a and 2b, Fig 1.1)?
3. How can surface contamination (CFU per cm^2) be translated to contamination on a weight basis (CFU per g)?

The grinding process is crucial, because meat of different origins, with different contamination levels, are mixed to make one large amount of minced beef. Any possible contamination on any part of the meat will be spread through the whole lot. This way, a larger amount of meat will become contaminated, the result being a larger number of consumers at risk.

When the variation of the microbial contamination from portion to portion is determined, a problem is that besides the ‘true’ variation, the method itself introduces much variation of the measurements [47] (Fig. 1.2). This variation obscures the information about the actual situation, and should be recognised, in particular when the data are used in quantitative microbiological risk assessment studies.

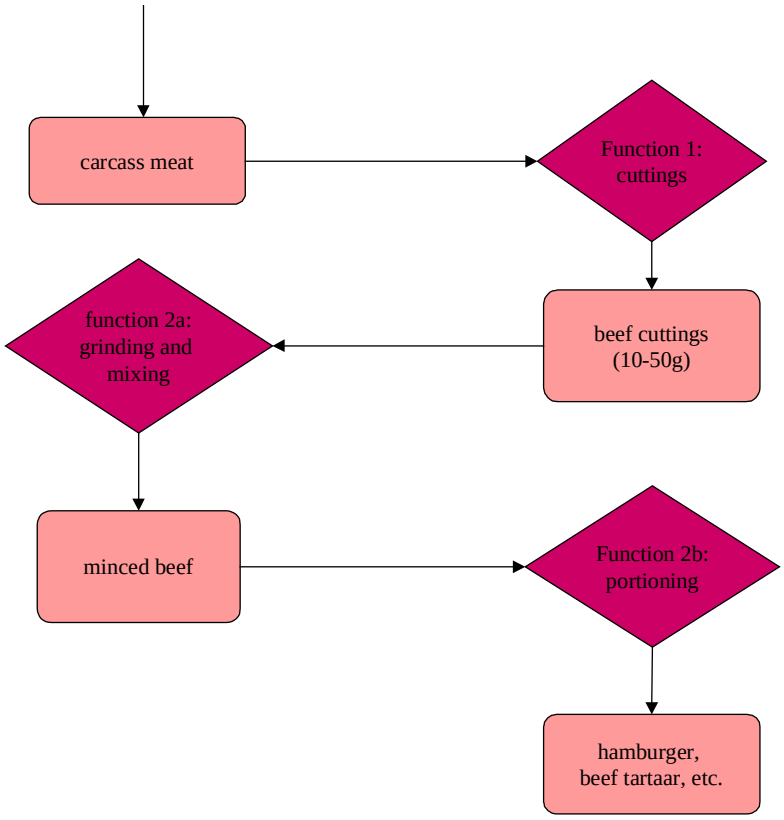

Fig. 1.1. The preparation of minced beef products.

The present study attempts to address the questions mentioned above, where they apply to the production of minced beef (Fig. 1.1). Using data available in the literature (Chapter 2), the occurrence of STEC O157 on carcasses (*post mortem*) and in meat is reviewed. Three examples of sources for variation of data due to the analysis, relevant to this study are discussed in Chapter 3, 4 and 5. In Chapter 3, media are compared to enumerate naturally occurring flora of *E. coli*, coliforms and Enterobacteriaceae, and a nalidixic acid-resistant *E. coli* O157 strain. In Chapter 4, the variation that results after homogenisation of meat is determined. In Chapter 5, random errors that occur during the preparation of bacterial counts are explored. Theoretical distributions are verified with empirical data. In Chapter 6, the effect of grinding on the distribution of *E. coli* O157 and naturally occurring microflora is described.

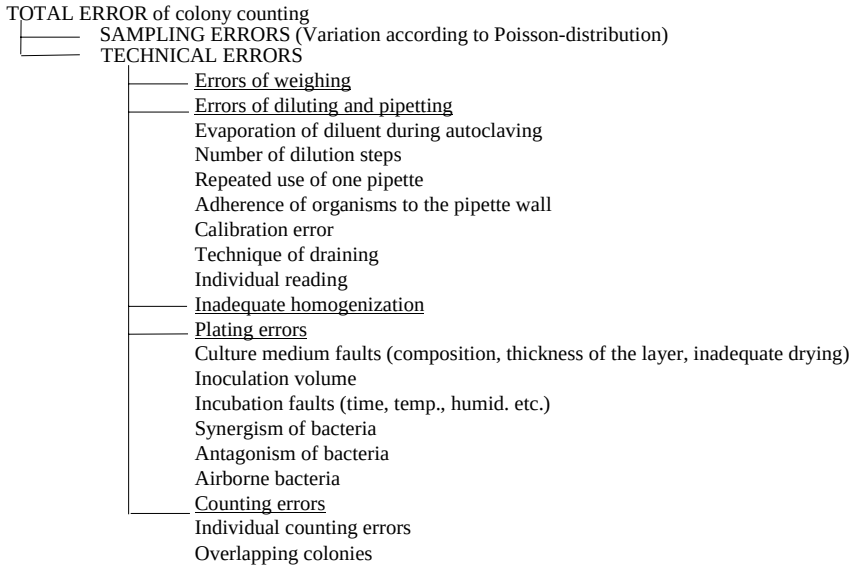

Fig. 1.2. Possible errors in colony counts (Müller and Hildebrandt, 1990 [47]).

2. Occurrence and distribution of STEC O157 on beef carcasses and meat - a review of literature

2.1 Introduction

This section gives an overview of the literature concerning the presence and distribution of STEC O157 on carcasses and in meat. The study is limited to beef. Many papers lack sufficient data on STEC O157. Where possible, data on *E. coli*, coliforms, or other related organisms are used to fill in missing information.

2.2 Occurrence and distribution of STEC O157 on beef carcasses

2.2.1 STEC O157 on beef carcasses in the Netherlands

No data available on this subject.

2.2.2 STEC O157 on beef carcasses in other countries

In Table 2.1 an overview of available literature about the occurrence of STEC O157 on beef carcasses is given. The prevalence ranges from 0 to 2% of the individual carcasses.

2.2.3 Comparison of anatomical locations

McEvoy *et al.* [45] have followed beef carcasses throughout the carcass dressing process, and sampled multiple sites of each of the carcasses. On three of the four contaminated carcasses (36 carcasses studied) STEC O157 could be isolated from all over the carcass at all stages of the dressing process.

E. coli is often used to assess hygiene practices at the slaughter line. Table 2.2 shows the distribution of *E. coli* on steer carcasses in a Canadian beef packing plant which processes 280 carcasses per hour [23]. After skinning, or cutting and tying of the bung in the case of the anal area site, the hock, anal area, and rump sites are relatively heavily contaminated with *E. coli*. In comparison, the waist and both back sites were lightly contaminated. In fact most samples from these sites did not yield *E. coli* in this study. After carcass splitting, the butt, anal area and rump sites are heavily contaminated, whereas the caudal back and waist sites are lightly contaminated. After trimming and washing the rump clearly is the most heavily contaminated site. In general, the variations from site to site decreases during processing, starting after skinning with a range –0.32 to 2.78 log CFU/100 cm^2 and resulting after washing with a range –0.14 to 1.65 log CFU/100 cm^2. Another article on the same subject from these authors showed similar results [24]. Differences can be found from plant to plant: the number of *E. coli* per 100 cm^2 from randomly selected sites on the hindquarters ranged from 2.07 +/- 1.44 log CFU/100 cm^2 on one plant to 0.19 +/- 0.94 log CFU/100 cm^2 on another [25].

Table 2.1. Overview of literature on the occurrence of STEC O157 on beef carcasses.

prevalence	method	country	comments	Ref.
0/750 (0%)	direct, Petrifilm kit-HEC, blot, validation against ref.method	US	although validated, culture /detection without enrichment (!?) questionable.	[7]
lot: 5/30 (95% ci: 5.6-34.7%) indiv: 6/330 (ci: 0.7-3.9%) mean/lot positive: 1.9 (0.2-3.7%)	GN-broth, IMS, CT-SMAC and BCM (a chromogenic agar)	US	4 abattoirs, July/August, This prevalence at the end of the carcass dressing process, which includes antimicrobial treatment, effects of processing, see Table 2.3 data on fecal and hide contamination association, see Table 2.3	[17]
0/384 (0%)	mECn?, Petrifilm kit-HEC	US	decontamination study, culture without enrichment?, not clear, but likely not very sensitive (see ref. [7])	[30]
1/236 (0.4%)	mTSB, SMAC	US	Method, no IMS. A sample of 1 carcass consisted of pooled samples from 10 sites, total 250cm2. 1/12 of this pooled sample was used for STEC O157 detection.	[38]
1/120 (0.8%)	VIDAS, SMAC of Petrifilm	B	Total of 5 abbatoirs investigated. Vidas is alternative immunoseparation	[41]
0/31 (0%)	mTSB, SMAC	US	Pooled samples, source unclear	[37]
no positives	-	US	Two slaughter plants, 40 samples each of carcass, clod, lean trim, and conveyor surfaces	[39]
4/36 (11%)	EEn, CT-SMAC	Irl	Swab samples at several sites, during different stages of processing	[45]
2/125 (1.6%)	enrichment, IMS, CT-SMAC	Can	Swab samples post processing,	[52]
ca. 0.5%	not mentioned	UK	Review article, contains elsewhere unpublished data. Over 4000 carcasses sampled	[59]
4/893 (0.45%)	Petrifilm kit-HEC, mECn enrichment, no IMS	Aus	'export carcasses', 49 slaughter plants all over Australia. 12 month period of sampling, plants were sampled several times in the year. In discussion it is said that similar prevalences were found in the US, but no reference was given, perhaps internet: http://www.aphis.usda.gov/vs/ceah/cahm	[67]

- Abbrev.: ci = confidence interval; GN = Gram negative broth; IMS = immunomagnetic separation; BCM = Biosynth Chromogenic Medium; mECn = modified *E. coli* broth with novobiocin; mTSB = modified Tryptone Soya Broth; SMAC = sorbitol MacConkey Agar; VIDAS = ?, a machine for immunoseparation, EEn = *Enterobacteriaceae* and *E. coli* broth.

Table 2.2. Means (x) and standard deviations (s) of the log values for sets of 25 E. coli counts per 100 cm^2 and the number of samples (n) in which E. coli was not detected. Each set of values was obtained from an equivalent site on each of 25 carcasses, which were randomly selected from the carcasses passing through a beef dressing process [23].

Carcass site	after skinning			after splitting			after washing		
	x	*s*	*n*	*x*	*s*	*n*	*x*	*s*	*n*
1. Hock	2.78	0.76	0	1.18	0.81	2	0.44	0.8	7
2. Butt	1.25	1.27	6	2.58	0.88	0	1.00	1.07	5
3. Anal area	2.72	1.03	1	2.82	0.89	0	0.93	1.00	5
4. Rump	2.01	1.18	3	2.12	1.21	1	1.65	1.28	4
5. Caudal back	0.20	0.99	15	0.44	0.86	8	0.69	0.76	5
6. Waist	-0.32	0.34	18	-0.28	0.37	17	-014	0.45	14
7. Cranial back	-0.13	0.67	17	1.18	0.86	3	0.69	0.94	5
8. Neck	0.75	1.01	6	0.85	1.12	8	0.64	0.64	4
9. Caudal brisket	1.02	1.15	7	0.88	1.09	5	0.5	0.95	10
10.Cranial brisket	1.17	0.78	3	1.55	1.26	3	0.55	0.87	6

2.2.4 Effect of processing steps

Already in section 2.2.3 the effect of processing was mentioned. In addition to the data of McEvoy *et al*. [45] from Ireland, recent data from Elder *et al.* [17] from the US provide insight in the fate of STEC O157 during carcass dressing. In contrast to the Irish work, the results from the US indicated that the presence of STEC O157 is significantly reduced during carcass dressing (Table 2.3). This is likely explained by the fact that in the US study an antimicrobial intervention took place during dressing. Details on this intervention were not mentioned in the article. A significant positive correlation was observed between pre- and postharvest lot prevalence. It is interesting to note that these authors have found a much higher prevalence than previously estimated. On a much smaller scale Chapman *et al*. [9] found that 7/23 (30%) carcasses of the animals that were positive for STEC O157 preharvest (fecal swab) were positive postharvest, whereas 2/25 (8%) of the carcasses of animals that were negative pre-harvest yielded STEC O157 postharvest ($\chi^2 = 3.96$, $P<0.05$).

Table 2.3. The presence of STEC O157 on beef carcasses during slaughter. The present data are the results of a study on four plants, carried out in July-August 1999 [17]. Before 'postprocessing' an antimicrobial intervention took place (no details mentioned). The "postprocessing" samples were taken after the carcasses entered the cooler. Confidence intervals are given in ref [17].

			carcass		
	fecal	hide	preevisceration	postevisceration	postprocessing
individuals	91/341	38/355	148/341	59/332	6/330
- percentage	27.8	10.7	43.4	17.8	1.8
lot	21/29	11/29	26/30	17/30	5/30
- percentage	72.4	37.9	86.7	56.7	16.7
positives/lot (%)	26.2	13.0	43.4	18.3	1.9
- range (%)	0-100	0-89	0-100	0-78	0-22

2.3 Occurrence and distribution of STEC O157 in meat products

2.3.1 STEC O157 in the Netherlands

Two reports on the occurrence of STEC O157 in meat in the Netherlands exist [32 33]. Table 2.4 summarizes the results. The prevalence of STEC O157 ranges from 0 to 1.5%. Although methods have not been compared on the same samples, it is likely that the method had great influence on the obtained results. In particular the introduction of immunomagnetic separation (IMS) and CT-SMAC (method C, Table 2.4) has improved the sensitivity of the isolation method [10 69], and this can explain why in 1996 and 1997 STEC O157 was isolated more frequently than before.

Table 2.4 Isolation of STEC O157 in raw beef at retail in the Netherlands [33].

product	period	prevalence (%)	method*	ref
Raw minced beef	1992-1995	0/1000	A	32
	1992-1995	0/201	B	32
	1996	4/264 (1.5)	C	33
	1997	2/307 (0.7)	C	33
Raw beef	1996	0/61	C	33
	1997	0/162	C	33
Raw minced mixed beef and pork	1992-1995	2/770 (0.3)	B	32
	1996	1/255 (0.4)	C	33
	1997	1/147 (0.7)	C	33

*Method A: enrichment in mTSBn, culture on SMAC; Method B: enrichment in mECn, culture on Petrifilm HEC-kit with immunodetection; Method C: enrichment in mECn, immunomagnetic separation (IMS), culture on CT-SMAC.

2.3.2 STEC O157 in other countries

Several studies outside the Netherlands have been undertaken to isolate STEC O157 from foods at retail. Many studies failed to isolate STEC O157, likely because of the method used (no immunomagnetic separation, use of sorbitol MacConkey Agar (SMAC) without selective substances [62]), or the period chosen for the study (winter months [42]). These studies were left out of this review.

In contaminated minced beef samples, the numbers range from less than 0.3 g^{-1}, to about $6.3 \cdot 10^3$ g^{-1} (Table 2.5).

Table 2.5. Review of selected literature on the presence of STEC O157 in beef outside the Netherlands

product	occurrence	country	method	details	ref.
hamburger/ minced beef	3/58 (5%)	E	IMS	--	4
beefburger	<0.3-2300 CFU/g	UK	IMS	Case-associated samples, says nothing about prevalence, only about bacterial loads.	5
beef mince, sausage, other	overall: 36/3216 (1.1%)	UK	IMS	Product effect, seasonal effect, one-year study, shop-effect P˜0.05	8
beef	6/164 (3.7%)	US	HGMF-immunoblot	--	14
beef	3/107 (2.8%)	US	ELISA	MPN showed that samples contained approx. 0.4 to 1.5 CFU/g	50
beef/pork mixed mince	0/60	D	EiaFoss and reference method	one sample regarded as false-positive by EiaFoss	57
beef	100 to 6200/g	US	HGMF- immunoblot	Case-associated food samples.	64

2.3.3 Changes in *E. coli* counts in minced beef during preparation and at retail

Table 2.6 gives an overview of observational studies on the presence of *E. coli* in beef at different stages of processing. These include an extensive study on the microbiological quality of hamburgers sold in the Netherlands carried out, unfortunately, already 20 years ago.

Gill and McGinnis [22] have followed the changes in the microflora, including *E. coli*, on beef trimmings from collection at slaughter until sale at retail (Fig 2.1). A particular increase in *E. coli* counts and variation is seen in samples taken from displayed beef, in comparison to freshly prepared ground beef.

Work from Chapman *et al.* [8] provides some information about possible differences between retailers. The data indicated that some shops tend to have more contamination than others. When the data were analysed by a χ^2-test, a small effect of was found, which was just statistically significant at 5%-level (P = 0.043). However, when Poisson or Poisson(Gamma)

distributions were used to analyse the data, the statistics (log-likelihood test) did not pass the 5% level (P = 0.052). The extent of cross-contamination that occurs at the retail premises, i.e. caused by insufficient disinfection of the utensils, including the meat grinder, might explain possible differences between retailers [2 35]. In this study, not much is known about the independence of the samples. It is likely that two samples of the same lot can be positive for STEC O157, having a great influence on the scoring of a positive outlet.

Table 2.6. Review of literature on the presence of E. coli and related organisms in minced beef during its preparation and at retail.

processes described	country	organism	comments	ref
beef processing plant, ingredients, contact surfaces and air sampled	US	*E. coli*	not much differences in coli-counts during preparation, only mean logs given	16
beef trimmings at several stages of the preparation of ground beef	Can	*E. coli*	24-sample-sets, log-normal distribution assumed, x and s given. see Fig 2.1.	22
meat used for hamburger production	Can	*E. coli*	each sample consists of meat from 10 samples of ca 100g, from which minced beef was prepared (check in ref)	26
hamburger	NL	Salmonella, *E. coli*	effect of heating, retail samples, 20-year old study. *E. coli* counts generally about 10% of Entero-counts. Correlations between *Enterobacteriaceae* counts and the presence of Salmonella is given	60

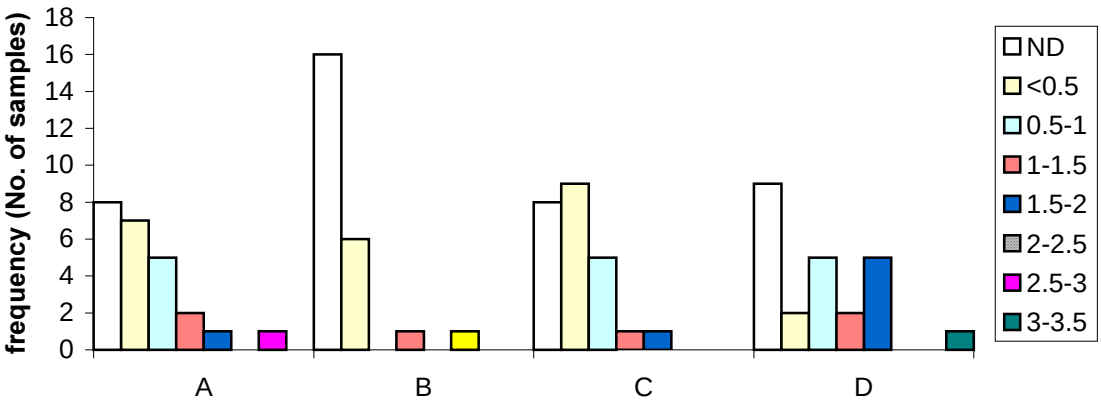

Fig. 2.1. The distribution of counts of E. coli in sets of 24 samples of (A) beef trimmings collected at a slaughtering plant; (B) vacuum packaged beef trimmings delivered to a retail outlet; (C) trimmings ground at the retail outlet; and (D) ground beef on display at the retail outlet. ND indicates the number of samples in which E. coli was not detected. A Canadian study [22].

Figure 2.2 shows the difference in *Enterobacteriaceae* counts of hamburgers sold raw or pre-cooked [60]. Although pre-cooked, most hamburgers still contain *Enterobacteriaceae*.

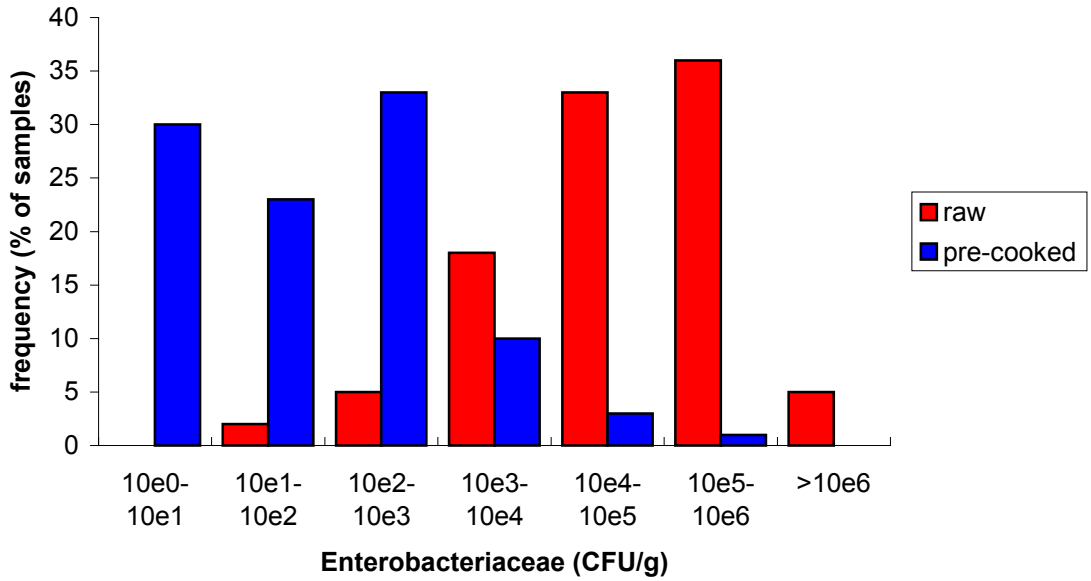

Fig. 2.2. Distribution of Enterobacteriaceae in raw and pre-cooked hamburgers sold in the Netherlands in 1980.

