research for
man and environment

RIJKSINSTITUUT VOOR VOLKSGEZONDHEID EN MILIEU
NATIONAL INSTITUTE OF PUBLIC HEALTH AND THE ENVIRONMENT

RIVM report 286555 002

Validation of Microbiological Methods
Enumeration of *Clostridium perfringens*
according to ISO 7937 (second edition, 1997)

S.M. Schulten, E. Benschop, N.J.D. Nagelkerke,
K.A. Mooijman

February 2001

This investigation has been performed by order and for the account of the Directorate-General of the National Institute of Public Health and the Environment and of the Measurements and Testing Programme of the EU (contract number SMT4-CT96-2098), within the framework of project no. V/286555/03 (MGB 132), validation of ISO 7932: Enumeration of *Clostridium perfringens* in foods and animal feeding stuffs.

RIVM, P.O. Box 1, 3720 BA Bilthoven, telephone: 31 - 30 - 274 91 11; telefax: 31 - 30 - 274 29 71

Abstract

The method for the enumeration of *Clostridium perfringens* in foods and animal feeding stuffs, as described in ISO 7937 (1997), was validated in the context of a European project. A collaborative study was organised with the aim of attaining precision, in terms of repeatability (r) and reproducibility (R), of this method. Seventeen laboratories in 13 European countries examined cheese, meat, dried animal feed and a reference material according to a standardised protocol. All samples were artificially contaminated to achieve the desired inoculation levels (blank, low, medium, and high) and homogeneity. Two techniques using 1) lactose sulfite medium (ISO 7937) and 2) lactose-gelatine medium and motility-nitrate medium (optional method in EN 13401), were included in this study for confirmation of presumptive *C. perfringens.* This enabled detection of possible differences in the performance of the two confirmation methods. The precision characteristics were calculated using both ISO 5725 (1994) and pr EN ISO 16140 (2000). Repeatability (r) and reproducibility (R) were calculated for each sample type, inoculation level and confirmation method separately. The precision depended on the type of sample analysed. The best performance was found when analysing the RMs.

Abbreviations and symbols

CEN	European Committee for Standardization
ISO	International Organization for Standardization
EN	European Standard
pr EN ISO	draft European and International standard
AFSSA	Agence Française de Sécurité Sanitaire des Aliments
CECALAIT	Centre d'Etude et de Controle des Analyses en Industrie Laitiere
MAFF-CSL	Ministry of Agriculture, Fisheries and Foods, Central Science Laboratory
RIVM-MGB	National Institute of Public Health and the Environment, Microbiological Laboratory for Health Protection
SOP	Standard Operating Procedure
RM	reference material (capsule)
cfp	colony forming particle
HCMP	highly contaminated milk powder
NCTC	National Collection of Type Cultures
NCDO	National Collection of Dairy Organisms; Reading, England
D&S	Duncan and Strong medium
FTG	fluid thioglycollate medium
LG	lactose-gelatin medium
LS	lactose-sulfite medium
MN	motility-nitrate medium
MN&LG	motility-nitrate and lactose gelatin medium
MRS	de Man, Rogosa and Sharp agar/broth
TSC	tryptose-sulfite-cycloserine agar
T_{repl}	indicator for the variation between replicate counts
T_{dupl}	indicator for the variation between samples
r	repeatability value
R	reproducibility value
RSD_r	repeatability value relative to contamination level
RSD_R	reproducibility value relative to contamination level

Contents

Samenvatting 6

Summary 7

1. Introduction 8

2. Materials and methods 11

2.1 Preparation of test materials *11*
2.1.1 Introduction 11
2.1.2 Cheese samples 11
2.1.3 Meat samples 13
2.1.4 Dried animal feed samples 13
2.1.5 Reference material 15

2.2 Homogeneity and stability of test materials *15*
2.2.1 Cheese samples 15
2.2.2 Meat samples 16
2.2.3 Dried animal feed samples 16

2.3 Design of the collaborative trial *17*
2.3.1 Dispatch of test materials 17
2.3.2 Standard Operating Procedure 17

2.4 Data analysis trial *18*
2.4.1 Screening test results 18
2.4.2 Performance within each laboratory 19
2.4.3 Comparison of confirmation techniques 20
2.4.4 Precision data from trial 20

3. Results and discussion 21

3.1 Homogeneity and stability of test materials *21*
3.1.1 Cheese samples 21
3.1.2 Meat samples 22
3.1.3 Dried animal feed samples 23