2.4 Discussion

Despite the large amount of literature that has been published in the last decades, there is limited quantitative data about the spread of STEC O157 in the meat production chain, particularly after slaughter. This is mainly caused by the relatively low prevalence, which hampers the collection of sufficient data and draw meaningful conclusions.

Indicator flora may be used to understand more about the contamination of STEC O157 in meat. This is particularly useful when processes are to be controlled according to HACCP-concepts, because the origin of the contamination, contents of the gastrointestinal tract, is similar. When the contamination from this source on the meat can be controlled, this is beneficial to the control of STEC O157 as well. However, direct correlations, for example between high *E. coli* counts and the presence of STEC O157, are usually not found [38].

Direct translation of data of *E. coli*, coliforms and *Enterobacteriaceae*, as presented here, to STEC O157 contamination will be difficult. Reason for this is that in the presented data groups of organisms are counted, which originated from many sources. In the case of STEC O157, usually just one strain will be found, coming from a limited number of sources (for example, a low number of carcasses from one herd). Thus, it is expected that STEC O157 is present in clusters, much more than *Enterobacteriaceae*, coliforms and *E. coli*.

It is generally assumed that bacteria in meat are lognormally distributed [6]. Although statistical evidence for this assumption is not given, it is widely used, for example, to evaluate

the contamination of total aerobic plate counts, coliforms and *E. coli* in several stages of the beef dressing process [23], evaluation of the hygienic performances of hamburger production [26] [29]. For the purposes used in the literature cited, the lognormal distribution is satisfying. However, for QMRA purposes distribution functions that are based on mechanistic considerations, are preferred. Such published data are scarce.

During prolonged cold storage, the distribution of aerobic mesophilic microflora (APC) in minced beef shifts from a more homogeneously to more a clustered distribution [3]. In fresh products a Poisson distribution may be assumed, but after storage a more lognormal one is appropriate, at least for APC. This may be different for the distribution of *E. coli* O157. The main difference is that the variation of the contamination of APC numbers on meat from different sources is likely to be smaller than the variation of STEC O157 contamination on this meat. Measurements should be carried out to determine this specifically. This may be dependent on the temperature during storage. The shift from random distribution, as observed with APC is explained by bacterial growth. Although the minimal growth temperature of STEC O157 has been estimated as low as 4°C [53] or 5°C [49] the numbers of STEC O157 remain constant at 7 or 15°C for at least 5 days [33]. Therefore for STEC O157, the distribution is unlikely to change during cold storage.

This literature review shows that the prevalence of STEC O157 in retail meats is usually around 1%. When STEC O157 is present on carcasses, it can usually be found on most anatomical locations, and at all stages of the slaughter and deboning process. Data about the STEC O157-situation in slaughtering and deboning plants in the Netherlands are lacking.

3. Productivity of media for the enumeration of a nalidixic acid-resistant, non-toxigenic strain of *Escherichia coli* O157

3.1 Introduction

STEC O157 infections have been associated with a wide range of foods, and the organism has shown to survive well in many of them. Some of these foods provide mild conditions to the micro-organism, but strains of STEC O157 have also shown to survive well in low-pH foods, such as filet Americain [33], apple cider [70], and fermented dry sausage [12].

Naturally contaminated samples are scarce and difficult to control. Therefore, the fate of STEC O157 in a product or process can best be studied in *in-vitro* experiments with artificially contaminated foods. In order to be able to quantify STEC O157 in such experiments a suitable method is required. Aim of this part of the study was to develop a method for the enumeration of STEC O157 in survival experiments.

The choice of the right selective agar medium is crucial. Firstly, a medium has to be sufficiently specific to allow reliable discrimination between the naturally occurring flora and the target organisms. Numerous agar media have been developed specifically for STEC O157. A recent survey of 70 laboratories showed that 25 different media are in use by these laboratories for the isolation of STEC O157 [15]. However, most media, including Sorbitol MacConkey Agar (SMAC) with or without additional selective substances, CHROMagar O157, Rainbowagar O157 or 'BCM' O157:H7 agar, do not succeed to reduce the number of aerobic mesophilic organisms from minced beef by more than one log cycle in comparison to Brain Heart Infusion Agar (BHIA) or Tryptone Soya Agar (TSA) [61]. Despite the fact that most media have distinct elective properties due to the addition of chromogens, the target organism can be overgrown because of insufficient selectivity, causing underestimation of the actual number of STEC O157 present.

Secondly, most selective media developed for STEC O157 do not support the growth of sub-lethally injured target-organisms [43 44]. Using SMAC directly, a more than 1000-fold reduction of the number of STEC O157 in comparison to TSA was observed in some cases [44]. Resuscitation of injured cells in liquid media (e.g. BPW) cannot be performed in quantitative studies. Solid repair on TSA, with subsequent transfer on a selective medium or covering the surface of TSA with a selective agar is a good, though labour intensive alternative.

As selective media SMAC, several variants of CHROMagar O157 and Eosin Methylene Blue agar (EMB) were chosen for evaluation. SMAC was chosen because of its widespread use, CHROMagar O157 was chosen for its excellent elective properties [61 68], and EMB was chosen because it has shown good productivity for injured and non-injured target organisms in comparison to other selective media [11-13 20 61]. Besides, good results have been

obtained with EMB previously for the recovery of spiked STEC O157 organisms from drinking water, manure and grass silage [56]. We used a non-toxigenic nalidixic acid-resistant *E. coli* O157 strain. By adding nalidixic acid to the agar media, the selectivity and specificity can be improved enormously, whereas the inability to produce toxins makes the handling of the organism safer. The ability of the chosen media to enumerate the nalidixic acid resistant strain in the presence of microflora of minced beef, and the ability to culture sub-lethally injured organisms was evaluated.

3.2 Materials and Methods

3.2.1 Microorganism and maintenance

Escherichia coli O157 strain rr98089, phage type 34, has been isolated previously from cow feces [55]. The strain harbours the *eae* and *ehly*-genes and shows enterohemolysis on enterohemolysin agar, but does not carry genes for shiga toxin-production. The strain was adapted to nalidixic acid, by spreading 100 µl of a culture grown in brain heart infusion broth (BHI, Oxoid; incubation 18h, 37°C) onto tryptone soya agar (Oxoid) with nalidixic acid (12.5 mg l^{-1}, Sigma). After incubation (24h, 37°C) a colony of nalidixic acid adapted cells (now designated rr98089R) was transferred into BHI with nalidixic acid (BHI-N) (24h, 37°C). 750 µl volumes were mixed with 250 µl sterile glycerol (87%, Sigma) and stored at –80°C. Prior to the experiments a loopful of the stored culture was inoculated and grown in BHI-N (24h, 37°C).

3.2.2 Media

CHROMagar O157 (CA, CHROMagar EE222, ITK Uithoorn, NL) was prepared according to the instructions of the manufacturer. CHROMagar O157 with 12.5 mg nalidixic acid per liter (CAN) was prepared by adding 1 ml l^{-1} of a 1000× stock of nalidixic acid to CA. This stock was prepared by dissolving 125 mg in 4 ml 0.4 M NaOH, adding 6 ml MilliQ water and filtersterilisation of this solution through a 0.2 µm syringe filter (Acrodisc). BBL CHROMagar O157 (CABBL, Becton Dickinson) was a ready-for-use product, based on CHROMagar O157, but supplemented with potassium tellurite, cefixime and cefsoludin. On CA and CAN *E. coli* O157 forms pink colonies, on CABBL *E. coli* O157 forms pink-brown colonies.

Eosine methylene blue agar with nalidixic acid (12.5 mg l^{-1}; EMB) was prepared by adding sodium pyruvate (Sigma; 5 g l^{-1}) and nalidixic acid, 1 ml 1000× stock per liter, to Levine's EMB (Oxoid) after sterilisation. On EMB *E. coli* O157 forms black colonies with a green metallic gleam.

Sorbitol MacConkey agar with nalidixic acid (12.5 mg l^{-1} ; SMAC) was prepared by adding nalidixic acid, 1 ml 1000× stock per liter, to SMAC (Oxoid) after sterilisation. *E. coli*

O157 forms cream colonies. CA, CAN, EMB and SMAC were surface dried 30 min at 50°C and prepared 1 to 3 days before use.

Violet bile glucose agar (VRBG, Oxoid) and Petrifilm EC (PF; 3M) were prepared and used according to the instructions of the manufacturer. For the interpretation of PF the AOAC-protocol was used (see instruction manual). In contrast to most *E. coli,* most *E. coli* O157 form blue colonies. *E. coli* O157 is indistinguishable from coliform colonies, which are purple and show gas formation. All media were incubated at 37°C for 48h and examined after 24 and 48h.

3.2.3 Enumeration of rr98089R in minced beef

Twelve 10-g portions of minced beef, obtained from a local retailer, were prepared in stomacher bags. Six pairs of minced beef portions were inoculated with approximately 5.000, 10.000, 20.000, 50.000 or 100.000 cfu or with sterile peptone saline (PS, 0.85% (w/v) NaCl [Sigma] and 0.1% (w/v) peptone (Difco); 100 µl), respectively. The samples were designated to one of the levels of contamination, randomly. Tenfold dilutions of the samples were prepared by adding 90 ml PS and homogenised in a stomacher for 2 minutes. From this homogenate, a subsequent 10-fold dilution was made in 9 ml PS. Bacterial counts (100 µl dilution/plate) were made in duplicate on CA, CAN, VRBG, PF, SMAC and EMB. Random colonies were confirmed as *E. coli* O157 by latex-agglutination (Oxoid).

3.2.4 Enumeration of rr98089R after acid/salt stress

100 µl of a BHI-N culture was added to 25 ml Injury broth [44] in triplicate. Injury broth consists of tripticase soya broth (TSB), to which 13.5% (w/v) sodium chloride and 1% (v/v) lactic acid is added. The pH is adjusted to 4.9 by 1M NaOH. Three vials of TSB served as unstressed controls (Control broth). During the experiment viable counts were made on TSA. After 12 days incubation at 5°C bacterial counts were determined by duplicate plating on TSA, CA, CAN, CABBL, EMB and SMAC. Random colonies were confirmed as *E. coli* O157 by a latex-agglutination. This experiment was also performed with a lower number of CFU: 100 µl of a 1:1000 dilution, resulting in approximately 4.5 $\log_{10}$ CFU/ml.

3.2.5 Statistical analysis

The Sign-test and Friedman-test were used to analyse the data of the counts of rr98089R in minced beef (Appendix 2). Paired *t*-tests were used to compare the counts of acid/salt stressed cells on different media. Non-paired *t*-tests were used to analyse the difference in survival of rr98089R in Injury broth and Control broth.

3.3 Results

3.3.1 Enumeration of rr98089R in minced beef

Figure 3.1 shows the relations between the selective media for the enumeration of rr98089R spiked in minced beef at variable levels of contamination. The data points closely followed the line of equality, which indicated that all media performed equally. This was confirmed by the Sign- and Friedman-tests (See Appendix 2) (P>0.05). However, on CA and SMAC some false-positive colonies were isolated from the unspiked control samples.

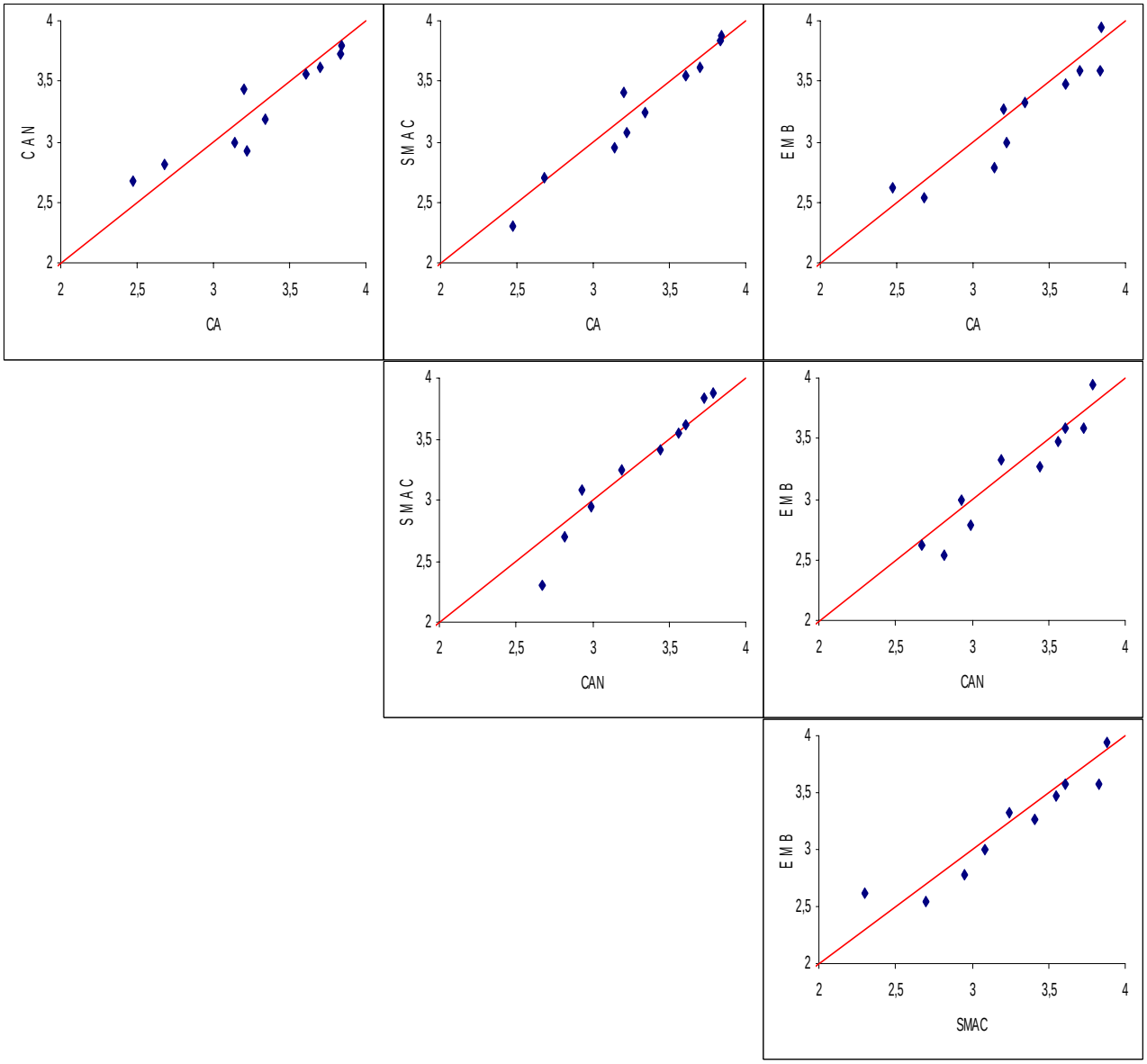

Fig. 3.1. Comparison of selective media for the enumeration of unstressed nalidixic acid-resistant Escherichia coli O157 from minced beef. Numbers indicate log_{10} CFU/g.

Besides, on CA the background flora was present in numbers that were often higher than the numbers of strain rr98089R. The numbers on EMB tended to be lower than the counts on CA or CAN, but these differences were not significant.

Figure 3.2 shows the comparison of CA and CAN with PF and VRBG. The relation between these counts is not very strong. The results of the counts on PF and VRBG are not suitable for the estimation of *E. coli* O157. The plate counts are presented in Appendix 2, Table App.-2.1 and 2.2.

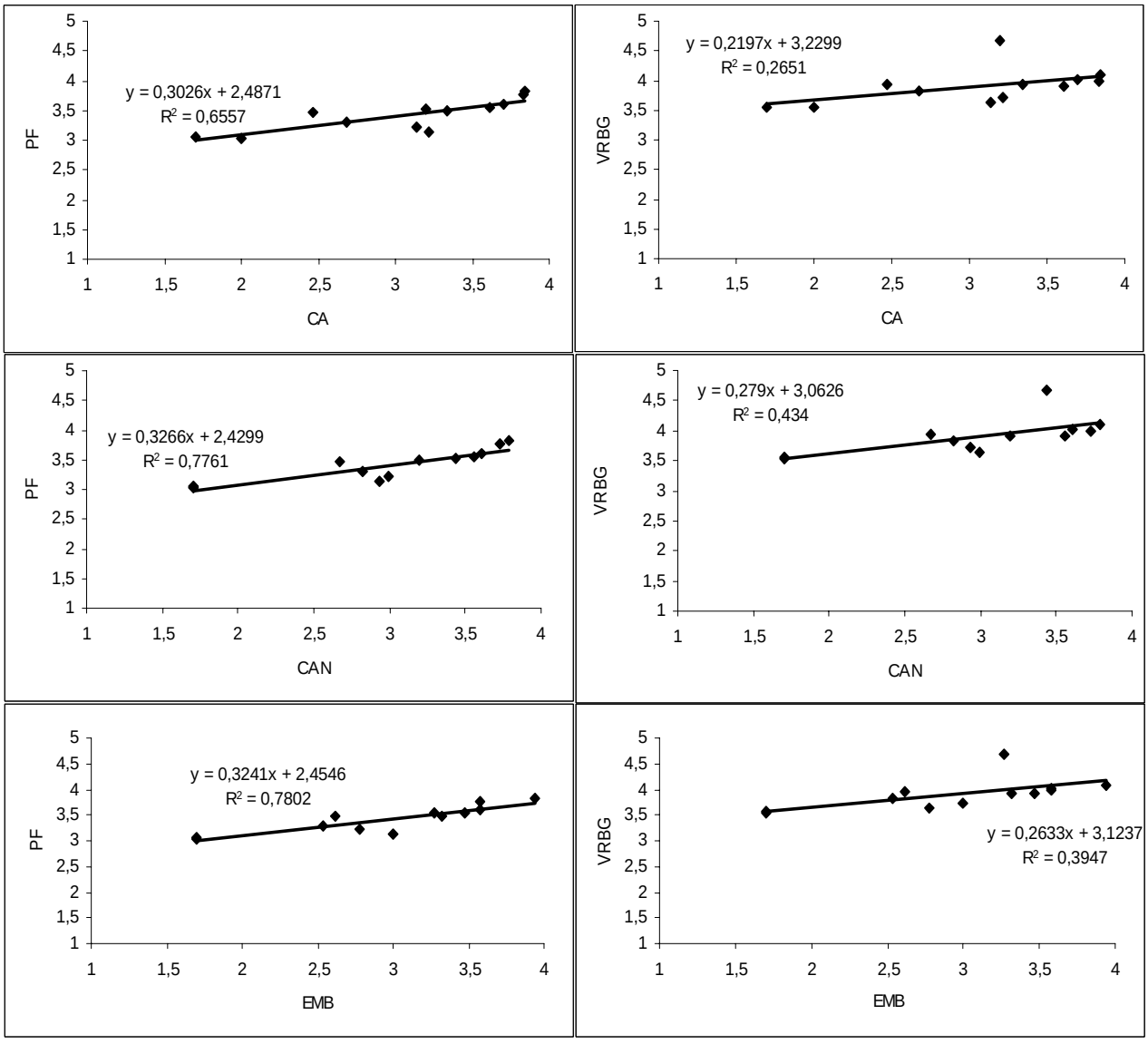

Fig. 3.2. The counts (log_{10} CFU/g) on Petrifilm EC (PF) and Violet Bile Glucose Agar (VRBG), in comparison to the counts on media specific for nalidixic acid resistant Escherichia coli O157 (Chromagar O157 with or without nalidixic acid (resp. CA and CAN), and EMB)).

3.3.2 Counts of rr98089R after acid/salt stress

Table 3.1 shows the recovery of strain rr98089R from Injury broth on different media. Of the selective media EMB performed significantly better than the other media, and although the recovery on EMB was lower than on TSA, the differences in these experiments were not statistically significant. CA and CAN performed equally well, which indicates that the addition of nalidixic acid does not influence the recovery of strain rr98089R. SMAC and CABBL showed the lowest recovery.

Similar results were obtained from the experiments with Control broth (Table 3.2). However, because the results from the Control broth experiments generally showed much higher standard deviations, statistically significant differences were hardly found between the media.

Table 3.1. Enumeration of acid/salt stressed E. coli O157 strain rr98089R in Injury broth on selective media and recovery in comparison to TSA, at high and low inoculum. Mean values (± standard deviation) followed by the same letter are not statistically different at 5%-level.

	high inoculum		low inoculum	
	log CFU/g	Recovery (%)	log CFU/g	Recovery (%)
TSA	7.05±0.11a	100	4.56±0.04v	100
EMB	6.89±0.02a	70	4.44±0.04v	75
SMAC	6.66±0.16abc	45	3.65±0.11wx	13
CA	6.81±0.04b	59	3.93±0.15yz	24
CAN	6.84±0.03ab	62	3.94±0.12xy	24
CABBL	6.56±0.07c	45	3.24±0.27wz	5

Table 3.2. Enumeration of acid/salt stressed E. coli O157 strain rr98089R in Control-broth on selective media and recovery in comparison to TSA, at high and low inoculum. Mean values (± standard deviation) followed by the same letter are not statistically different at 5%-level.

	high inoculum		low inoculum	
	log CFU/g	Recovery (%)	log CFU/g	Recovery (%)
TSA	5.85±0.47a	100	3.99±0.13xy	100
EMB	5.62±0.48a	63	3.59±0.22x	45
SMAC	5.37±0.64a	37	3.45±0.37xy	39
CA	5.49±0.68a	54	2.95±0.35z	12
CAN	5.31±0.31a	32	2.80±0.14z	7
CABBL	5.15±0.99a	36	3.36±0.27y	28

Figure 3.3 shows the survival of strain rr98089R in Injury broth in comparison to Control broth (TSB). During the experiments, the pH of the Injury broth dropped from pH = 4.9 at preparation to pH = 4.6 at the end of the experiment, both in inoculated and sterile broths. The pH of the Control broth was stable at pH = 7.2. At both levels of inoculation (approximately 10^7 and $10^{4.5}$ CFU ml^{-1}) rr98089R survived significantly better in Injury broth than in Control broth (*t*-test: $P = 0.0026$ and 0.0001, respectively).

Figure 3.4 illustrates the comparison of media for the enumeration of strain rr98089R in Injury and Control broths at low inoculum level. On all selective media except EMB, the results of the plate counts of the subsequent decimal dilutions of the Injury broth incubations

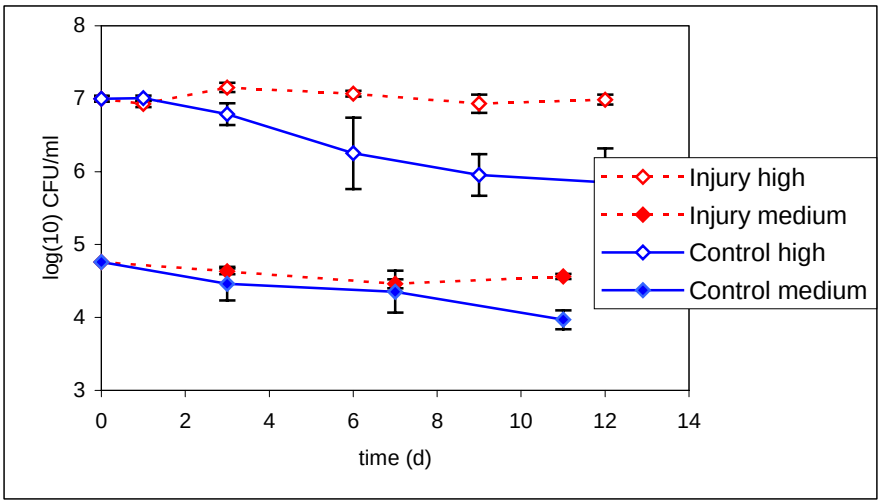

Fig. 3.3. Survival of E. coli O157 in Injury Broth and Control broth at a high and low inoculum level at 5°C. Viable counts were determined on TSA.

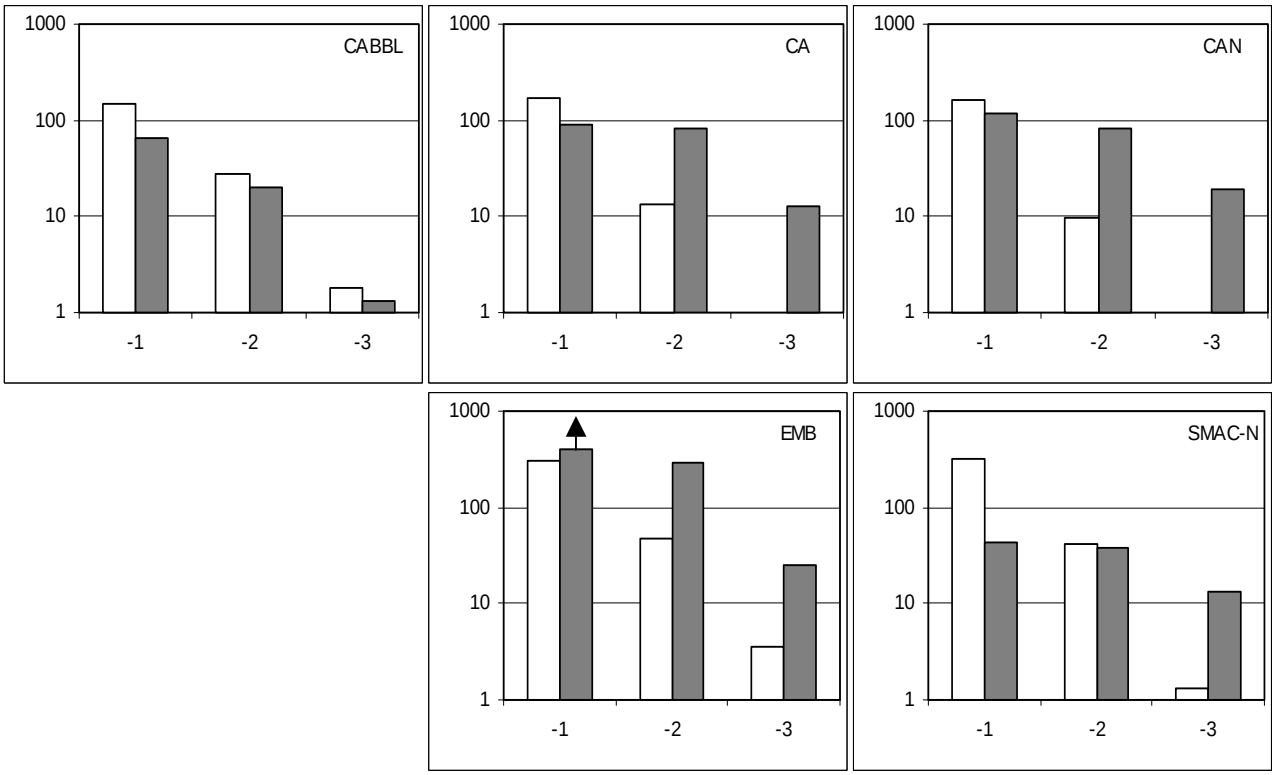

Fig. 3.4. The number of CFU per plate in decimal dilutions of control broth (white bars) and Injury broth (grey bars). The arrow indicates that the number of CFU was too high to count.

did not show a 10-fold reduction between the –1 and –2 dilutions. The plate counts of the –1 dilution were much lower than expected. This problem did not occur with the control counts which did show an approximate 10-fold decrease from the dilutions –1 to –2, to –3, respectively, as expected (Figure 3.4). Data are given in Appendix 2, Table App.-2.3 – -2.6.

3.4 Discussion

All selective media supported the growth of non-stressed rr98089R equally well. However, on CA and SMAC some false positive colonies were recovered from unspiked beef. This might have resulted in some overestimation of the number of target-organisms in the spiked samples as well. On the other hand, much background flora was present on CA. Although the background flora could in most cases be distinguished from colonies of strain rr98089R, there is a risk of the target-organisms being overgrown, causing underestimation.

It is not possible to estimate if the selective media supported maximum productivity, because in this experiment a "golden standard" for productivity was not available. Because of the background flora present, TSA could not be used as such. Data from Calvero and Beuchat [11] indicate that not much difference exists between the recovery of unstressed *E. coli* O157 from minced beef on TSA, MEMB and SMAC. In some cases small but statistically significant differences were found in their study, but these varied from strain to strain and, more importantly, from experiment to experiment.

The presence of nalidixic acid did not influence the recovery of both stressed and unstressed cells of rr98089R. The addition of nalidixic acid proved very useful to prevent the growth of background flora. This is a great advantage, even if the target flora can be distinguished from the non-target flora by the use of chromogens.

For the recovery of stressed cells from Injury and Control broth, EMB was the best performing medium. This has been shown by others before [11-13 20 61]. EMB was the only selective medium that supported the growth of stressed rr98089R from Injury broth at all levels of dilution (Figure 3.4). Although Clavero and Beuchat modified EMB to specifically recognise STEC O157, we preferred the original formulation, only supplemented with sodium pyruvate and nalidixic acid. The problem with modified EMB (MEMB), as it was composed by Clavero and Beuchat [11] is that sorbitol is used instead of lactose. Advantage of this is that *E. coli* O157 can be distinguished from other *E. coli*, because, in contrast to most *E. coli* strains, *E. coli* O157 does not ferment sorbitol within 24h. However, on the sorbitol containing MEMB, *E. coli* O157 colonies vary in colour [61], whereas the use of lactose instead of sorbitol gives clear black colonies, with a green metallic gleam. This facilitates comfortable counting and easy discrimination from non-*E. coli* colonies.

On other media the growth of cells from the –1-dilution were probably inhibited by residual lactic acid and/or sodium chloride from the Injury broth. In combination with inhibiting substances in Chromagar media and SMAC, and perhaps a temperature shock

(from 5°C in the Injury broth to room temperature of the agar), this may have inhibited sub-lethally injured organisms. The temperature shock alone cannot explain this difference, because the counts of the Control broth clearly followed 10-fold reductions at every dilution step. The fact that this problem did not occur with EMB, advocates the use of EMB for the recovery of stressed cells of strain rr98089R.

An important observation was that strain rr98089R survived better in Injury broth than in Control broth at 5°C. Injury broth was composed to mimic dry fermented sausage. These observations are in agreement with observations of Uljas and Ingham [65]. These authors observed that STEC O157 survived at 4°C in apple juice of pH 3.5 without any loss of viable bacteria, whereas the numbers dropped with more than one log-cycle in apple juice of pH 6.5. Similar observations were described in TSB with lactic (pH 4.5), citric (pH 3.5) or malic acid (pH 3.5), but not in TSB with lactic acid at pH 3.5 [65].

These observations are in contrast to common sense, namely that bacteria survive better under neutral conditions than under stressful conditions. Reduced pH and wateractivity would inhibit survival of bacteria. Moreover, the pathogen modelling program (PMP) of the USDA predicts better survival of STEC O157 under neutral conditions at 5°C. Our observations indicate that caution should be taken for the use of mild preservation methods for foods. More work should be done to understand this phenomenon.

This study showed that for the enumeration of non-stressed cells in minced beef, each agar medium investigated is suitable, but CAN and EMB are preferred. However, for the enumeration of acid/salt stressed rr98089R cells EMB is the selective agar medium of choice.

4. Variations caused by homogenisation of ground beef samples

4.1 Introduction

Since its introduction in 1972 [58], the stomacher is widely accepted as an instrument to homogenise samples for bacteriological examination. Since that time, many authors evaluated the stomacher. In most studies, the stomacher was compared to the blender method. In general, both methods perform equally well for most kinds of samples including pork, beef and poultry [21 63] and many other foods [1 18 36 54]. In comparison to the blender, the stomacher tends to give higher counts for milk powder, but lower counts for sausages [54]. Jay and Margitic [36] observed a higher recovery of total aerobic, but in particular of gram-negative microorganisms from beef, including hamburger meat, by stomacher than by blender. In general, problems can occur in foods, which contain high concentrations of fat (see ref. [54] for review).

To our best knowledge, the assessment of systematic error (bias) of both methods, i.e. when the bacterial counts structurally tend to result in lower or higher values than the 'true value', has never been attempted. Here, the location of the micro-organisms in the sample suspension after homogenisation is important. After homogenisation, the sample suspension contains two fractions: fluid and slurry. Usually, only the fluid fraction can be handled in the process of the analysis, particles of the slurry only clog up the pipette-tip. It is assumed that after homogenisation the microorganisms are equally divided over both fractions, so that a sample of the fluid is representative for the whole sample suspension. If this is not the case, for instance, if most microorganisms remain attached to the particles in the slurry, the number of microorganisms in the sample will be underestimated.

Aim of this study was to estimate this systematic error for samples of minced beef. The systematic error was estimated for both the stomacher and the blender, and for different types of microflora, including total aerobic mesophilic flora, *Enterobacteriaceae,* coliforms and *E. coli*. Beef from a local retailer was used, so that the naturally present microflora, with presumably the natural characteristics of contamination and attachment of microorganisms, could be studied. Plate counts of both the fluid and slurry fractions were prepared. This way, the location of the microorganisms could be measured, and the true number of microorganisms present in the sample could be estimated. This estimate was denominated 'complete plate count' (CPC) and served as a 'golden standard'. The differences between the counts of the fluid (denominated 'standard plate count' (SPC)) alone, and the estimate of the CPC gives an indication for the systematic error.

4.2 Materials and Methods

4.2.1 Experimental set-up

Experiment 1

Minced beef was purchased from a local retailer, and divided into 4 10-g samples. 90 ml peptone saline was added to each of the samples. Two samples were homogenised in a stomacher (2 min, normal speed) and the other two samples were homogenised in a blender (10 sec. low speed, 50 sec. high speed). Subsequently, the four homogenates were filtered through a net filter to separate the fluid from the slurry fraction. The volume and weight of the fluid and slurry fractions were determined. The slurry was diluted with peptone saline (1:1) and homogenised in an ultra-turrax (30 sec., high speed). Serial dilutions of the fluid and diluted slurry fractions were plated on Tryptone Soya Agar (TSA), Petrifilm *E. coli* (PF (3M)) and Sorbitol MacConkey agar with 4-methylumbelliferyl-β-D-glucuronide (SMAC-MUG). All media were incubated 24 h at 37°C. TSA was used to determine the total aerobic plate count (APC), PF was used to enumerate coliforms (colonies with gas-production) and *E. coli* (blue colonies with gas production). SMAC was also used to enumerate *E. coli* (purple colonies with fluorescence under UV-illumination).

Experiment 2

The second experiment was carried out as experiment 1, except that "antiflex" was used to filter the homogenate, instead of "net filter".

Experiment 3

The third experiment was carried out as experiment 1, except that:
(1) tea strainers were used to filter the homogenates;
(2) a total of 10 samples (5 stomached, 5 blendered) were tested, and
(3) bacterial counts were made on TSA, Violet Red Bile Glucose agar (VRBG) and PF.
VRBG was used to enumerate *Enterobacteriaceae.*

4.2.2 Calculations

'Standard calculation of the plate count' (colony forming units (CFU) g^{-1}; *SPC*) was calculated as follows:

$$SPC = C_{fluid} \times 10^{-D}$$

with D is the $\log_{10}$ dilution factor of the counted plates, C_{fluid} is the mean number of colonies of the duplicate plates with dilution *D*. For example (See Appendix 3), TSA-counts experiment 3, sample A): $D_{fluid} = -4$; $C_{fluid} = (67 + 72)/2$.

$$\Rightarrow SPC = 7.0 \times 10^{5}\,\text{CFU g}^{-1}$$

To quantify the systematic error caused by used methods for homogenisation, a "Complete calculation of the plate count" (CFU/ml; CPC) was calculated as follows:

$$CPC = \frac{\left(\left(C \times 10^{-D} \times V\right)_{fluid} + \left(2 \times C \times 10^{-D} \times V\right)_{slurry}\right)}{V_{fluid} + V_{slurry}}$$

With C and D as in the previous equation and V as volume of the fluid or slurry fraction respectively. It is assumed that for the slurry g = ml. A factor 2 is introduced for the slurry samples, because the slurry was diluted 1:1 to allow further processing. Example (the same sample as above): D_{fluid} = -4; C_{fluid} = (67 + 72)/2; V_{fluid} = 81 ml; D_{slurry} = -4; C_{slurry} = (117 + 123)/2; V_{slurry} = 13 ml.

$$\Rightarrow CPC = \left(\left(7.0 \times 10^{5} \times 81\right) + \left(2 \times 1.2 \times 10^{6} \times 13\right)\right) \times \left(\frac{1}{81+13}\right) = 1.3 \cdot 10^{6}\,\text{CFU g}^{-1}$$

The %-error of the estimation of CPC by SPC, was calcutated as follows:

$$\%-Error = \frac{SPC - CPC}{SPC} \times 100\%$$

CPC is regarded the ‘golden standard’. The sign indicates overestimation (+) or under-estimation (-), respectively. Example (the same sample as above):

$$\Rightarrow \%-Error = \frac{7.0 \cdot 10^{5} - 1.3 \cdot 10^{6}}{7.0 \cdot 10^{5}} \times 100\% = -81\%$$

In this example, the result of *SPC* is an *under*estimation of *CPC*. In fact, *CPC* = 1.81 x *SPC*.

4.2.3 Statistical analysis

To test if any systematic error occurred by using either the stomacher or blender method for homogenisation of a sample, it is assumed that the bacterial counts of the fluid and slurry (CFU ml^{-1}) are equal:

$$H_0 : C_{fluid} \times D_{fluid} = C_{slurry} \times D_{slurry}$$

T-tests were used for statistical analysis, with $\alpha = 0.05$.

Alternatively, the systematic error, calculated as mentioned above, was used in the statistical analysis:

$$H_0 : \left(\frac{\sum (Error)_i}{n} \right) = 0$$

The following null-hypothesis was used to test if the blender and stomacher methods for homogenisation of the samples performed equally:

$$H_0 : \left(\frac{\sum (SPC)_i}{n} \right)_{stomacher} = \left(\frac{\sum (SPC)_i}{n} \right)_{blender}$$

t-tests were used to compare blender and stomacher homogenisation, $\alpha = 0.05$.

4.3 Results

Experiment 1

The results of APC of experiment 1 are shown in Appendix 3, Table App.-3.1. Coliform and *E. coli* counts were too low for meaningful calculations. The APC counts in the slurry were higher than the APC in the fluid, but this difference was not statistically different from zero, for both stomacher ($P = 0.468$) and blender ($P = 0.475$). This indicates that the systematic error of both methods --underestimation of the CPC-- was not statistically different from zero. The difference between the systematic errors of the stomacher and blender was not statistically different ($P = 0.495$), either.

Experiment 2

The results of APC of experiment 2 are shown in Appendix 3, Table App.-3.1. Again, coliform and *E. coli* counts were too low for meaningful calculations. In contrast to the results of experiment 1, the APC counts in the slurry were lower than the counts in the fluid, which resulted in an overestimation of the CPC counts. This overestimation was not statistically different form zero, for both stomacher ($P = 0.100$) and blender ($P = 0.444$). The difference between the systematic error of the stomacher and blender was not statistically different ($P = 0.682$), either.

Experiment 3

The results of the total APC, *Enterobacteriaceae* and coliforms are shown in Appendix 3, Table App.-3.1, 3.2 and 3.3, respectively. The *E. coli* counts were to low for meaningful calculations. The results are summarised in Table 4.1.

The APC of the slurry was significantly higher than the APC of the fluid for both stomached and blendered samples ($P = 0.014$ and 0.015, respectively). This difference is illustrated in Figure 4.1 (APC). Both methods showed a significant systematic error. The stomacher showed an underestimation of -38% ($P = 0.012$), whereas the blender gave -8.2%

underestimation (P = 0.002). The difference between the systematic error of the stomacher or blender was not significant (P = 0.057).

The *Enterobacteriaceae* counts in the slurry of the stomached samples were significantly higher than the counts in the fluid (Figure 4.1 (Entero's); P = 0.043). This caused a systematic underestimation of -15%, which was not statistically significant (P = 0.051). The *Enterobacteriaceae* counts in the slurry and the fluid of the blendered samples were not significantly different (P = 0.134). Hence, the systematic error of 1.4% was statistically insignificant (P = 0.129). The difference between the systematic error of the stomacher and the blender was statistically significant (P = 0.036), though.

The coliform counts in the slurry were significantly lower than the counts in the fluid in both the stomached (P = 0.005) and the blendered samples (P = 0.021; Figure 4.1 (Coliforms)). This resulted in a statistically significant overestimation of 10.1% (P = 0.003) and 3.8% (P = 0.014) for the stomached and blendered counts, respectively. The difference between the systematic error of the stomacher and the blender was statistically significant (P = 0.021). In general, the samples that were homogenised by the blender method yielded less slurry than the samples that were stomached.

Table 4.1. Summary of the results of experiment 3. Estimation of the systematic error of two methods of sample homogenisation (stomacher or blender), for the determination of three types of bacterial populations.

Microflora	log CFU/g	systematic error (%)			
		stomacher		blender	
		mean	st.dev.	mean	st.dev.
APC	5.9	-38.4*	25.5	-8.2**	2.4
Enterobacteriaeae	3.7	-15.2	10.9	1.4	1.8
Coliforms	2.3	10.1**	4.0	3.8*	2.0

*. P<0.05; ** P<0.01.

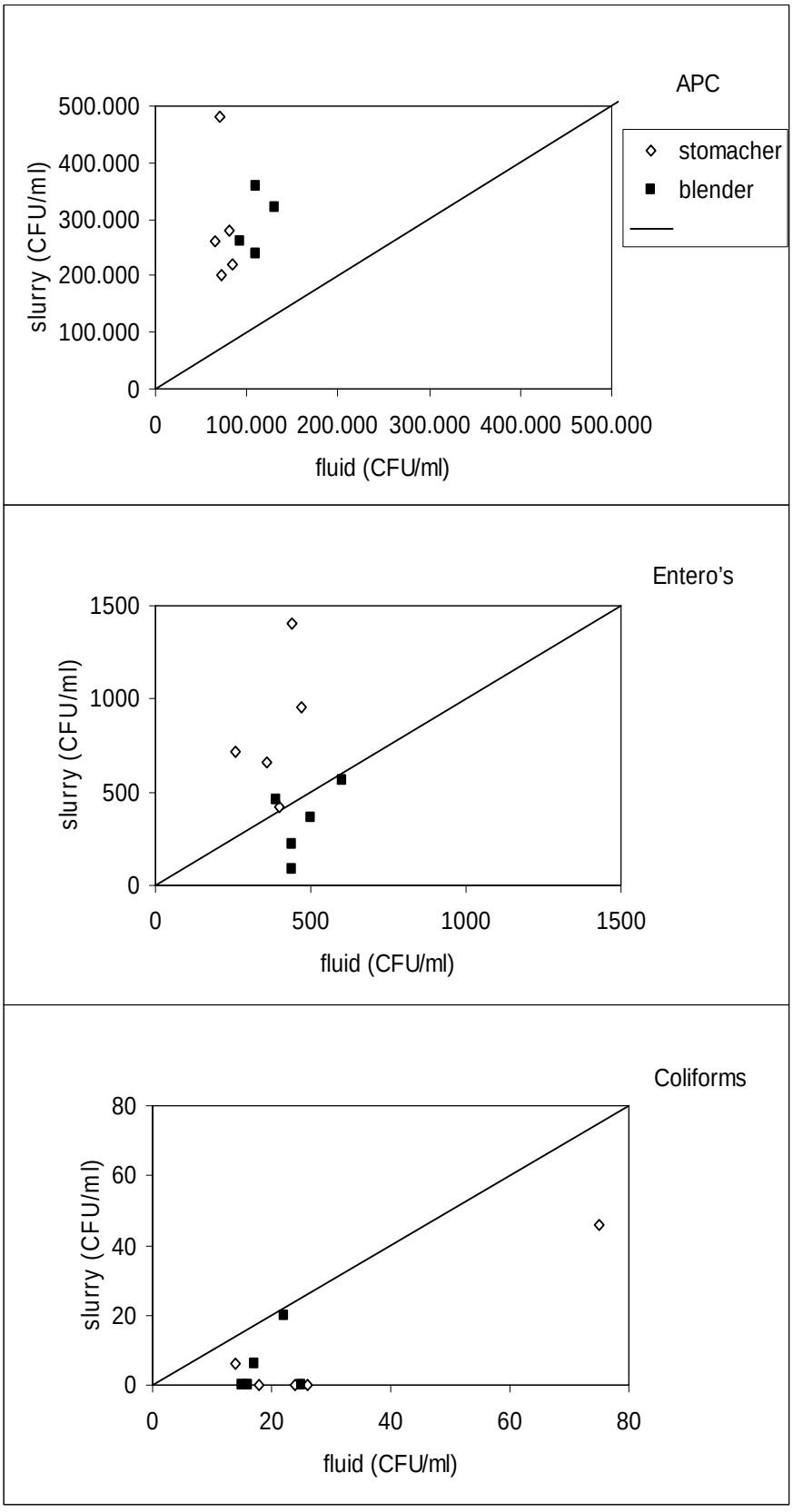

Fig. 4.1. Comparison of the APC, Enterobacteriaceae (Entero's) and coliforms in the fluid and the slurry of samples homogenised by blender (■) or stomacher (◊). The line indicates where the number in the fluid and slurry are equal.