3.2 General results of the trial *24*
3.2.1 Participants 24
3.2.2 Dispatch of test materials 24
3.2.3 Media 24
3.2.4 Incubation conditions of media 25
3.2.5 Laboratory temperature 26
3.2.6 Use of pipettes 26

3.3 Statistical analysis of trial data *26*
3.3.1 Screening data 26
3.3.2 Performance within each laboratory 27
3.3.3 Comparison of confirmation techniques 27
3.3.4 Precision data from trial 29

4. Conclusions and recommendations to ISO/CEN 32

Participants and acknowledgements 33

References 35

Appendix 1 Mailing list 37

Appendix 2 General information 38

Appendix 3 Standard Operating Procedure (SOP) 40

Appendix 4 Test report 51

Appendix 5 Results collaborative trial 60

Appendix 6 Duncan’s test results 67

Appendix 7 Deviating test results 74

Appendix 8 SAS protocol 75

Appendix 9 Repeatability and reproducibility values 81

Appendix 10 Media 83

Samenvatting

De validatie van de telling van *Clostridium perfringens* in voedingsmiddelen en diervoeders, zoals beschreven in ISO 7937 (1997), is uitgevoerd in het 4[e] kaderprogramma van het 'Standards, Measuring and Testing project' van de Europese Unie (SM&T project nr. SMT4-CT96-2098). De resultaten zijn bedoeld voor publicatie door ISO in de ISO standaard en door de Europese Unie (CEN) in de corresponderende CEN methode EN 13401 (1999).
Zeventien laboratoria uit 13 Europese landen hebben deelgenomen aan het ringonderzoek dat werd georganiseerd om de prestatiekenmerken van deze methode te bepalen in termen van herhaalbaarheid (r) en reproduceerbaarheid (R). Kaas, vlees en gedroogd diervoeder werden als voedingsmatrices gebruikt. Voor elke matrix werden 8 monsters onderzocht: 2 blinde duplicaten voor elk van 4 concentratie niveaus (blanco, laag, midden, hoog). Alle monsters zijn kunstmatig besmet om de gewenste niveaus en homogeniteit te bereiken. Daarnaast zijn twee referentiematerialen (RM) in het ringonderzoek meegenomen: capsules met melkpoeder, dat kunstmatig is besmet met *C. perfringens*. De testmaterialen zijn in drie laboratoria bereid en gecontroleerd op homogeniteit en stabiliteit om vast te stellen dat de materialen geschikt waren voor gebruik. De monsters zijn met een koeriersdienst naar de deelnemers gestuurd. De monsters zijn geanalyseerd volgens een gestandaardiseerd protocol. Twee methoden voor de bevestiging van verdachte *C. perfringens* kolonies zijn in deze studie meegenomen om mogelijke verschillen in resultaat te bepalen: 1) lactose sulfiet medium (zoals in ISO 7937 (1997) is voorgeschreven) en 2) lactose-gelatine medium + beweeglijkheids-nitraat medium (alternatieve bevestiging in EN 13401 (1999)).
De prestatiekenmerken zijn berekend volgens zowel ISO 5725 (1994) als volgens pr EN ISO 16140 (2000). De herhaarbaarheid (r) en reproduceerbaarheid (R) zijn voor elk monster type, concentratieniveau en bevestigingsmethode afzonderlijk berekend.
De precisie varieert per monster type. Het is niet duidelijk of dit een gevolg is van de matrix of van de verschillende manieren van kunstmatig besmetten van de monsters. De analyse van de RMs leverde de beste prestatiekenmerken op. De precisie varieerde per besmettingsniveau. Beide bevestigingsmethoden gaven vergelijkbare resultaten.
Aan ISO en CEN wordt aanbevolen om:

- De prestatiekenmerken, zoals gevonden in dit ringonderzoek en berekend volgens pr EN ISO 16140 (2000) op te nemen in ISO 7937 (1997) en EN 13401 (1999);
- Beide methoden voor de bevestiging van *C. perfringens*, die in dit ringonderzoek zijn getest, op te nemen in ISO 7937 (1997), waarbij de keuze aan de gebruiker wordt gelaten welke van de twee methoden te hanteren. Hiermee zouden ISO 7937 (1997) en EN 13401 (1999) worden geharmoniseerd.