4.4 Discussion

When bacterial numbers are calculated, it is assumed that the bacterial population in a homogenised sample is distributed evenly over the fluid and slurry fraction. In order to test this assumption, the 'true' size of the bacterial population had to be known. In our experiments, we chose to use the natural contamination instead of an artificial contamination. The disadvantage was that we did not have exact knowledge about the size of the investigated population prior to the experiment, but the advantage was that the investigated population had 'natural' characteristics. For example, bacteria may not only be present at the surface of the meat, but can also have penetrated the muscle tissue, by passing between muscle fibres, through degradation of non-collagenous layers by proteolytic enzymes [27 28]. Although we did not study intact meat, such aspects may influence the location and release of bacteria from minced beef during homogenisation, as well. In order to estimate the systematic error of both methods, the actual number of bacteria in the sample was calculated from the results of the fluid and the slurry ('complete plate count' (CPC)). This number was regarded as the 'golden standard'.

The results give insight into the total error that may occur during homogenisation. For example, the breakdown of cell clumps (aggregates) is not studied in particular. It may be possible that during homogenisation of the slurry in the stomacher, cell clumps that exist after blendering or stomaching are destroyed, so that the number of CFU in the slurry becomes higher. Additionally, pipetting errors, errors in the volumes of diluents used, may have caused additional error, too, but the relative importance of these errors is thought to be low in this experiment.

In the three experiments over- or underestimation of the total aerobic plate counts (APC) varied and might depend on the experimental set-up. Main difference between these experiments was the method by which the fluid and slurry was separated, namely by 'net filter', 'antiflex' or teastrainers, respectively. The teastrainer and 'net filters' probably mimic the pipette-tip best, because it does not absorb fluid, and the distance of the wires is approximately the same as the opening of the pipette-tip. Thus, experiment 1 and 3 likely represent the normal situation best.

For both methods the systematic error is dependent on the type or size of the population counted. With both methods a large population of aerobic mesophilic organisms (APC), 5.9 $\log_{10}$ g^{-1}, was underestimated, whereas the small coliform population 2.3 $\log_{10}$ g^{-1}, tended to be overestimated. We could not find a likely explanation for the underestimation of the APC counts. The overestimation of the coliform counts might have been caused by the lower counts of the slurry, due to the presence of much debris on the agar surface at the lowest dilution. Previously, Gerats and Snijders [21] did not find differences between means and variations of bacterial counts of 110 samples of meat after

homogenisation by stomacher or blender, for APC, *Enterobacteriaceae* of Gram-negative microflora.

The extend of the under- or overestimation was larger with the stomacher method than with the blender method. This could likely be explained by the observation that the blender method yielded a fluid fraction that was generally larger than that of the stomacher method. Therefore, the counts of the fluid (SPC) took a greater part in the CPC calculations for the blender samples than for the stomacher samples. As a consequence, the SPC counts and the CPC counts of the blender samples were more similar, and the systematic error was smaller. The difference between the systematic errors of the stomacher and the blender could thus be explained by the higher yield of fluid of the blender method. Apparently, the blender is better able to produce particles that can pass teastrainers than the stomacher. Such particles can be handled by pipette-tips.

In Table 4.2 the SPC and CPC results are shown as expressed in $\log_{10}$ CFU g^{-1}. This table shows that the systematic error, which was found as high as 81% (TSA-counts of sample A), does not influence the results so much on a log-scale.

In conclusion, the systematic error of the bacterial count, caused by homogenisation of a sample of minced beef depends on the size or type of the bacterial population and the method of homogenisation. The systematic errors of the stomacher are higher than the systematic errors of the blender. In addition, the blender causes less variation than the stomacher. In practice these differences are negligible when compared to variations between subsamples.

Table 4.2. Comparison of log-transformed observed (SPC) and expected (CPC) counts.

	sample	TSA		VRBG		Petrifilm	
		SPC	CPC	SPC	CPC	SPC	CPC
stomacher	A	6.1	5.8	3.8	3.6	2.1	2.1
	B	5.9	5.9	3.6	3.6	2.9	2.9
	C	6.0	5.9	3.5	3.4	2.4	2.4
	D	6.0	5.9	3.6	3.6	2.3	2.4
	E	6.0	5.8	3.7	3.7	2.2	2.3
blender	F	6.0	6.0	3.6	3.6	2.4	2.4
	G	6.1	6.0	3.6	3.6	2.3	2.3
	H	6.1	6.0	3.6	3.6	2.2	2.2
	I	6.1	6.0	3.7	3.7	2.2	2.2
	J	6.1	6.1	3.8	3.8	2.2	2.2

5. Variations caused by diluting and plating

5.1 Introduction

When a production lot of hamburgers is sampled for microbiological examination, the results of the counts will show a probability distribution, which is the sum of the actual variation in the microflora from hamburger-to-hamburger and the variations due to the method of enumeration. Many steps of the process of plate counting can cause variation in the results (Fig 5.1).

In 1922, Fisher *et al.* [19] demonstrated that under ideal circumstances, the distribution of parallel plate counts is completely random, and this has been confirmed by many others (reviewed in [34 47]). A random distribution can be mathematically described by the Binomial distribution, or alternatively, by the Poisson distribution.

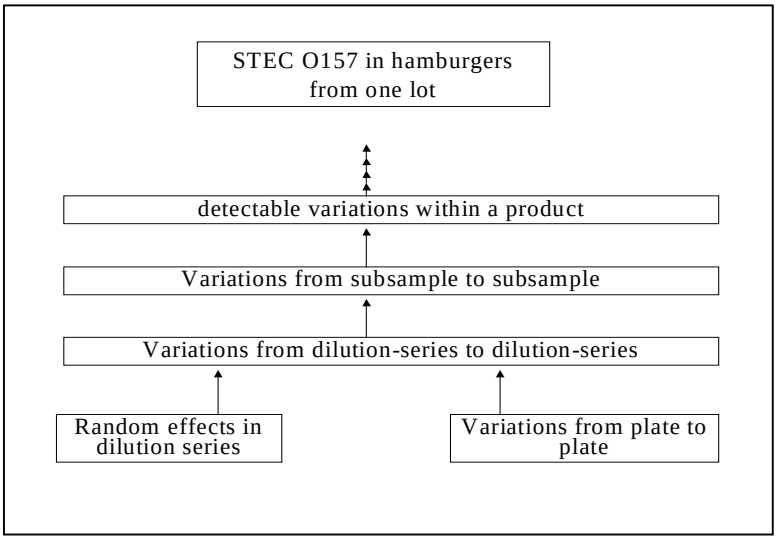

Fig. 5.1. Sources for variation (random errors) in the results of the enumeration of microorganisms in a product.

Sources of additional error include, for example, variations in temperature and volumes of dilution buffers, inaccuracies of pipettes, inaccuracies of personnel, colony forming units that originated from aggregates of more than one viable cell, or variations in the physiological conditions of individual organisms [47]. As a result, many authors found that microbiological enumerations of a variety of products was described best by a normal or log-normal distribution, rather than the Poisson distribution [34 40]. However, normal or log-normal distributions are descriptive rather than mechanistic distribution functions, and do not give

insight into the variation of the data beyond randomness. Exactly this information is of great relevance for data to be used in quantitative microbiological risk assessment models [48].

Aim of this part of the study was to evaluate if the variations in the plate counts of minced beef can be described by the Poisson distribution. The Gamma distribution is introduced to explore variations of the Poisson parameter λ, in order to describe differences between the empirical cumulative distribution function (EDF) and the Poisson distribution. The evaluation is made for each step in the process of enumeration and is discussed in the order of the direction of the arrows in Figure 5.1.

5.2 Materials and Methods

5.2.1 Bacterial enumeration

Violet red bile glucose agar (VRBG; Oxoid) was used to enumerate *Enterobacteriaceae*. Pour plates were prepared into which 1 ml of diluted sample was mixed. Petrifilm EC (3M) was used to enumerate *Escherichia coli* and coliforms. On each Petrifilm plate 1 ml of a diluted sample was brought. Details of incubation and interpretation are given in paragraph 3.2.

5.2.2 Sample preparation

From an amount of whole beef, purchased at local retail outlets, surface samples were taken by a cork borer (5 cm^2). The samples were homogenised with 15 ml peptone (1% w/v) saline (0.85% w/v; PS) in a stomacher (2 min at medium speed). Subsequent decimal dilutions were made in PS. 1-ml volumes of the homogenates and dilutions were added to the agar media.
From an amount of minced beef or hamburger, purchased at local retail outlets, 10-g samples were homogenised with 90 ml PS in a stomacher (2 min). Bacterial counts of whole and minced beef were determined in various strategies (Table 5.1; Figure 5.2).

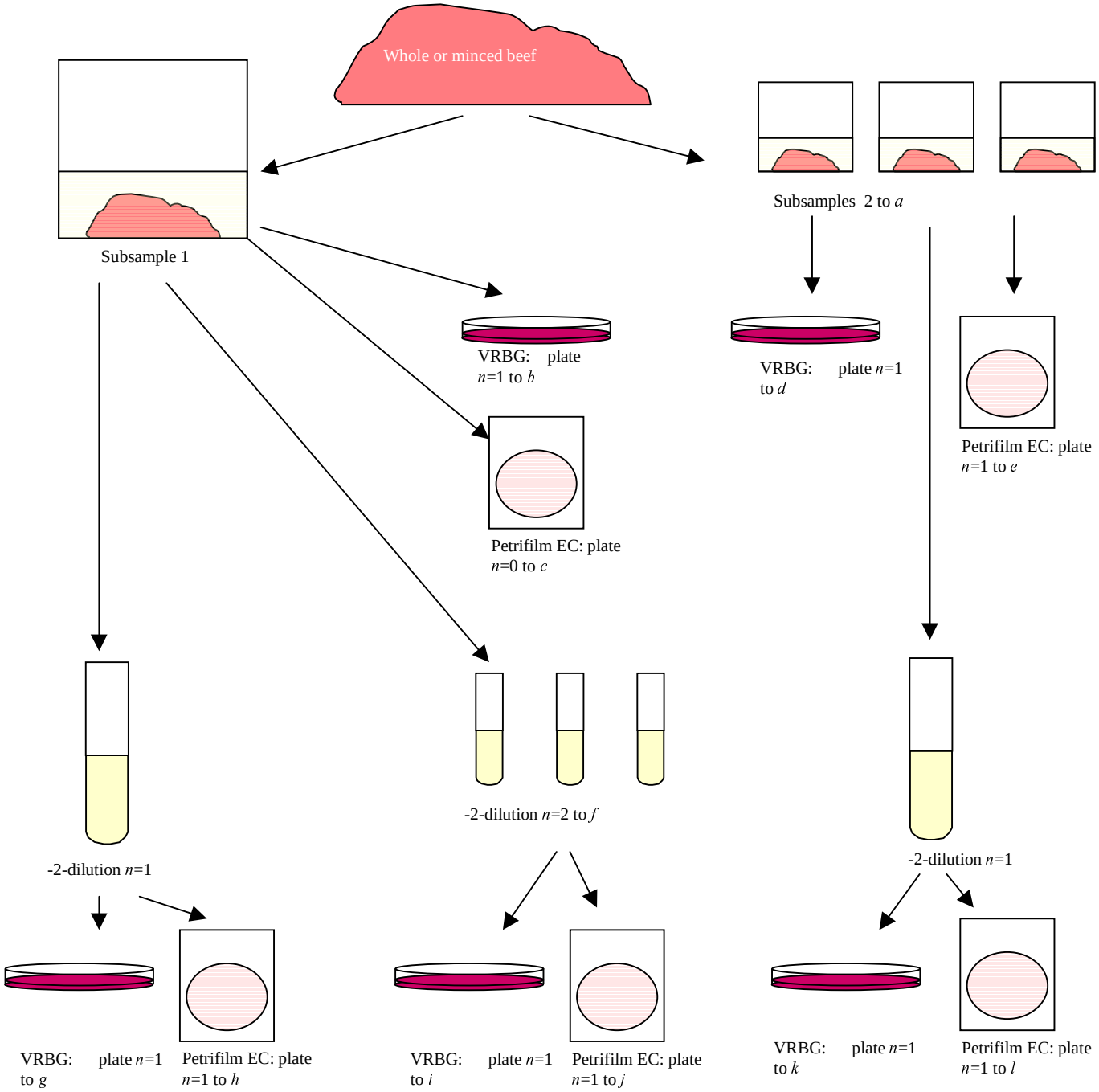

Fig. 5.2. Experimental set-up. The values of a to l are mentioned in table 5.1, for the respective experiments.

Table 5.1. Experimental set-up. The values of a to l for each of the six experiments described in this chapter, and mentioned in Fig 5.2.

	appendix	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*j*	*k*	*l*
Exp. 1. hamburger	5.1	1	7	-	-	-	7	7	-	1	-	-	-
Exp. 2. whole beef	5.2	4	7	-	1	-	7	7	-	1	-	1	-
Exp. 3. minced beef	5.3	4	7	-	2	-	7	7	-	1	-	2	-
Exp. 4. whole beef	5.4	4	7	-	2	-	7	7	-	1	-	2	-
Exp. 5. minced beef	5.5	7	10	7	2	2	7	3	3	3	3	2	2
Exp. 6. minced beef	5.6	7	7	7	2	2	7	3	3	3	3	2	2

5.2.3 Mathematical approximations to the empirical distribution function (EDF)

All calculations were carried out in MS Excel.

5.2.3.1 Empirical distribution function (EDF)

The EDF is drawn as follows. A number of *n* data are collected and sorted in increasing order, with *i* from 1 to *n*. The cumulative probability is calculated for each of the data as *i/n*.

5.2.3.2 Poisson distribution with fixed λ.

The Poisson distribution has one variable: λ. Mean and standard deviation can be derived from the value λ as follows:

$$mean = \lambda$$
$$s.d = \sqrt{\lambda}$$

Poisson curves were fit by estimating λ through several approaches. The first approach was to use the mean count of the empirical data as estimate for the Poisson variable λ. The second approach was using the smallest square method. With this method the differences between the cumulative probability of the theoretical and the empirical distribution function are squared for each observation, and the sum of these squares is minimised by adjusting the parameter λ by using the solver function in Excel.

5.2.3.3 Poisson distribution with Gamma-distributed λ

Instead of a fixed value λ, a statistical distribution can be used for λ to fit the Poisson curve. The Gamma-distribution, with parameters α and β was used for this. These variables can be estimated by the maximum likelihood method or the smallest square method. With the maximum likelihood method the sum of the natural logarithm of probability for each observation is multiplied by –2. This product is minimised by changing λ by using the solver function in Excel. The solver function in Excel is used to estimate these parameters, with $0.001 \leq \alpha \leq 171$ and $\beta \geq 0.001$. These constraints were defined to prevent that the solver performs calculations beyond the capacity of Excel. As a consequence, it is assumed that if $\alpha > 171$, then $\alpha \rightarrow \infty$. Mean and standard deviation can be derived from the values α and β as follows:

$$mean = \alpha\beta$$
$$s.d. = \alpha\beta^2$$

5.2.3.4 Binomial distribution

The Binomial distribution, with parameters n and p, was fit to the EDF by calculating the total number of CFU in a suspension, and the fraction that was plated. Mean and standard deviation can be derived from the values of n and p as follows:

$$mean = np$$
$$s.d. = \sqrt{np(1-p)}$$

The values for n and p are calculated when the amounts of the sample and subsample and the mean number of colonies in the subsamples are known. For example, from a volume of 10 ml, an amount of 0.1 ml is spread on agar plates. As a result the value p equals 0.1/10 = 0.01. When the *mean* number of colonies is 25, n can be calculated as follows:

$$n = \frac{mean}{p}$$
$$\Rightarrow n = \frac{25}{0.001} = 2{,}500$$

5.2.3.5 Normal distribution

The Normal distribution has two variables: μ and σ. μ is estimated by calculating the mean CFU from the data; σ is estimated by calculating the standard deviation of the data.

5.2.3.6 Log-normal distribution

The Lognormal distribution has two variables: μ^* and σ^*. μ^* is estimated by calculating the mean of the data after transformation to the natural logarithm. σ^* is estimated by calculating the standard deviation of the data after transformation to the natural logarithm.

5.2.4 Statistical analysis

Statistical differences between parallel subsamples or between parallel dilutions were analysed by ANOVA. The theoretical distribution functions were compared to each other by using the maximum likelihood test. The theoretical distribution functions were compared to the EDF by Kolmogorov-Smirnov. For the Kolmogorov-Smirnov tests the *P*-values were calculated at http://www.io.com/~ritter/JAVASCRP/NORMCHIK.HTM.

5.3 Results

Data collected in the experiments described above were used to evaluate the distribution of the enumerations in every step of the process. The counts of whole beef were, however, not useful for our purposes, because of the low numbers and many negative results that were obtained (Appendix 4, Table App.-4.2 and App.-4.4).

5.3.1 Variation from plate to plate

5.3.1.1 Theoretical approach

Figure 5.3 shows theoretical curves for Poisson (with λ = mean) and Binomial distributions. In this example it is assumed for the binomial distribution that from 10 ml of a dilution 1 ml is drawn (p = 0.1) and n = mean/p. Figure 5.3 demonstrates that with p = 0.1 a small difference exist between Poisson and Binomial, independent of the mean, which ranges from 10 to 100 CFU per plate.

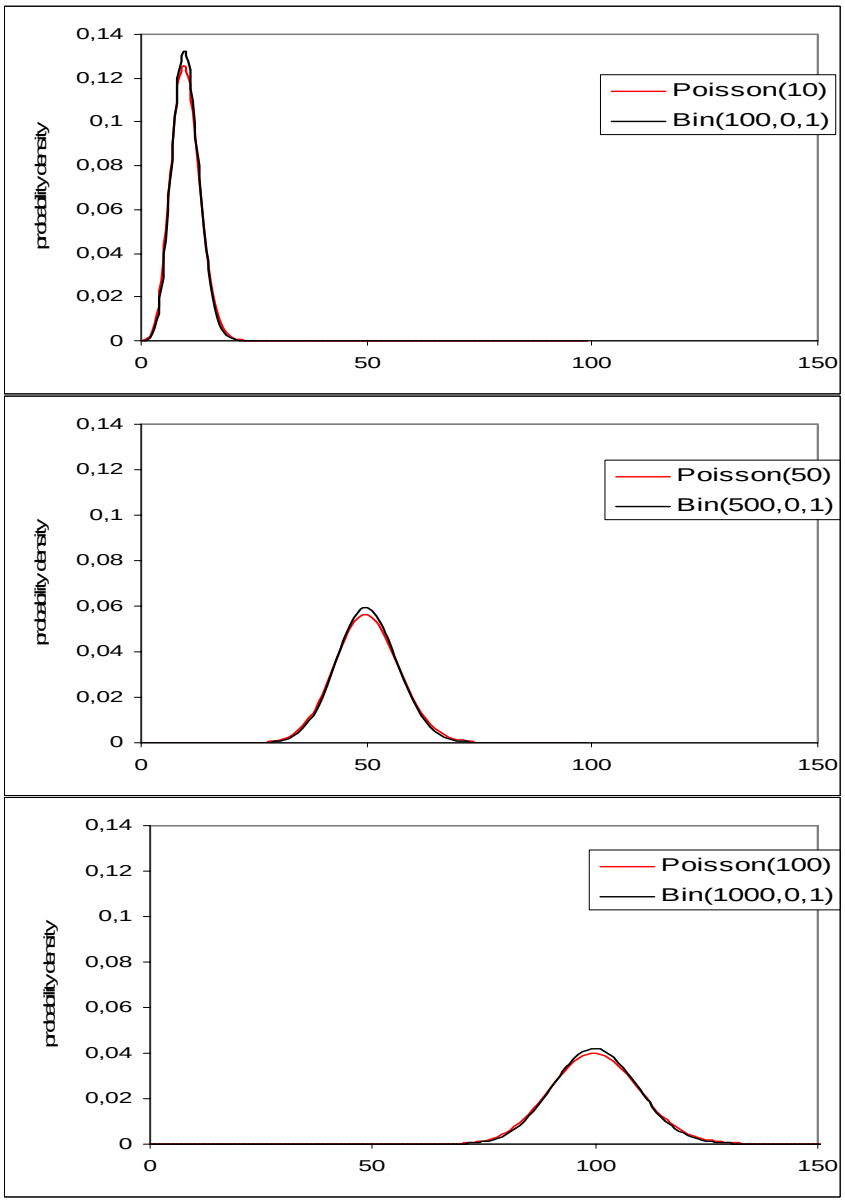

Fig. 5.3. Comparison of the Poisson and Binomial distribution at different mean values.

Figure 5.4 demonstrates that when *p* decreases, with *np* = constant, the Poisson distribution approaches the binomial distribution. In this Figure it was assumed that 1 ml samples were taken from 10, 100 or 1000 ml, respectively. This Figure shows that for larger volumes the Poisson curve and binomial curves are almost identical. For comparison of plate counts from dilutions of 10 ml volumes the Binomial is more correct, but Poisson is probably an acceptable alternative in every case. It is a rule of thumb that with $n > 50$ the approximation of the Binomial distribution by the Poisson distribution is acceptable.

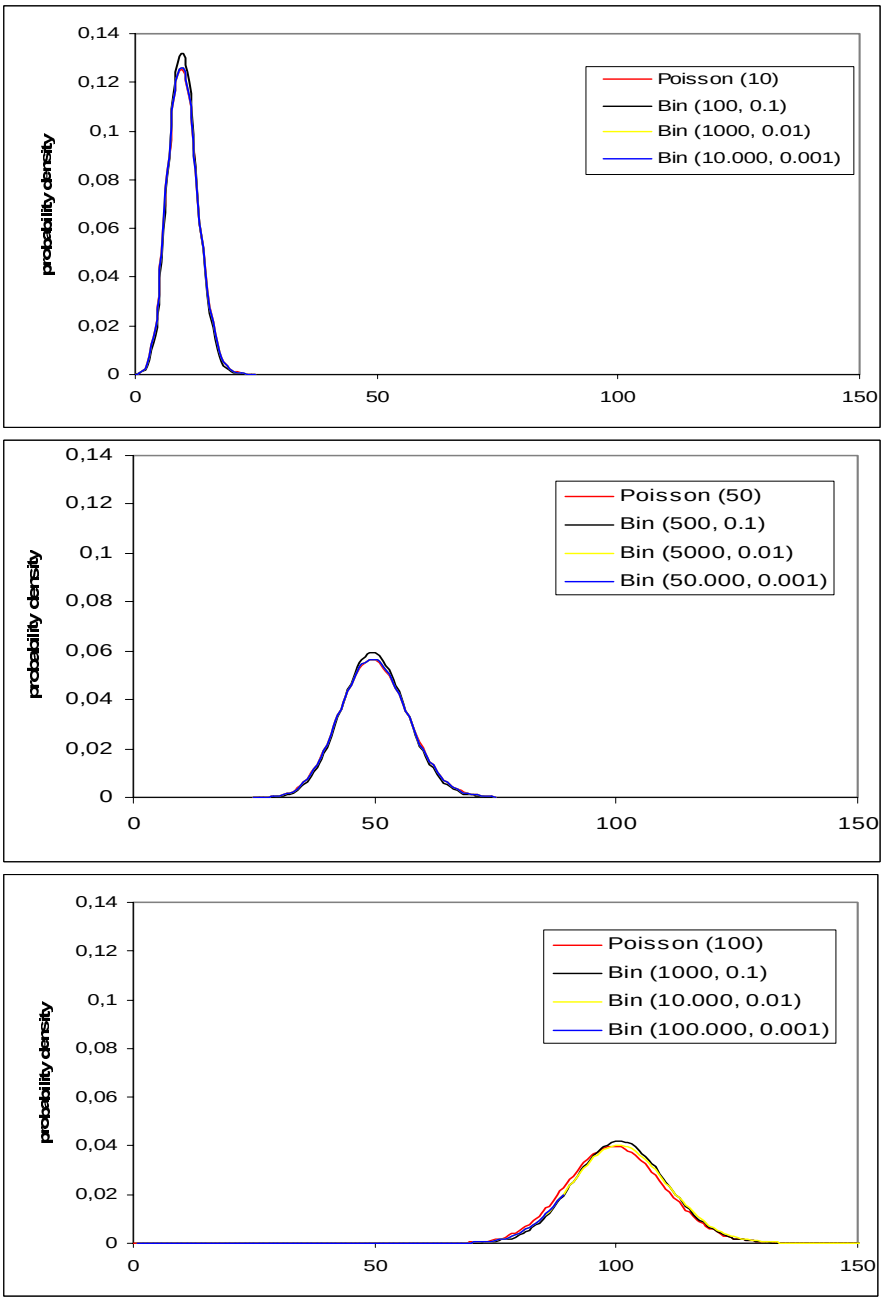

Fig. 5.4. The effect of the value of p, with np = constant, on the shape of the distribution curve, and comparison with the Poisson distribution curve.

5.3.1.2 Verification with experimental data

Empirical data were used to test the hypothesis that the Poisson distribution is an acceptable function for the distribution of plate counts from 1 ml of a suspension of 10 ml, as mentioned above, and a good alternative for the Binomial distribution. The EDF of the data of the of Enterobacteriaceae counts of the –2 dilution of minced beef (Appendix 4, Table App.-4.3) were compared to the Binomial (n = 107.1; p = 0.1) and Poisson distributions (λ = mean, in this example: λ = 10.71). Additionally, Normal (μ = 10.71; σ = 3.20), and Lognormal (μ^* = 2.33; σ^* = 0.336) distribution curves, and a Poisson curve with a Gamma distributed λ were produced (Fig. 5.4). The variables of the Gamma-distribution, α and β, were $\rightarrow \infty$ and $\rightarrow$ mean/∞. All shown distributions are similar to each other (P>0.05) and each distribution is not statistically different from the EDF (Table 5.2). Therefore, the assumption that plate counts are Poisson distributed is acceptable.

Table 5.2. Kolmogorov-Smirnov tests for Goodness-of-fit of some selected distributions on the empirical distribution presented in Figure 5.5.

	Kolmogorov-Smirnov	
	D*	*P***
Poisson	0.291	0.255
Poisson(Gamma)	0.211	0.474
Binomial	0.300	0.235
Normal	0.228	0.424
Lognormal	0.252	0.351

*D:Kolmogorov-Smirnov Statistic
***P:* Probability

In the following experiment four different fits were used to estimate λ. Fitting was carried out by estimating λ by four different methods:

Fit 1: fixed λ, where λ = mean;

Fit 2: fixed λ, where λ is estimated by smallest square method;

Fit 3: Gamma-distributed λ, where the parameters of the Gamma distribution (α and β) are estimated by the smallest square method, and

Fit 4: Gamma-distributed λ, where α and β are estimated by the maximum likelihood method.

Data-set Appendix 4, Table App.-4.5 was used in this example. Fig 5.5 shows the respective curves.

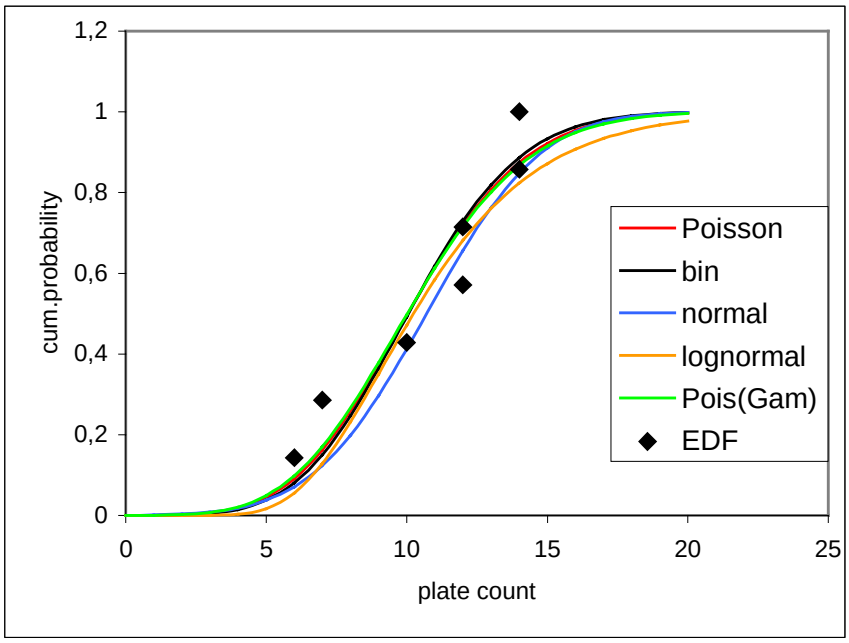

Fig. 5.5. Variation from plate-to-plate from one dilution. Comparison of the empirical distribution function (EDF) with Poisson, Binomial, Normal, Lognormal and Poisson(Gamma) distributions.

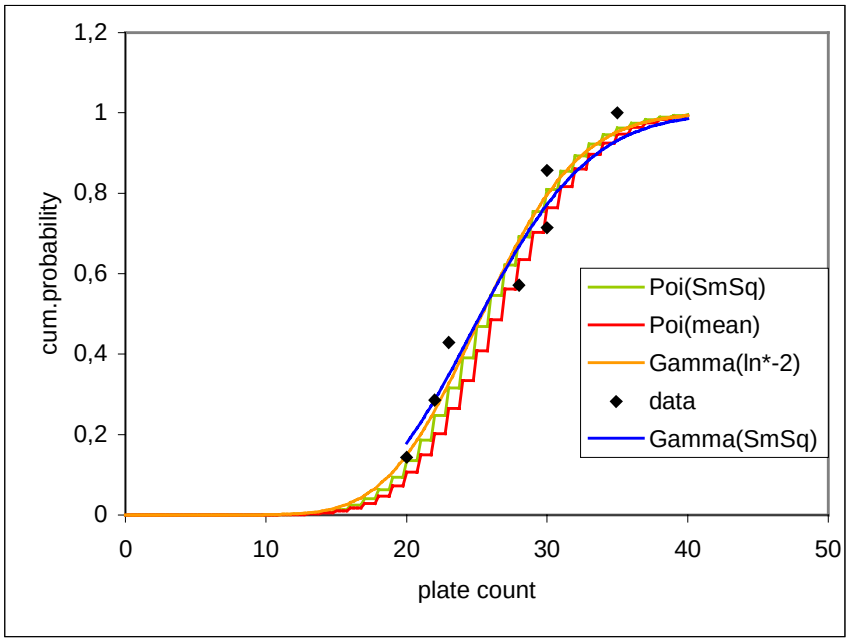

Fig. 5.6. Comparison of different strategies (see text) for fitting the Poisson distribution to the EDF.

The approximations using the smallest square method, fit 2 (with $\lambda = 26.08$) and fit 3 (with $\alpha = 56.001$ and $\beta = 0.45309$) tended to the left. The standard Poisson curve (Fit 1) fitted better to the EDF than the curves of Fit 2 and 3 ($P<0.05$ for both Fit 2 and 3, in comparison to Fit 1). However, none of the fits was significantly different from the EDF (Figure 5.6, Table

5.3). The limited amount of data indicate that the standard Poisson (Fit 1) is an acceptable estimation. Where the standard Poisson is not suitable, the Poisson(Gamma) fit (Fit 4) according to the loglikelihood approach is a good alternative.

Table 5.3. Kolmogorov-Smirnov test for Goodness-of-fit of some selected distributions on the EDF presented in Figure 5.5.

Poisson with λ estimated		Kolmogorov-Smirnov	
		*D**	*P**
Fit 1	fixed λ (mean)	0.207	0.487
Fit 2	fixed λ (SmSq)	0.263	0.323
Fit 3	Gamma (Loglklhd)	0.192	0.534
Fit 4	Gamma (SmSq)	0.254	0.347

**D*: Kolmogorov-Smirnov statistic.
***P*: Probability.

5.3.1.3 The variation of the results when the mean of duplicate plates for a sample is used

By using @ Risk software, a simulation of duplicate plating was carried out. 1002 single and 501 mean outcomes from duplicate plates with λ = 10 were generated according to Poisson distribution (Figure 5.7). While the means for single and duplicate platings are the same (mean = 9.988), the standard deviation is reduced by a factor √½ (s.d. = 3.218 and 2.244 for the single and duplicate platings, respectively), as expected.

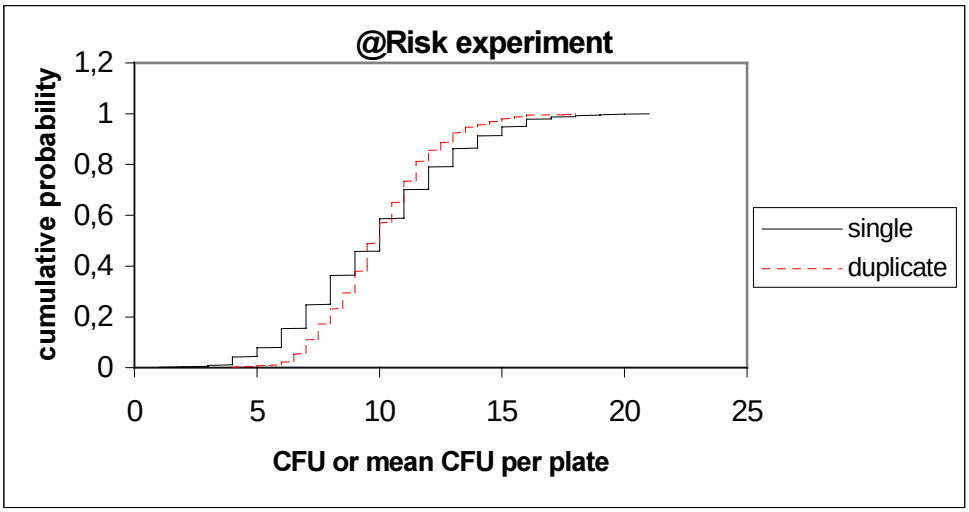

Fig. 5.7. The effect of duplicate platings on the variation of the results.

5.3.2 Effect of decimal dilution on results

5.3.2.1 Theoretical approach

When decimal dilutions are made, each dilution stage is a random event, that influences the number of CFU in subsequent dilutions. The number of CFU present in the highest dilution is determined by the number of CFU in the sample, and the random events that happened in the previous stages of the procedure. During this procedure, the number of CFU that is eventually

present in the used diluted cell suspension may deviate from the number of CFU that was expected. By calculating repeated Poisson progresses for each dilution step, this procedure is modelled. Alternatively, a simplified Poisson curve was drawn, with:

$$X = \lambda \cdot 10^{D}$$

X is the number of CFU g^{-1} in the original sample, and D is the $\log_{10}$ dilution rate.
Consequently, the standard deviation of X in the simplified Poisson is calculated as:

$$sd_x = \sqrt{\lambda \cdot 10^{D}}$$

Figure 5.8 shows the simplified Poisson (λ/100) is not equal to a repeated Poisson (one for each dilution step).

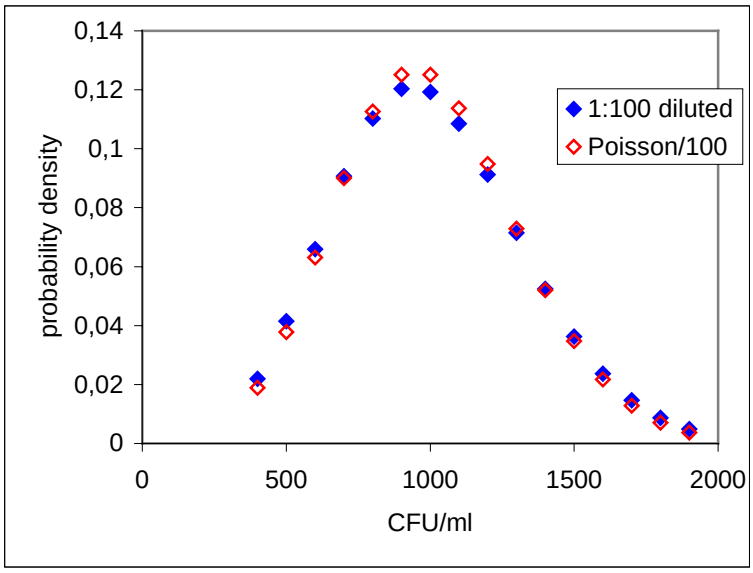

Fig. 5.8. Comparison of the almost) exact curve that describes the distribution of the plate count (repeated Poisson) with a Poisson curve that is adjusted to the dilution by incorporating a factor 100.

Figure 5.9 shows that with every dilution step the uncertainty of the estimated bacterial counts increases. In this example the standard deviation of the sample, the 1:1, 1:10 and the 1:100 dilution is √1000 = 31.6, 10√100 = 100 and 100√10 = 316, respectively. The repeated Poisson is a bit wider than the simplified Poisson curve, but the simplified version can be an acceptable modification.

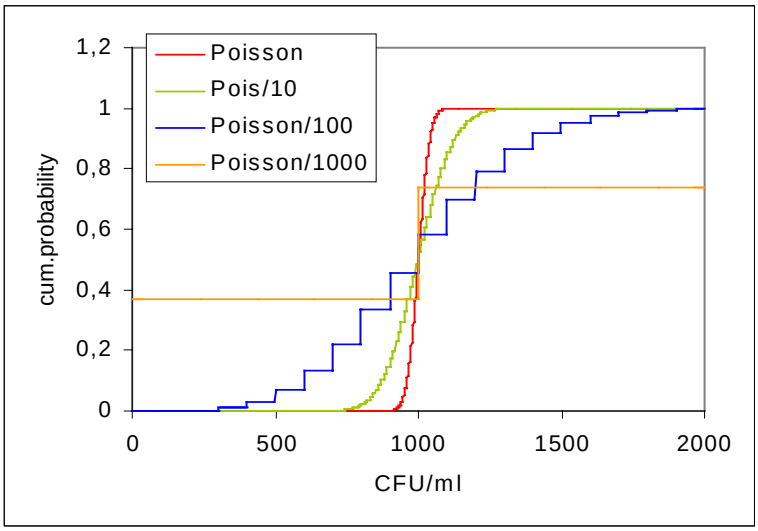

Fig. 5.9. Effect of dilution on the outcome of the variation of the plate counts.

5.3.2.2 Verification with experimental data

Figure 5.10 shows the phenomenon mentioned above, but now with experimental data (Dataset Appendix 4, Table App.-4.3). The EDF of the VRBG counts of the –1, -2 and –3 dilutions of minced beef are drawn, and the respective Poisson curves (fixed λ) with appropriate dilution factors were fitted. The observed standard deviations (based upon Normal distribution), were 138, 382 and 671, for the –1, -2 and –3 dilutions, respectively. When the standard deviation is calculated for the Poisson distribution adjusted to the dilution, the standard deviations were $10\sqrt{(1200/10)} = 110$, $100\sqrt{(1254/100)} = 354$ and $1000\sqrt{(684/1000)} = 827$, respectively. The observed and calculated figures are fairly similar, so that the simplification of the Poisson appears to be acceptable.

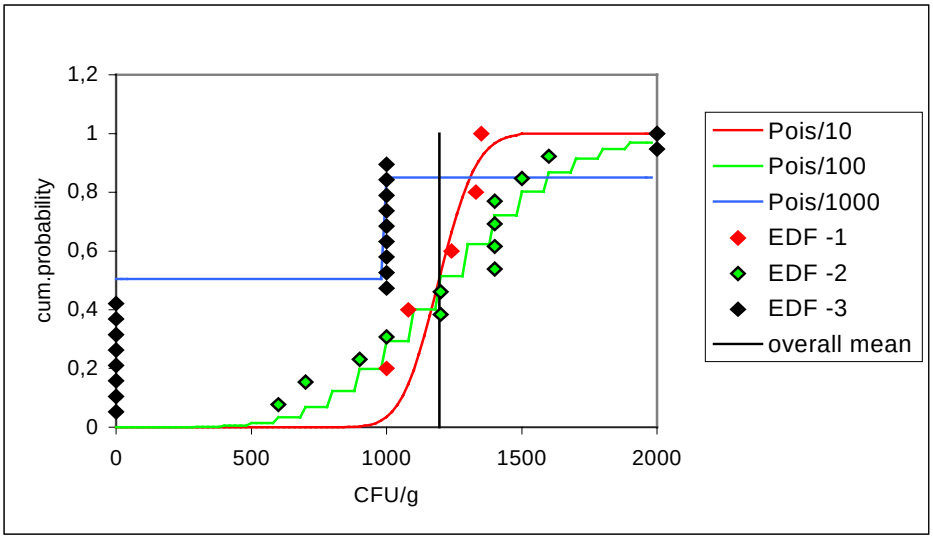

Fig. 5.10. Experimental data to illustrate the effect of dilution on the outcome of the variation of the plate counts.

5.3.3 Variation in plate counts from parallel dilution series.

In section 5.3.2 it was already mentioned that each dilution step influences subsequent results: when a relatively low number of CFU is transferred to the next dilution, the results of the plate counts of this dilution will tend to lower counts compared to the count of the original sample. In this section this effect is explored a bit more, by comparing parallel dilutions from one sample.

VRBG-counts of Dataset Appendix 4, Table App.-4.5 are used. The results of this experiment are drawn in Figure 5.11. Error bars are added to the datapoints (i = 1 to n, mean CFU/plate for each of n dilution series) and indicate standard deviation. As discussed above, a Poisson distribution can be assumed, and the standard deviation can be defined as $s.d. = \sqrt{\lambda_i}$. Figure 5.11 also shows three fits to the EDF, the Poisson (λ = mean), Poisson (λ fitted with maximum likelihood test: λ = 25.43) and Poisson (Gamma distributed λ). The estimated parameters for the Gamma-distribution were $\alpha \rightarrow \infty$, and $\beta \rightarrow$ mean/∞, which indicates that the fit of the Poisson (λ = mean) can not be improved.

All three fits were not significantly different from each other (P>0.05). Moreover, none of the fits were significantly different from the EDF (P>0.05). ANOVA showed that the plate counts of the respective dilution series were not significantly different (P>0.05).

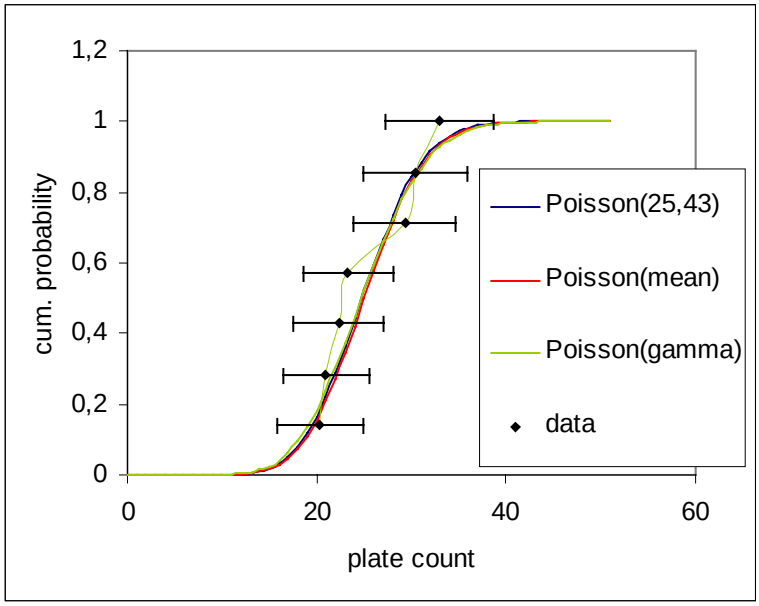

Fig. 5.11. Variation in plate counts from parallel dilution series.

5.3.4 Variation from subsample to subsample within a large sample

In the previous section the variation between different dilution series of one subsample was established. This should help to determine the variation from sub-sample to sub-sample of, for example a production lot of minced beef. In this section the variation between subsamples

from one source, for example a production lot of minced beef is studied. In Figure 5.12, an example of such an experiment is shown (Dataset Appendix 4, Table App.-4.5, VRBG-counts). Error bars indicate standard deviation. For the calculation a Poisson distribution for each subsample was assumed. Seven subsamples were drawn from an amount of minced beef and plate counts were made on VRBG. Poisson and Poisson(Gamma)-fits were made to the EDF. The standard Poisson and Poisson(Gamma) showed a good fit to the EDF (P = 0.50 for both). For the Poisson(Gamma)-fit, the parameters were estimated as α = 99.5347, β=0.292791. Both fits were not significantly different from each other (P = 0.605). Differences found between the subsamples were only just not statistically significant (ANOVA: P = 0.057).

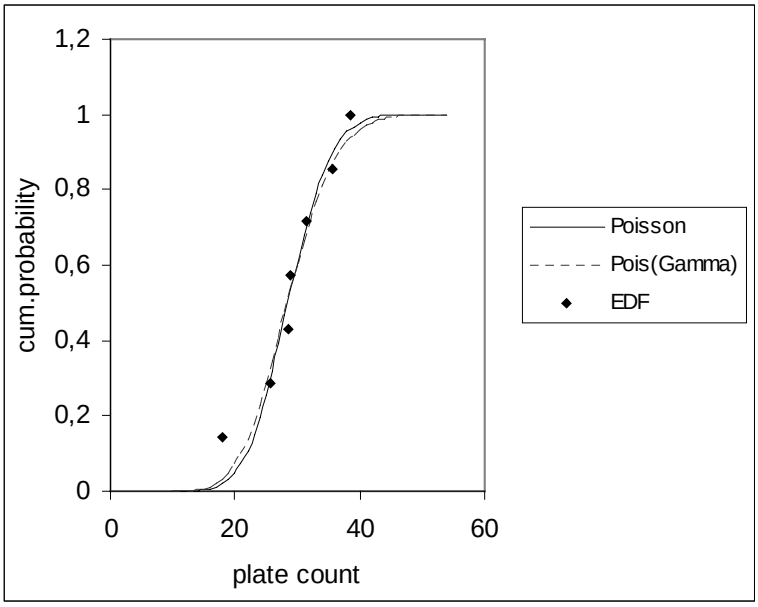

Fig. 5.12. Variation in plate counts from subsample to subsample within a large sample.

This was not the case with the *E. coli*-counts in the same experiment (Dataset Appendix 4, Table App.-4.5, *E. coli*-counts). Figure 5.13 shows that one of the samples had a much higher *E. coli* count than the other samples. ANOVA showed a significant subsample effect (P<0.001). This indicates that differences found between subsamples are not caused by method variation, but apparently *E. coli* was present in clusters. As a result, the Poisson fit was significantly different from the EDF (P<0.001). The Poisson(Gamma) distribution, with parameters α = 1.7701 and β = 20.572) was not statistically different form the EDF, although based on n = 7 samples the P-value tends to be very low (P = 0.07). The results indicate that clustering of *E. coli* in minced beef can occur, and that in such a case appropriate fits are difficult to find.

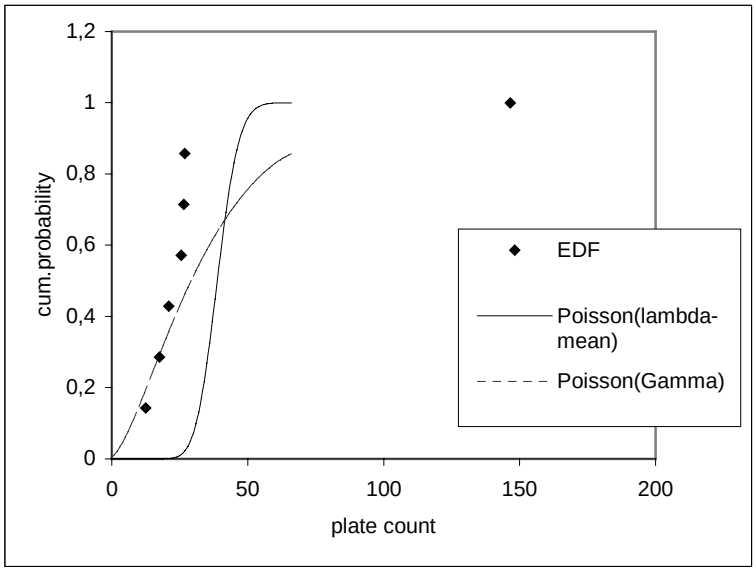

Fig. 5.13. Variation from subsample to subsample within a large sample. The effect of an outliner on the fitting of theoretical curves.

Because of the clustering found in the above mentioned experiment, the experiment was repeated (*E. coli* counts Dataset Appendix 4, Table App.-4.5). This time, both Poisson and Poisson(Gamma), with $\alpha \rightarrow \infty$; $\beta \rightarrow mean/\infty$) curves are good approximations to the EDF (*P*-values of 0.53 and 0.41, respectively). No differences were found between both theoretical curves (P = 0.574) or between subsamples this time. Thus, there was no evidence for clustering in this sample.

5.4 Discussion

Poisson distribution is an acceptable model to describe the variation of colony counts from different subsamples from one lot. However, the Poisson curve has to be adjusted to the level of dilution that was used. Incorporating a factor 10^D can make a simplification. The fact that the Poisson distribution requires just one parameter makes its application in quantitative microbiological risk assessment models easy. In addition, the mechanistic nature of the Poisson distribution gives a good basis for modelling [31].

Table 5.4. The effect of random effects during the preparation of bacterial enumerations on the variation of plate counts at different levels of dilution, and a comparison between the actual and measured variations.

statistics	actual number of CFU	measured number of CFU		
		-1 dilution	-2 dilution	-3 dilution
mean	10,000	10,000	10,000	10,000
s.d.	100	316	1,000	3,162
lower 95% limit	9,804	9,380	8,000	4,000
upper 95% limit	10,196	10,620	12,000	16,000
+/- error (%)	2	6	20	62

An example is given in Table 5.4. In this example, the actual number of CFU in a certain sample is 10,000 CFU g^{-1}. The results of a perfect method would range between 9,804 and 10,196 CFU (95% confidence interval), an error of ± 2%. However, at this level it is impossible to count, and even the –1 dilution is not countable. Nonetheless, at the –1 dilution the error at this level has increased to ± 6%. In this example, the –2 dilution should be countable, but only due to random effects, the error has already increased to ± 20%. At the level of the –3 dilution, the error has increased to ± 62%, but at this level the numbers are to low to count. The difference between the actual variation of 2% and the measured variation of at least 20%, makes it difficult to estimate the actual variation between subsamples. In other words, due to the uncertainty of the method used, the variability of the bacterial concentrations between subsamples is difficult to estimate.

Using duplicate plates (Figure 5.7) reduces this uncertainty. It should be noted that with this approach it was assumed that each sample was replaced. When, for example, duplicate 1-ml samples are drawn from 10 ml suspension, without replacement, the variation of the means of the duplicate platings will be further reduced.

Nonetheless, clustering of *E. coli* was detected in one of the experiments. In this experiment one of the samples contained 3.4 $\log_{10}$ CFU/g, whereas the average load was approximately 2.4 $\log_{10}$ CFU/g. In this experiment an outliner of 2.7 $\log_{10}$ CFU/g would already have been detected. From our results it cannot be said if such clustering occurs frequently. In a second experiment to verify this, such a clustering was not detected again, but many more of such experiments have to be carried out before anything can be stated.

Berg and Hildebrandt [3] found that immediately after grinding the total mesophilic aerobic plate count of minced beef is randomly distributed. However, after storage for 7 days at 5°C, ANOVA showed that in many lots clustering had occurred [3]. At the start of the experiment the Poisson distribution could be used to describe the variations from sub-sample to sub-sample, but after a week storage, the lognormal distribution was more appropriate. If growth should explain the clustering of *E. coli* in this experiment, this would suggest that the meat was subjected to temperatures higher than about 4°C. This is the minimum growth

temperature (T_{min}) calculated by extrapolation of empirical data [53], although the lowest T_{min} observed, at least for STEC O157, is 7°C [49].

At most of the stages in the preparation of bacterial enumerations, the variation that occurs (the stochastic processes that occur) can be described by Poisson distributions. Strict random variation from subsample to subsample cannot be distinguished, because the variation that occurs due to the method used is much larger. The Poisson(Gamma) distribution proved useful to model variations of bacterial counts within a batch beyond randomness. The sources for additional variation, either method or sample-to-sample variation, can be distinguished with ANOVA.

6. Variations of the microflora in minced beef as affected by the grinding of meat

6.1 Introduction

An important step in preparing hamburgers, minced beef, steak tartare, is the grinding of the beef. In this step the meat is also mixed and, if necessary, seasoned. In practice the meat is passed through the grinder two or three times. Since the meat for producing minced beef is usually originating from several different sources, the contamination that exists on the starting material is far from randomly distributed. Some parts may carry the contamination, while most parts do not. During the grinding and mixing, however, the contamination will be distributed into the whole lot. It is no surprise that the standard deviation of the distribution of the contamination will be decreased after grinding [40].

In this part of the study, it is hypothesised that after grinding the microflora in ground beef is distributed randomly. The effect of the grinding process on the distribution of the microflora will be studied on the naturally present microflora consisting of coliforms and *Enterobacteriaceae*, and on artificial local contamination of STEC O157. Poisson and Gamma-Poisson-fits are calculated, and the difference between them is statistically tested (log-likelihood test). It is assumed that if the Gamma-Poisson is significantly better than the Poisson, that the Poisson is not an appropriate distribution. The distribution of λ, according to Gamma-distribution, can be an approximation for the variability of the contamination. Wherever possible, Normal and Lognormal distributions will be calculated. The Goodness-of-fit of the theoretical distributions to the EDF is determined by Kolmogorov-Smirnov statistics.

6.2 Material and Methods

6.2.1 Micro-organism

Nalidixic acid resistant *E. coli* O157 rr98089R (see Chapter 3).

6.2.2 Bacterial enumeration

For the enumeration of coliforms Petrifilm EC was used (see Chapter 4). Violet Red Bile Glucose Agar (VRBG) was used to enumerate *Enterobacteriaceae* (see Chapter 4). CHROMagar O157 with nalidixid acid (CAN) was used to enumerate the nalidixid acid resistant strain of *E. coli* O157 rr98089 (see Chapter 3). One-ml volumes were applied to these media.

To allow the counting of low numbers, 1 ml portions of the homogenate was spread over three CAN plates. The total number of colonies of these three plates represented one plate count for the lowest dilution.

6.2.3 Experimental set-up

For all experiments beef was purchased from Utrecht University.

Distribution of natural microflora

Surface samples (Cork borer samples) were taken from the surface of the meat to determine the initial contamination of microflora. Then, the meat was cut into pieces of ca 50 g each, and ground in industrial meat grinder equipment (3mm-hole plates). From the resulting minced beef 25 samples of approximately 10g were taken. The remaining meat was mixed briefly and ground again. The sampling, mixing and grinding steps were repeated twice. This resulted in a total of 100 samples: 25 samples before grinding and 25 samples after grinding once, twice or three times, respectively. Coliforms and *Enterobacteriaceae* were enumerated.

Distribution of a semi-clustered STEC O157 contamination

The meat was cut into pieces of approximately 10 g. On 10 spots in the meat 100 µl of a bacterial suspension was inoculated. The inoculated meat was grinded in a Hobart Laboratory Scale meat grinder (3 mm-hole plates). 25 samples of the resulted minced beef were drawn, after which the meat was gently mixed by hand (using sterile gloves), and grinded again. Again 25 samples were taken from the produced minced beef. STEC O157 was enumerated.

Distribution of a strictly clustered STEC O157 contamination

In the first experiment, the meat was cut into pieces of approximately 10 g each. One of the pieces was inoculated with 100-µl inoculum, and added to the rest of the meat. The meat was ground twice and sampled after both steps as mentioned above.

In the second experiment, the meat (ca 1-kg) was cut into pieces of approximately 10 g each. From this amount of meat portions one portion of 100 g and two portions of approximately 300 g were made. The first portion (100g) was ground and STEC O157 was enumerated from them. Subsequently one 300 g-portion was ground once. The first piece of meat that was placed in the grinder was inoculated with 10 µl inoculum, containing $1.2 \cdot 10^6$ CFU. After grinding all parts of the equipment were rinsed in peptone saline (90 ml) to determine the number of CFU that remained in the equipment.

After this, the parts were autoclaved and the machine was cleaned and disinfected thoroughly. Then the second portion of 300 g was ground twice. Again he first piece of meat that was placed in the grinder was inoculated with 10 µl inoculum, containing $1.2 \cdot 10^6$ CFU. After grinding all parts of the equipment were rinsed in peptone saline (90 ml) again.

6.2.4 Curve fitting

All calculations were made in MS Excel 97 spreadsheet. The EDF was approximated by several different distribution functions, including Poisson(λ) with fixed λ (Poisson), Poisson (λ) with Gamma(α,β)-distribution of λ (Poisson(Gamma)), and Normal and Lognormal distributions.

The log-likelihood method was used to estimate α and β for the best fit of the theoretical distribution functions to the EDF. In the solver-function to estimate α and β, the constraints were $171 \geq \alpha \geq 0.001$; $\beta \geq 0.001$. If $\alpha = 171$, it is assumed that $\alpha \rightarrow \infty$.

In some experiments the size of the samples varied greatly. Instead of 10-g samples, the weight of the samples was in the range of approximately 6 to 25 g. Since in most of the experiments the results were calculated as the number of CFU/plate, this figure had to be adjusted to the sample weight. This was done as follows:

In Excel, a standard Poisson distribution function is described like this:

= POISSON (*Pc; λ;* 0)

with *Pc* = the number of colonies on an agar plate (in this experiment Pc = CFU/ml homogenate or –1-dilution), and λ = average plate count in the experiment (all samples). When the weight of the samples is not constant, this should be considered in the calculations.

The concentration X (CFU/g) in the sample is:

$$X = Pc \times \frac{wt + 90}{wt}$$

with *wt* is the weight of the sample (g), 90 is the amount of peptone saline added to the sample (ml = g), *X* is the total number of CFU.

X is calculated for all samples. The average X for all samples is an estimate for λ_m. Now the expected number of CFU on the agar plate of the individual samples (λ_i) has to be adjusted to the sample weight again. This is calculated as follows:

$$\lambda_i = \lambda_m \times \frac{wt_i}{wt_i + 90}$$

The likelihood/probability of the observed outcome under Poisson distribution can now be calculated by using λ_i:

= POISSON (Pc;λ_i; 0)

6.2.5 Statistical analysis

In this study the null-hypothesis was that after grinding the beef once or more, the microflora was randomly distributed in the minced beef. As a result the Poisson distribution should fit the EDF.

For comparison of the hypothetical distribution function curves to each other, the maximum likelihood test with χ^2 was used. The Kolmogorov-Smirnov test ($\underline{D}$) was used to determine the goodness-of-fit of the hypothetical distribution functions to the EDF. To calculate P an internet programme was used[1]. Critical value 5%.

6.3 Results and Discussion

Naturally occurring microflora

The results of the plate counts are given in the tables of Appendix 5.1.

Table 6.1 shows the results of the comparison of the Poisson fits with fixed λ or with a Gamma-distributed λ) at several stages in the grinding process.

Before grinding the Poisson distribution with a Gamma-distributed λ (Poisson(Gamma)) was a significantly better approximation to the observed coliform and *Enterobacteriaceae* counts than the Poisson distribution with a fixed λ. This indicates that on whole beef the coliform and *Enterobacteriaceae* microflora is not distributed randomly.

[1] http://www.io.com/~ritter/JAVASCRP/NORMCHIK.HTM#TheMeaning

Table 6.1. Evaluation of the Poisson distribution of the natural microflora of coliforms and Enterobacteriacae in beef, after grinding once, twice or three times. P indicates the probability of the Poisson and Poisson(Gamma) being similar in the log-likelihood test.

microflora	grinding step	Poisson(Gamma)			Poisson	*P*
		α	β	*D**	*D*	
coliforms	before	0.937	6.410	292.7	144.2	3*10(-34)
	once	3.836	3.827	87.47	89.76	0.130
	twice	5.908	3.512	104.5	106.9	0.116
	three times	17.762	1.397	101.8	102.3	0.488
Enterobacteriaceae	before	0.929	6.667	146.2	298.5	5*10(-33)
	once	1.138	15.350	97.91	111.6	0.0002
	twice	11.771	1.642	98.83	99.45	0.432
	three times	7.869	2.818	103.3	101.8	0.209

*Sum –2 ·Log-likelihood.

This can also be concluded from Table 6.2, which shows that before grinding, the Poisson distributions for the coliform and *Enterobacteriaceae* counts differ significantly from the EDF.

Table 6.2. Kolmogorov-Smirnov goodness-of-fit analysis of Poisson and Poisson(Gamma) distributions to model the distribution of the number of coliforms and Enterobacteriaceae in beef before grinding and after grinding once, twice or three times.

microflora	grinding step (n)	Poisson		Poisson(Gamma)		Normal		Lognormal	
		D	*P*	*D*	*P*	*D*	*P*	*D*	*P*
coliforms	before (25)	0.4088	0.0001	0.2457	0.0408	0.3392	0.0023	0.1520	0.2838
	once (24)	0.1767	0.1993	0.1807	0.1853	-	-	-	-
	twice (25)	0.1404	0.3413	0.1825	0.1678	-	-	-	-
	three times (24)	0.1622	0.2417	0.1043	0.5552	-	-	-	-
Enterobact	before (25)	0.3758	0.0006	0.2190	0.0781	0.3069	0.0069	0.2483	0.0382
	once (24)	0.2820	0.0176	0.1558	0.2825	-	-	-	-
	twice (25)	0.1885	0.1493	0.1783	0.1819	-	-	-	-
	three times (24)	0.1563	0.2800	0.2094	0.1057	-	-	-	-

For the coliform counts, the Poisson(Gamma) and additionally, the Normal distribution could not approximate the EDF, either. Only the Lognormal distribution was statistically acceptable (Table 6.2). This is illustrated in Figure 6.1. These differences could be explained by two data-points.

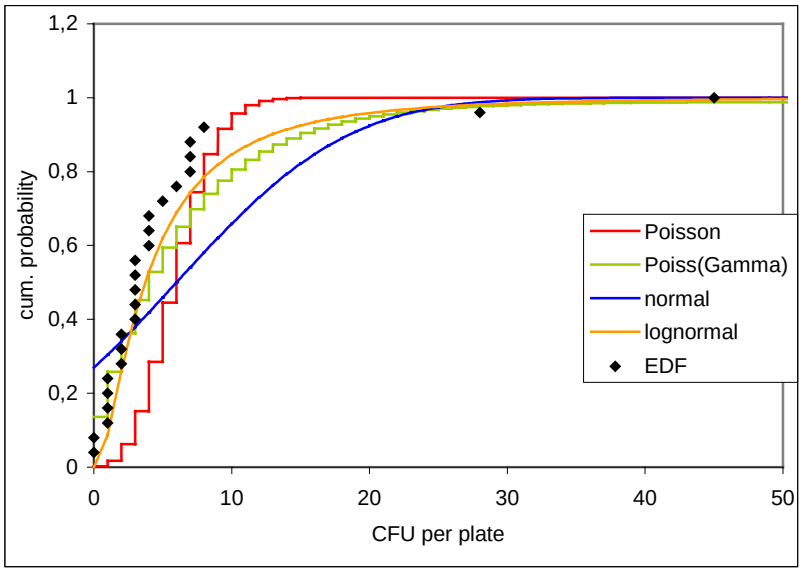

Fig. 6.1. Distribution of the coliforms on beef before grinding. Comparison of four fits to the EDF.

Figure 6.2 shows the Gamma-distributions of the underlying parameter λ. As expected, the variation of the distribution of λ becomes smaller after grinding. This figure also shows that λ tended to increase during the experiment, likely because of small clusters falling apart.

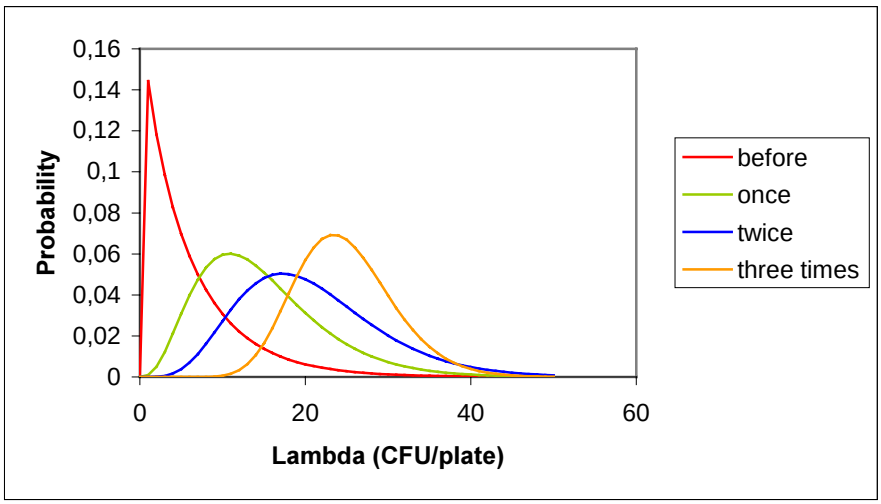

Fig. 6.2. The effect of grinding on the distribution of the underlying parameter λ for the variation of the coliforms in minced beef as described by the Poisson(Gamma) distribution.

The EDF of the *Enterobacteriaceae* results, however, was fitted successfully by the Poisson(Gamma), whereas the standard Poisson and the Normal and Lognormal distributions were significantly different from the EDF (Table 6.2; Figure 6.3).

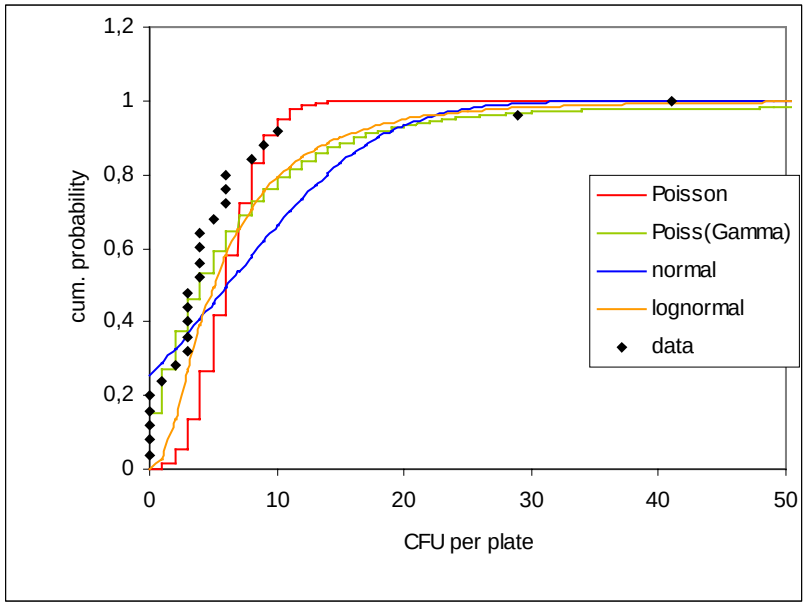

Fig. 6.3. The distribution of Enterobacteriaceae in beef before grinding. Comparison of four distribution functions to the EDF.

Figure 6.4 shows the Gamma-distributions of the underlying parameter λ. The variation of the distribution of λ becomes smaller after grinding at least once. The third grinding step did not have much influence anymore. After grinding once, the difference between Poisson(Gamma) and the standard Poisson was no longer statistically significant for the coliform counts. The *Enterobacteriaceae* counts, however, were still approximated by the Poisson(Gamma) better than by the standard Poisson after the first grinding stage (Table 6.1). As a result, the standard Poisson was not acceptable (Table 6.2).

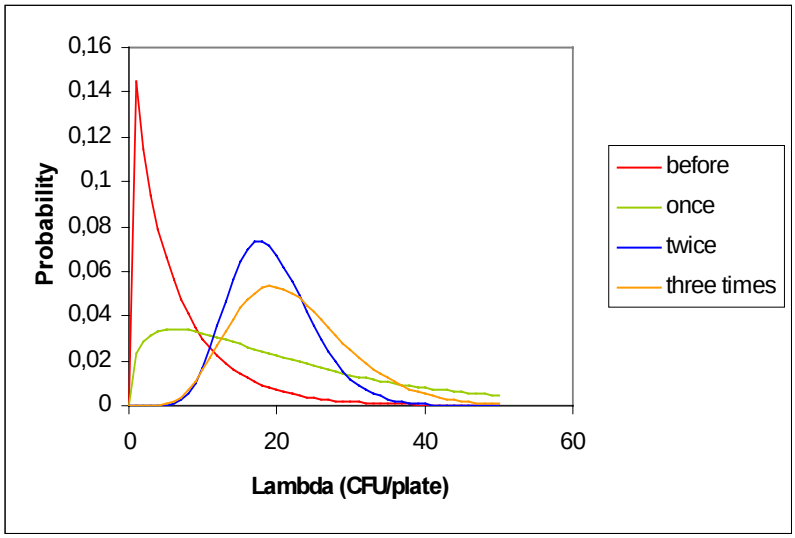

Fig. 6.4. The effect of grinding on the distribution of the underlying parameter λ for the variation of the Enterobacteriaceae in minced beef as described by the Poisson (Gamma) distribution.

Subsequent grinding steps resulted in a better mixing of the *Enterobacteriaceae*, with the differences between Poisson(Gamma) and the standard Poisson being no longer statistically significant. Both Poisson and Poisson(Gamma) were no longer significantly different from the EDF.

These results indicate that before grinding, and possibly after the first grinding step, the microflora is not distributed randomly. Only after two grinding steps the distribution is more or less random. As a consequence, it should be expected that an STEC O157 contamination in the starting material is not distributed randomly but clustered.

Semi-clustered E. coli O157 contamination

The tables in Appendix 5.2 give the results of the experiments with *E. coli* O157 added to the beef in a semi-clustered manner.

Figure 6.5 shows the results of the plate counts of a semi-custered contamination of STEC O157 after grinding once or twice, and the Poisson and Poisson(Gamma) fits. After the first grinding step the counts show a large variation, but after the second time grinding a much narrower range was found.

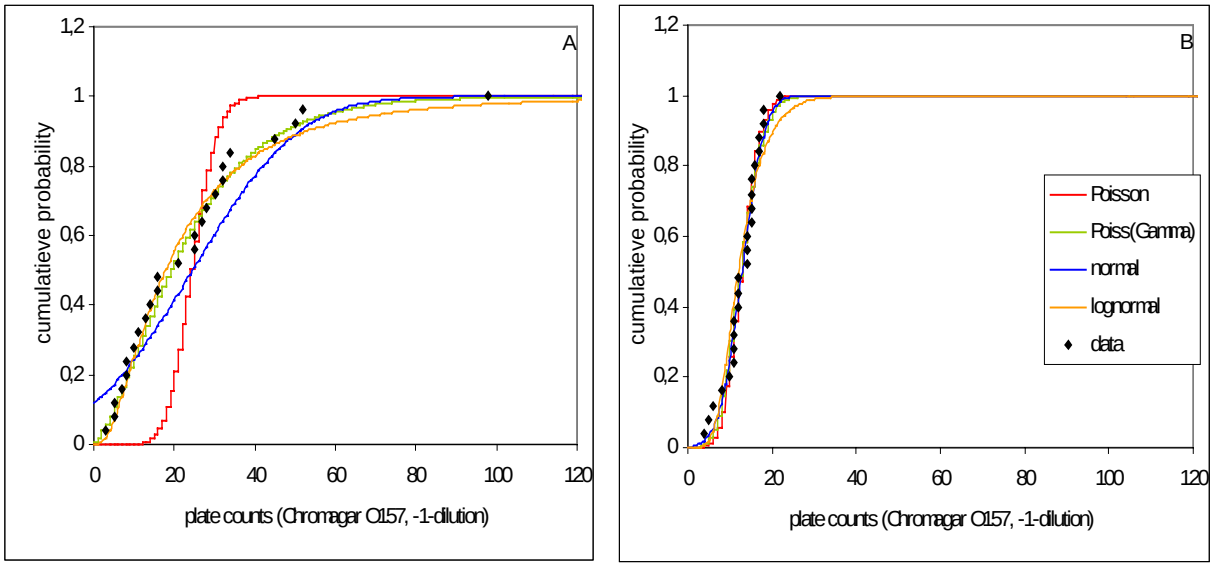

Fig. 6.5. The distribution of E. coli O157, inoculated in a semi-clustered manner, after grinding once (A) or twice (B) and comparison of the empirical distribution function (EDF) with theoretical distribution functions.

Table 6.3 shows the comparison of the Poisson and Poisson(Gamma) fits. After the first grinding step a large difference was found between both fits, but after the second grinding step the fits did not differ significantly anymore.

Table 6.3. Comparison of the variation of E. coli O157, inoculated (semi-clustered) to beef before grinding, to Poisson distribution.

grinding step	Poisson(Gamma)			Poisson	*P*
	α	β	$\underline{D}$*	$\underline{D}$	
once	1.879	13.034	207.2	473.6	9.2*10(-60)
twice	30.936	0.4176	144.2	145.7	0.226

*$\underline{D}$ = sum –2.log likelihood

Table 6.4 shows that the standard Poisson was unsuitable to model the distribution of STEC O157 in the minced beef after just one step of grinding, but that this distribution could be approximated by the fitted Poisson(Gamma) with α = 1.879 and β = 13.034. After the second step the standard Poisson was not significantly different from the EDF, and neither was the Poisson(Gamma), with α = 30.936 and β = 0.4176. The Normal and Lognormal distributions gave acceptable approximations to the EDF.

Table 6.4. Kolmogorov-Smirnov analysis of Poisson and Poisson(Gamma) fits to the EDF of semi- clustered contamination.

grinding step (n)		Poisson	Poisson (Gamma)	Normal	Log-normal
once (24)	$\underline{D}$	0.4356	0.0757	0.1654	0.0935
	P	<0.0001	0.7236	0.2289	0.6091
twice (25)	$\underline{D}$	0.1633	0.1468	0.0865	0.1232
	P	0.2373	0.3100	0.6511	0.4333

Strictly clustered E. coli O157 contamination

Figure 6.6 shows the results of the plate counts of a semi-clustered contamination of STEC

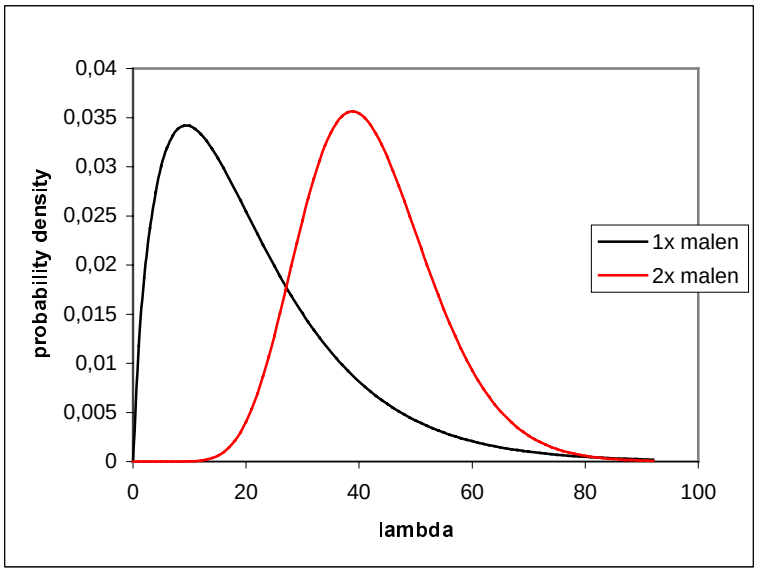

Fig. 6.6. The distribution of λ, describing the variation of STEC O157, initially present in one cluster, in minced beef after grinding once or twice.

O157 after grinding once or twice, and the Poisson and Poisson(Gamma) fits. After the first grinding step the counts show a large variation, but after the second time grinding a much narrower range of counts was found.

Table 6.5 shows the comparison of the Poisson and Poisson(Gamma) fits. After the first grinding step a large difference was found between both fits. After the second grinding step the Poisson(Gamma) fit was still better than the Poisson.

Table 6.5. Comparison of the variation of E. coli O157, inoculated (clustered) to beef before grinding, to Poisson distribution.

grinding step	Poisson(Gamma)			Poisson	*P*
	α	β	D*	D	
once	1.791	11.84	310.1	766.6	3*10(-101)
twice	13.19	3.184	199.1	467.1	3*10(-60)

D = sum –2.loglikelihood. Estimate within capacity that was allowed in Excel, However, the data are out of range for more accurate Excel calculations.

Table 6.6 shows that the standard Poisson was unsuitable to model the distribution of STEC O157 in the minced beef after just one step of grinding, but that this distribution could be approximated by the fitted Poisson(Gamma) with $\alpha = 1.794$ and $\beta = 11.836$. After the second step the standard Poisson was still significantly different from the EDF, but the Poisson (Gamma), with $\alpha = 13.190$ and $\beta = 3.184$, was an acceptable approximation.

Table 6.6. Kolmogorov-Smirnov analysis of Poisson and Poisson(Gamma) fits to the EDF of strictly clustered contamination.

grinding step (n)		Poisson	Poisson (Gamma)	Normal	Log-normal
once (24)	D	0.6275	0.1298	0.3302	0.1527
	P	<0.0001	0.4104	0.0039	0.2962
twice (25)	D	0.2509	0.1046	0.0912	0.1109
	P	0.0357	0.5416	0.6229	0.5040

Further experiments were carried out to determine the profile of the contamination of the meat that passes the grinder after the contaminated meat has passed. Appendix 5.3 gives the data from this experiment. Figure 6.7 shows the levels of contamination of subsequent fractions of the meat that passed the grinder. After grinding once, the first part of the meat contains high numbers of *E. coli* O157 organisms. The number of CFU drops rapidly when more minced meat is produced. This gives a large variation in the counts/g in the fractions. Nonetheless, all fractions, up to the 25th and last sample are contaminated. Additionally, *E. coli* O157 was detected in most parts of the grinder (Fig. 6.8), except the knives. It was observed that in many parts (mincer hull, ring, plate, screw, but not on the knives) considerable amounts of meat remained (in this experiment approximately 10% of the meat, which equals ca. 30g). Of course, after grinding twice, there is much less variation in the STEC O157 concentration (Fig. 6.7).

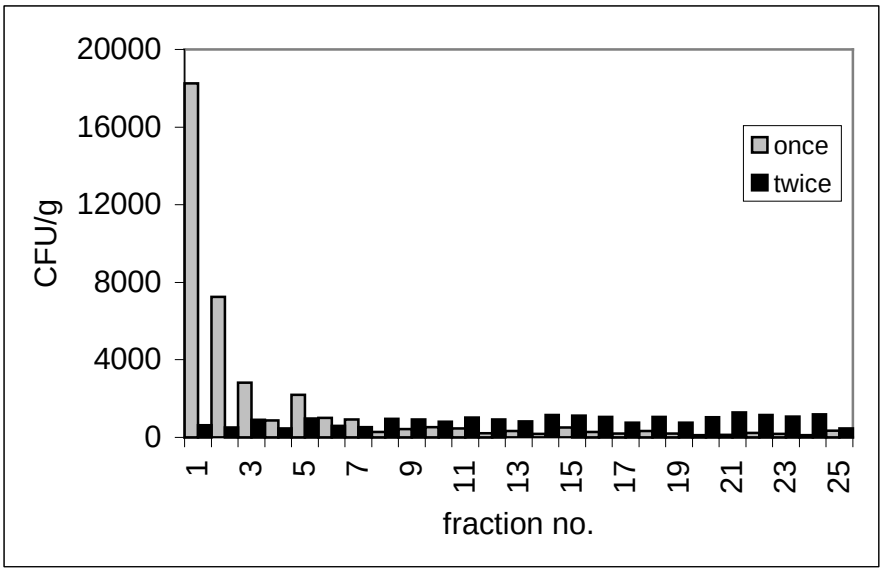

Fig. 6.7. Concentration of E. coli O157 in subsequent samples of minced beef after grinding once or twice.

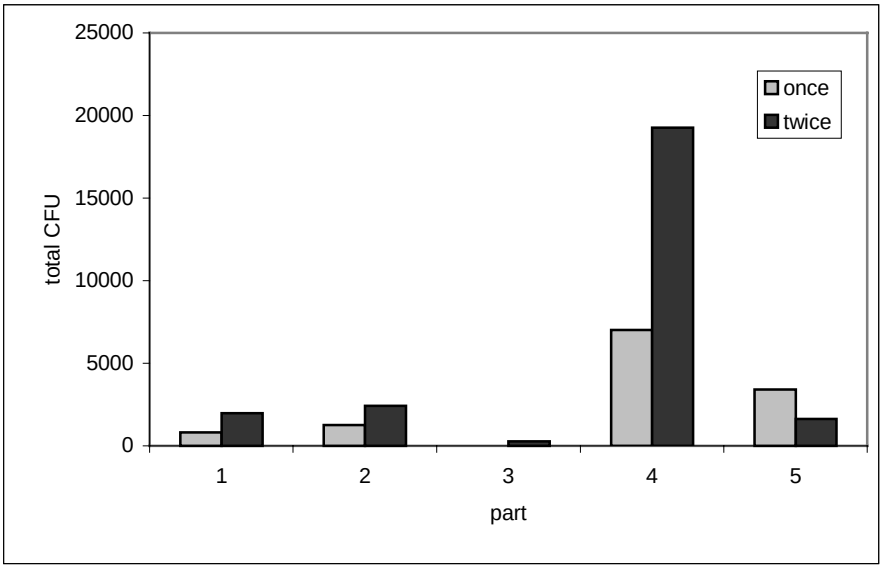

Fig. 6.8. Recovery of E. coli O157 from parts of the equipment after grinding once or twice.

7. Conclusions

The Poisson distribution with Gamma-distributed parameter λ is useful to explore variations in data beyond randomness. While this study has demonstrated that the Poisson distribution is acceptable to model the variation of plate counts (Chapter 5), the Gamma-distribution curves of λ provide insight into the variation of the levels of contamination of the samples from one lot (Chapter 5 and 6). It should be noted that with the current methods a 'detection limit' for variability exists. The lognormal distribution proved once again to be a robust distribution function for the description of variations in the results, but does not give insight in underlying mechanisms (Chapter 5 and 6). The Poisson(Gamma) distribution presented in this study can provide this information.

The medium used for the enumeration of bacterial populations can cause significant systematic error (bias) in the data. For the enumeration of unstressed nalidixic acid resistant *E. coli* O157, CHROMagar O157 with nalidixic acid is preferred (Chapter 3). When sub-lethally injured cells and considerable background flora are expected, EMB with nalidixic acid is the medium of choice. The method for homogenisation causes systematic error, which depends on the level and type of the microflora studied. The stomacher method gave more variation in the results than the blender, but the systematic error of both methods did not seem to be of practical significance (Chapter 4).

The Poisson distribution is acceptable for modelling the variations in plate counts of the distribution of naturally present microflora of coliforms and *Enterobacteriaceae*, and of *E. coli* O157 that is inoculated onto the meat in a minimum of 10 clusters, after grinding at least twice (Chapter 6). When *E. coli* O157 is inoculated into the starting material in one cluster, some clustering remains after grinding twice.

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Appendix 2 Data from Chapter 3

Table App-2.1.Counts of the overnight BHI-N culture. Enumeration of unstressed rr98089R in minced beef

medium	Dilution	plate counts	average	log(10) cfu/ml
CA	-7	110/134	1.2*10(9)	9.09
CAN	-7	87/43	0.65*10(9)	8.81
MEMB	-7	178/182	1.8*10(9)	9.26

Table App-2.2. Plate counts of inoculated minced beef on 6 different media.

	CA		CAN		Petrifilm		VRBG	SMACN		MEMB	
nr.*	-1	-2	-1	-2	-1	-2	-2	-1	-2	-1	-2
1	>/>§	72/62	>/>	57/51	129/144	52/67	92/102	>/>	75/61	>/>	32/42
2	8/12	0/4	0/0	0/0	65(1)/54(3)‡	12/9	26/43	1/1	0/ND	0/0	0/0
3	>/>	49/32	424/367	33/39	278/242	35/36	84/77	>/>	35/36	>/>	26/33
4	>/>	6/11	103/93	8/8	112(1)/116	15/19	45/43	89/91	11/ND	>/>	3/9
5	216/188	12/17	82/87	3/7	140(1)/96(2)	15(1)/12	60/46	133/106	9/12	>/>	10/10
6	24/35	5/7	42/57	7/4	127(3)/166(7)	28/31(1)	82/93	>/>	2/ND	42/>	4/3
7	309/396	43/57	302/>	42/40	182(1)/222	41(1)/40	111/100	412/268	37/45	>/>	36/40
8	4/5	1/0	0/0	0/0	78(2)/72(4)	14/9	35/37	0/0	0/ND	0/0	0/0
9	219/221	11/12	154/153	15/14	180(3)/220(3)	34/27	75/92	>/>	12/23	>/>	23/19
10	>/>	60/79	>/>	66/57	270(1)/230(1)	34/27	75/92	>/>	83/67	>/>	82/92
11	37/59	8/4	61/70	2/2	100(2)/72(2)	19/21	67/67	51/49	6/ND	>/>	4/3
12	69/28	15/17	189/172	4/12	>(35)/>(31)	33(5)/34(5)	404/528	>/>	26	>/>	15/22

* Sample 1 and 10 were inoculated with approximately 10.000 cfu/g; 2 and 8 0 cfu/g; 3 and 7 5.000 cfu/g; 4 and 5 1.000 cfu/g; 6 and 11 500 cfu/g.

‡ Total coliforms, number of presumptive *E. coli* (not STEC O157) between brackets.

§ > to many CFU or impossible to produce a reliable count.

Friedman-test calculations for comparison of selective media to count unstressed rr98089R in minced beef.

Ranking:

sample no.*	medium			
	CA	CAN	SMAC	EMB
1	3.5	2	3.5	1
3	4	3	2	1
4	4	3	2	1
5	4	1	3	2
6	2	4	1	3
7	4	2.5	2.5	1
9	4	1	2	3
10	2	1	3	4
11	2	4	3	1
12	2	4	3	1
total (Rj)	31.5	25.5	25	18

*Samples 2 and 8 are ommitted, because no rr98089R was added.

$$\left.\begin{array}{l} b = 10 \\ t = 4 \end{array}\right\} \Rightarrow \text{Critical value}\,(\alpha = 0.05) = 128$$

$$\text{Statistic}: \; Q = \sum \left(R_j - \frac{1}{2} b(t+1) \right)^2$$

$$\Rightarrow Q = (31.5 - 25)^2 + (25.5 - 25)^2 + (25 - 25)^2 + (18 - 25)^2 = 92.15$$

$$\Rightarrow Q < 128$$

Conclusion: No statistical difference between the selective media.

Table App-2.3. Counts of the overnight BHI-N culture for high level inoculum in Injury and Control broth.

medium	Dilution	plate counts	average (CFU/ml)
TSA	-7	231/277/251	2.57*10(9)
	-8	19/33/36	

Table App.-2.4. Plate counts of high level inoculum in Injury broth and Control broth during incubation at 5°C.

Incubaction time (d)	Medium	dilution	Injury broth			Control broth		
			a	b	c	a	b	c
1	TSA	-4	855/1040	800/860	890/660	>/>	>/>	>/>
		-5	-	-	-	133/91	101/93	99/94
3	TSA	-4	>/>	>/>	>/>	>/>	>/>	>/>
		-5	128/116	160/134	170/155	72/103	52/36	71/51
6	TSA	-4	>/>	>/>	>/>	>/>	89/105	68/111
		-5	129/92	166/95	115/110	72/59	16/8	12/12
9	TSA	-4	>/>	>/>	>/>	174/205	55/53	75/68
		-5	83/80	139/93	77/55	-	-	-
12	TSA	-3	>/>	>/>	>/>	>/>	>/>	283/258
		-4	>/>	>/>	>/>	212/241	60/58	28/24
		-5	177/108	112/121	85/86	-	-	-
	EMB	-3	>/>	>/>	>/>	>/>	162/235	>/190
		-4	>/>	>/>	>/>	194/103	7/15	20/27
		-5	81/81	84/74	65/82	-	-	-
	SMAC	-3	>/>	>/>	>/>	>/>	112/88	85/112
		-4	>/>	>/>	>/>	111/144	18/5	18/15
		-5	23/36	72/53	51/53	-	-	-
	CA	-3	>/>	>/>	>/>	>/>	79/58	185/185
		-4	>/>	>/>	>/>	225/130	25/27	23/23
		-5	72/73	53/75	44/75	-	-	-
	CAN	-3	>/>	>/>	>/>	>/>	70/136	137/157
		-4	>/>	>/>	>/>	27/64	32/30	10/10
		-5	68/70	81/68	68/61	-	-	-
	CABBL	-2	-	-	-	>/>	>/>	26/32
		-3	>/>	>/>	>/>	>/>	38/31	1/64
		-4	>/>	>/>	>/>	214/187	8/11	9/6
		-5	35/35	38/50	36/29	-	-	-

Table App-2.5. Counts of the overnight BHI-N culture for medium level inoculum in Injury and Control broth.

medium	Dilution	plate counts	average (CFU/ml)
TSA	-7	143/143	1.43*10(9)
	-8	16/14	

Table App.-2.6. Plate counts of low level inoculum in Injury broth and Control broth during incubation at 5°C.

Incubaction time (d)	Medium	dilution	Injury broth			Control broth		
			a	b	c	a	b	c
3	TSA	-3	34/43	51/41	48/41	29/20	54/51	19/19
7	TSA	-2	280/262	>/>	360/330	124/116	>/>	246/182
		-3	13/14	23/30	36/26	15/6	54/36	20/7
11	TSA	-1	>/>	>/>	>/>	>/>	>/>	>/>
		-2	>/>	>/>	>/>	69/73	120/56	110/47
		-3	na/36	na/34	na/40	-/-	-/-	-/-
	EMB	-1	>/>	>/>	>/>	256/315	>/>	237/247
		-2	>/>	310/300	266/287	23/44	71/64	55/28
		-3	23/26	21/32	26/23	4/2	4/6	2/3
	SMAC	-1	48/67	36/46	43/24	192/174	>/>	>/>
		-2	34/63	32/45	29/21	34/24	75/78	12/21
		-3	17/14	14/14	9/17	0/1	1/2	1/3
	CA	-1	125/140	108/101	40/24	56/58	>/>	38/59
		-2	136/105	51/105	55/51	16/10	11/32	1/10
		-3	18/15	4/10	18/13	0/0	3/1	0/0
	CAN	-1	138/69	96/102	117/173	21/69	>/>	37/65
		-2	95/98	77/51	94/83	15/11	6/12	2/12
		-3	11/20	17/11	29/27	0/0	2/1	1/1
	CABBL	-1	26/26	56/45	114/22	8/4	>/227	132/118
		-2	11/22	9/11	31/38	12/13	71/22	2/45
		-3	3/1	0/1	3/0	2/3	0/1	4/1

Appendix 3. Data from Chapter 4

Table App-3.1. Comparison of the total aerobic counts (TSA, 37C, 24h) in fluid and slurry of the homogenates prepared by using a stomacher or blender.

experiment	treatment	sample	fluid		slurry		SPC	CPC	error
			ml	(CFU/ml)	g	(CFU/g)	(CFU/g)	(CFU/g)	(%)
exp 1	stom.	A	71	1.10e5	12	1.80e5	1.10e6	1.20e6	-8.4
		B	72	5.30e5	15	1.92e5	5.30e6	7.70e6	-31
	blen.	C	74	1.20e5	7	1.22e5	1.20e6	1.20e6	-0.1
		D	76	9.40e4	8	1.44e5	9.40e6	9.88e5	-4.8
exp 2	stom.	A	79	2.00e6	10.9	1.84e6	2.00e7	1.98e7	1.0
		B	76	2.30e6	10.2	2.18e6	2.30e7	2.27e7	1.1
	blend.	C	74	3.00e6	5.1	1.40e6	3.00e7	2.90e7	3.5
		D	69	1.90e6	7.4	1.76e6	1.90e7	1.89e7	0.7
exp 3.	stom.	A	81	7.00e4	13	4.80e5	7.00e5	1.27e6	-81
		B	84	7.20e4	10.4	2.00e5	7.20e5	8.61e5	-20
		C	85	8.40e4	10.6	2.20e5	8.40e5	9.91e5	-18
		D	81	8.10e4	13	2.80e5	8.10e5	1.09e6	-34
		E	82	6.60e4	12.7	2.60e5	6.60e5	9.20e5	-39
	blen.	F	83	9.30e4	3.8	2.60e5	9.30e5	1.00e6	-7.9
		G	86	1.10e5	4.8	3.60e5	1.10e6	1.23e6	-12
		H	86	1.10e5	4.3	2.40e5	1.10e6	1.16e6	-5.6
		I	88	1.10e5	5.4	2.40e5	1.10e6	1.18e6	-6.8
		J	87	1.30e5	5.4	3.20e5	1.30e6	1.41e6	-8.5

Table App.-3.2. Comparison of the number of Enterobacteriaceae (VRBG, 37C, 24h) in fluid and slurry of the homogenates prepared by stomacher or blender.

experiment	treatmen t	sample	fluid		slurry		SPC	CPC	error
			ml	(CFU/ml)	g	(CFU/g)	(CFU/g)	(CFU/g)	(%)
exp 3.	stom.	A	81	4.40e2	13	1.40e2	4.40e3	5.73e3	-30
		B	84	4.00e2	10.4	4.20e2	4.00e3	4.02e3	-0.6
		C	85	2.60e2	10.6	7.20e2	2.60e3	3.11e3	-20
		D	81	3.60e2	13	6.60e2	3.60e3	4.01e3	-12
		E	82	4.70e2	12.7	9.60e2	4.70e3	5.36e3	-14
	blen.	F	83	4.40e2	3.8	2.20e2	4.40e3	4.3e3	2.2
		G	86	3.90e2	4.8	4.60e2	3.90e3	3.94e3	-0.9
		H	86	4.40e2	4.3	8.40e2	4.40e3	4.23e3	3.8
		I	88	5.00e2	5.4	3.60e2	5.00e3	4.92e3	1.6
		J	87	6.00e2	5.4	5.60e2	6.00e3	5.98e3	0.4

Table App.-3.3. Comparison of the number of coliform counts (Petrifilm EC, 37C, 24h) in fluid and slurry of the homogenates prepared by stomacher or blender.

experiment	treatment	sample	fluid		slurry		SPC (CFU/g)	CPC (CFU/g)	error (%)
			ml	(CFU/ml)	g	(CFU/g)			
exp 3.	stom.	A	81	1.40e1	13	6.00e0	1.40e2	1.29e2	7.9
		B	84	7.50e1	10.4	4.60e1	7.50e2	7.18e2	4.3
		C	85	2.60e1	10.6	0.00e0	2.60e2	2.31e2	11
		D	81	2.40e1	13	0.00e0	2.40e2	2.07e2	14
		E	82	1.80e1	12.7	0.00e0	1.80e2	1.56e2	13
	blen.	F	83	2.50e1	3.8	0.00e0	2.50e2	2.39e2	4.4
		G	86	2.20e1	4.8	2.00e1	2.20e2	2.19e2	0.5
		H	86	1.50e1	4.3	0.00e0	1.50e2	1.43e2	4.8
		I	88	1.70e1	5.4	6.00e0	1.70e2	1.64e2	3.7
		J	87	1.60e1	5.4	0.00e0	1.60e2	1.51e2	5.8

Appendix 4. Data from Chapter 5

Table App.-4.1. Results of VRBG-counts of hamburger meat obtained from Digros (de Bilt), 17/11/1999.

homogenate (dilution –1)		dilution –2		dilution –3	
sample nr	plate counts	sample nr	plate counts	sample nr	plate counts
A	4	1A	0	1A	0
B	4	1B	0	1B	0
C	6	1C	0	1C	0
D	0	1D	2	1D	0
E	3	1E	0	1E	0
F	3	1F	0	1F	0
G	1	1G	0	1G	0
		2	0	2	0
		3	0	3	0
		4	1	4	0
		5	1	5	0
		6	0	6	0
		7	0	7-1	0
				7-2	0
				7-3	0
				7-4	0
				7-5	0
				7-6	0
				7-7	0

Table App.-4.2. Results of VRBG-counts of cork borer samples from whole beef obtained from Digros (de Bilt), 17/11/1999.

Enterobacteriaceae were not found in any of the samples. Three samples (5, 5, and 20 cm^2) yielded 2, 10 and 3 + 8 colonies respectively.

Table App.-4.3. Results of VRBG-counts of minced beef obtained from a local butcher's shop (23/11/1999), subsample 1.

homogenate (dilution –1)		dilution –2		dilution –3	
sample nr	plate counts	sample nr	plate counts	sample nr	plate counts
A	135	1A	12	1A	1
B	133	1B	14	1B	1
C	>	1C	7	1C	0
D	>	1D	10	1D	1
E	100	1E	12	1E	0
F	108	1F	6	1F	0
G	124	1G	14	1G	0
		2	14	2	1
		3	9	3	2
		4	16	4	1
		5	14	5	0
		6	15	6	1
		7	20	7-1	0
				7-2	1
				7-3	1
				7-4	0
				7-5	0
				7-6	1
				7-7	2

Table App.-4.3 (continued) Additional subsamples.

sample nr	-1		-2	
	A	B	A	B
sub 2	163	170	18	25
sub 3	>	>	23	22
sub 4	>	156	14	16

Table App.-4.4. Results of VRBG-counts of cork borer samples from whole beef obtained from a local butcher's shop (de Bilt), 23/11/1999.

Enterobacteriaceae were not found on any of the VRBG-plates, including those from three additional sub-samples.

Table App. 4.5 Results 2/12/1999. VRBG/Petrifilm

homogenate (dilution –1)				dilution -2			
sample	VRBG	Petrifilm		sample	VRBG	Petrifilm	
		E.coli	total coliforms			*E.coli*	total coliforms
1a	256	20	>	1a1	19	5	25
1b	226	30	>	1a2	16	4	29
1c	200	22	>	1a3	28		
1d	248	35	>	1b1	25		
1e	267	28	234	1b2	15		
1f	181	23	270	1b3	21		
1g	236	30	204	1c1	31		
1h	208	nd	nd	1c2	20		
1i	256	nd	nd	1c3	19		
1j	214	nd	nd	1d1	37		
				1d2	30		
				1d3	24		
				1e1	30		
				1e2	25		
				1e3	33		
				1f1	37		
				1f2	24		
				1f3	38		
				1g1	27		
				1g2	20		
				1g3	20		
2a	>	30	>	2a	29	2	24
2b	>	21	>	2b	34	0	13
3a	>	140	>	3a	34	11	29
3b	>	153	>	3b	43	13	27
4a	>	33	>	4a	30	1	20
4b	>	20	>	4b	28	2	22
5a	>	12	>	5a	14	2	19
5b	>	13	>	5b	22	4	22
6a	>	14	>	6a	43	2	24
6b	>	21	>	6b	28	2	29
7a	>	30	>	7a	24	1	22
7b	>	12	>	7b	33	1	10

Table App.-4.6 Results 21/12/1999. Exclusively Petrifilm.

homogenate (dilution –1)			dilution -2		
sample	Petrifilm		sample	Petrifilm	
	E.coli	total coliforms		*E.coli*	total coliforms
1a	18	21	1a1	2	2
1b	19	22	1a2	1	1
1c	21	23	1a3	1	1
1d	25	25	1b1	1	1
1e	20	23	1b2	2	3
1f	17	20	1b3	2	3
1g	14	16	1c1	0	0
			1c2	1	1
			1c3	2	2
			1d1	2	3
			1d2	1	1
			1d3	0	0
			1e1	0	0
			1e2	1	2
			1e3	1	2
			1f1	1	1
			1f2	1	2
			1f3	3	4
			1g1	5	7
			1g2	4	4
			1g3	2	3
2a	24	29	2a	3	3
2b	16	21	2b	0	0
3a	20	22	3a	1	1
3b	17	23	3b	0	0
4a	14	18	4a	3	3
4b	13	14	4b	1	1
5a	12	14	5a	2	3
5b	20	22	5b	1	1
6a	12	16	6a	2	3
6b	16	20	6b	2	2
7a	8	12	7a	0	0
7b	16	20	7b	2	2

Appendix 5. Data from Chapter 6

Appendix 5.1

Results of experiments with natural microflora in beef

Table App.-5.1A. The effect of grinding on the distribution of the natural contamination of coliforms (Petrifilm EC), Enterobacteriaceae (VRBG), and total aerobic colony count (PCA). This table shows the results of the corkborer-samples of the meat before grinding. Petrifilm can be used to enumerate E. coli, but E. coli was not found.

sample	Petrifilm (-1)	VRBG		PCA	
	coliforms*	-1	-2	-3	-4
1	7	4	1	>	136
2	3	4	0	>	46
3	2	2	0	75	7
4	4	3	0	>	159
5	1	0	0	>	134
6	0	0	0	159	11
7	7	6	0	116	8
8	2	6	0	>	88
9	5	3	0	186	12
10	6	3	0	143	<
11	28	29	2	95	9
12	7	10	0	>	694
13	4	8	0	>	32
14	4	3	0	>	370
15	3	9	0	>	230
16	3	3	0	51	<
17	45	41	0	>	82
18	3	6	0	>	24
19	2	4	0	>	34
20	1	1	0	>	89
21	3	0	0	115	11
22	8	5	0	>	63
23	0	4	0	70	3
24	1	0	0	57	9
25	1	0	0	>	122

Table App.-5.1B. The effect of grinding on the distribution of the natural contamination of coliforms (Petrifilm EC), Enterobacteriaceae (VRBG), and total aerobic colony count (PCA). This table shows the results after the first grinding step.

sample	weight (g)	Petrifilm (-1)	VRBG		PCA	
		coliforms*	-1	-2	-3	-4
1	20.5	0	5	0	219	16
2	19.6	2	6	1	>	41
3	9.0	1	0	0	164	<
4	22.1	4	5	0	199	17
5	10.9	1	0	0	78	7
6	18.3	2	4	0	185	12
7	16.8	0	3	0	234	26
8	10.6	5	9	0	78	7
9	20.6	3	2	0	360	23
10	9.0	nd	nd	nd	nd	nd
11	20.3	3	5	0	202	15
12	13.7	2	1	2	258	29
13	11.0	1	4	0	67	5
14	20.2	3	0	0	174	15
15	12.1	2	2	0	72	11
16	6.4	0	3	0	83	2
17	13.5	4	3	0	169	70
18	8.7	0	0	0	80	<
19	13.3	0	0	0	162	16
20	12.7	2	0	0	79	10
21	8.4	1	0	0	127	8
22	23.9	3	0	0	181	10
23	10.0	7	0	0	58	5
24	21.4	1	2	0	120	8
25	9.1	1	3	0	159	20

Table App.-5.1C. The effect of grinding on the distribution of the natural contamination of coliforms (Petrifilm EC), Enterobacteriaceae (VRBG), and total aerobic colony count (PCA). This table shows the results after the second grinding step.

sample	weight (g)	Petrifilm (-1)	VRBG		PCA	
		coliforms*	-1	-2	-3	-4
1	21.3	7	6	0	180	10
2	16.1	6	4	0	102	9
3	20.9	9	8	1	133	12
4	20.7	4	4	0	116	14
5	10.2	1	3	0	53	3
6	16.9	3	2	0	85	10
7	10.3	3	0	0	53	<
8	21.5	7	5	2	126	17
9	21.9	2	4	0	126	12
10	11.9	3	4	0	111	5
11	19.1	3	1	0	109	11
12	7.0	2	1	0	35	<
13	21.8	2	3	1	191	22
14	8.6	0	1	0	68	4
15	20.3	0	1	0	100	9
16	9.0	3	6	0	123	11
17	15.7	5	4	0	166	30
18	9.9	5	1	1	102	7
19	25.1	4	1	0	159	9
20	8.7	0	0	0	68	6
21	20.8	1	3	0	201	19
22	9.0	2	1	0	79	8
23	23.6	3	5	0	160	19
24	14.4	1	3	0	99	13
25	25.2	2	2	0	41	<

Table App.-5.1D. The effect of grinding on the distribution of the natural contamination of coliforms (Petrifilm EC), Enterobacteriaceae (VRBG), and total aerobic colony count (PCA). This table shows the results after the third grinding step.

sample	weight (g)	Petrifilm (-1)	VRBG		PCA	
		coliforms*	-1	-2	-3	-4
1	21.2	3	9	0	202	-
2	11.3	2	2	0	105	-
3	20.0	6	6	0	247	-
4	12.2	2	0	0	260	-
5	18.3	5	8	0	243	-
6	15.8	5	3	0	120	-
7	17.4	6	7	0	124	-
8	16.0	1	4	0	112	-
9	14.1	4	2	0	97	-
10	17.4	5	4	0	137	-
11	11.5	0	2	0	270	-
12	19.3	5	2	0	200	-
13	14.3	3	1	0	80	-
14	15.9	6	0	0	133	-
15	14.1	4	2	0	160	-
16	11.8	10	4	0	215	-
17	9.2	nd	nd	nd	nd	-
18	22.8	4	0	0	160	-
19	19.7	2	3	0	154	-
20	12.0	2	3	0	121	-
21	15.1	1	3	0	115	-
22	15.3	5	3	0	150	-
23	17.3	2	3	0	179	-
24	12.1	3	4	0	64	-
25	17.8	3	5	0	189	-

Appendix 5.2.

Results of experiments with semi-clustered contamination of E. coli O157 in beef

Table App.5.2A. Results of experiment with semi-clustered contamination of STEC O157 in beef. Counts of overnight culture of E. coli O157 rr98089 nalR in BHI at 37°C, on Chromagar O157 +nal, overnight 37°C.

	-7	-8	mean
A	184	20	2.05.10(9)
B	224	24	

*Table App.-5.2B. Results of experiment with semi-clustered contamination of STEC O157 in beef. Control counts of inoculated meat. Expected number in the experiment will be 2.05*10^5 per g.*

sample	-1	-2	-3	CFU/g
A	>/>	23/38	1/5	3.0.10(4)
B	>/>	113/80	4/8	9.7.10(4)
C	>/>	38/57	5/5	4.8.10(4)
D	>/>	41/18	2/3	3.0.10(4)
E	>/>	71/48	8/10	6.0.10(4)
			mean	5.3.10(4)

Table App.-5.2C. Results of experiment with semi-clustered contamination of STEC O157 in beef. Bacterial counts before grinding.

sample	VRBG	Petrifilm	PCA			
	-1	-1	-1	-2	-3	-4
1	0	0	>	>	36	2
2	0	0	>	77	5	0
3	0	0	>	>	33	2
4	1	0	>	>	50	9
5	1	0	>	>	31	2
6	0	0	>	>	36	1
7	0	0	>	>	45	9
8	1	0	>	>	25	2
9	0	1	>	>	50	10
10	1	0	>	>	59	2
11	0	0	>	264	24	4
12	1	1	>	>	>	51
13	1	0	>	>	53	4
14	1	1	>	212	21	0
15	1	0	>	>	50	6
16	0	0	>	224	27	1
17	3	0	>	>	72	16
18	0	1	>	>	44	3
19	0	0	>	76	8	0
20	8	1	>	>	83	12
21	2	0	>	>	48	5
22	1	0	>	>	60	14
23	0	0	>	>	76	12
24	0	0	>	>	80	13
25	2	1	>	174	14	1

Table App.-5.2.D. Results of experiment with semi-clustered contamination of STEC O157 in beef. E.coli O157 counts after grinding.

sample	grinding step					
	once			twice		
	-1	-2	-3	-1	-2	-3
1	71	7	0	135	11	4
2	247	10	0	123	16	0
3	188	8	0	142	15	0
4	200	5	0	180	14	1
5	204	14	0	156	6	1
6	54	3	0	181	12	0
7	365	30	0	81	5	0
8	172	13	2	33	11	0
9	>	27	2	96	11	0
10	141	5	1	177	14	2
11	>	16	0	169	11	0
12	>	32	1	161	8	1
13	>	45	3	143	4	0
14	>	98	2	117	12	0
15	258	28	2	138	17	2
16	184	34	0	128	18	4
17	52	11	1	139	15	2
18	>	52	6	117	14/15	--
19	119	21	2	106	15	0
20	>	50	4	201	18	0
21	>	32	0	114	15	0
22	95	8	0	120	22	1
23	>	25	0	130	12	0
24	136	25	0	125	10	0
25	>	16	2	196	17	1

Appendix 5.3.

Results of experiments with strictly clustered contamination of E. coli O157 in beef

Table App.-5.3A. Count of overnight culture of nalidixid acid resistent E. coli O157 rr98089 in BHI with nalidixid acid at 37°C

dilution	a	b	c	mean
-7	173	183	180	1.8.10(9)
-8	17	18	18	

Table App.-5.3B. Plate counts of E. coli O157 (Chromagar O157 with nalidixic acid, after 24h, 37°C).

sample	grinding step					
	once			twice		
	-1	-2	-3	-1	-2	-3
1	7	0	0	28	3	0
2	14	1	0	38	3	1
3	11	0	0	15	2	1
4	7	1	0	35	4	0
5	15	3	0	45	2	0
6	9	2	0	27	3	0
7	12	0	1	24	0	3
8	22	2	1	51	2	0
9	18	1	0	43	2	1
10	9	6	0	29	2	0
11	15	1	1	53	10	2
12	7	1	1	46	2	0
13	8	0	0	29	3	0
14	-	-	-	53	6	0
15	16	5	2	32	3	0
16	22	0	1	46	4	3
17	7	1	0	55	1	0
18	14	0	0	51	9	0
19	18	5	0	32	3	0
20	62	19	1	59	7	0
21	31	1	0	43	5	0
22	45	4	1	76	8	0
23	68	5	1	39	6	2
24	3	0	-	48	4	0
25	169	18	3	50	9	0