Summary

The evaluation of the enumeration method for *Clostridium perfringens* in foods and animal feeding stuffs, as described in ISO 7932 (1997), has been carried out in the 4th framework of a Standards, Measurement and Testing project of the European Union (SM&T project no SMT4-CT96-2098). Results are intended for publication by the International Organization for Standardization in the ISO standard and for the European Committee for Standardization (CEN) in the corresponding CEN method EN 13401 (1999).
Seventeen laboratories in 13 European countries participated in the collaborative trial that was organised to determine the precision, in terms of repeatability (r) and reproducibility (R), of this method. Cheese, meat and dried animal feed were used as food matrices. For each type of food 8 samples were examined: 2 blind duplicates at each of 4 concentration levels (blank, low, medium, high). All samples were artificially contaminated to achieve the desired inoculum levels and homogeneity. In addition two reference materials (RM) were included in the trial: capsules containing milk powder, artificially contaminated with *C. perfringens.* Test materials were prepared at three laboratories and verified on homogeneity and stability to ensure fitness for purpose. Samples were shipped to participants by express delivery services. Analyses of the test materials have been done according to a standardised protocol. Two techniques for confirmation of presumptive *C. perfringens* were included in this study to detect possible differences in performance: 1) lactose sulfite medium (as described in ISO 7937 (1997)) and 2) lactose-gelatine medium + motility-nitrate medium (alternative confirmation method in EN 13401 (1999)).
The precision characteristics were calculated using both ISO 5725 (1994) and pr EN ISO 16140 (2000). Repeatability (r) and reproducibility (R) were calculated for each sample type, inoculation level and confirmation method separately. The precision depended on the type of sample analysed. It is not clear whether this was due to the matrix or the different way of artificially contaminating the test materials. The best performance was found when analysing the RMs. The precision varied between the different contamination levels. Both confirmation techniques showed similar performance.
Recommendations to be made to ISO and CEN are:

- To include the precision data, found in this trial and calculated using pr EN ISO 16140 (2000), into ISO 7937 (1997) and EN 13401 (1999).
- To include both techniques for the confirmation of *C. perfringens*, as tested in this trial, into ISO 7937 (1997), leaving the choice at the user which one to perform. This will harmonise ISO 7937 (1997) with EN 13401 (1999).

1. Introduction

For acceptance of standards by the European Committee for Standardization (CEN), methods need to be validated. Project no. SMT4-CT96-2098, supported by the European Commission (4th framework Standards, Measurements and Testing Program (SM&T)), was elaborated to determine the precision data in terms of repeatability (r) and reproducibility (R) (quantitative methods) or performance characteristics (qualitative methods) of six International Organization for Standardization (ISO) methods. These were prioritised for acceptance by CEN as follows:

- ISO 7932 (1993): Enumeration of *Bacillus cereus*;
- EN ISO 11290 Part 1 (1997): Detection of *Listeria monocytogenes*
- EN ISO 11290 Part 2 (1997): Enumeration of *Listeria monocytogenes*
- EN ISO 6888 Part 1 and 2 (1999): Enumeration of coagulase positive *Staphylococci* (*Staphylococcus aureus* and other species)
- ISO 7937 (1997): Enumeration of *Clostridium perfringens*
- ISO/DIS 6579 (2000): Detection of *Salmonella*.

Three contractors were involved in this project:

- Agence Française de Sécurité Sanitaire des Aliments (AFSSA), France, also co-ordinator of the project;
- Ministry of Agriculture, Fisheries and Foods, Central Science Laboratory (MAFF-CSL), York, England
- National Institute of Public Health and the Environment, Microbiological Laboratory for Health Protection (RIVM-MGB), Bilthoven, The Netherlands.

Each contractor was responsible for the organisation of two of these six ISO methods:

- AFSSA: ISO/DIS 6579 (2000) and EN ISO 6888-Part 1 and 2 (1999);
- MAFF-CSL: EN ISO 11290-Part 1 (1997) and EN ISO 11290-Part 2 (1997);
- RIVM-MGB: ISO 7932 (1993) and ISO 7937 (1997).

All methods were validated by collaborative studies according to a similar design which conforms to the rules of the International Harmonised Protocol for Collaborative Studies [6]. Approximately twenty laboratories from various countries were invited to participate in each trial. Participants received detailed trial chronology, standard operating procedures (SOP) and a test report on which to record their results and return to the trial leader for analysis. Prior to each collaborative trial a pre-trial was organised between the three contractors to verify the SOP as written, identify any ambiguities and to establish that the test materials were fit for purpose. For each validation the method was challenged with three types of food from the dairy, meat and dried food groups. In addition a reference material (RM) was included to identify any serious errors in a participant's performance and to determine the maximum

precision possible in case of quantitative methods. Test materials used to validate the methods needed to be artificially inoculated to achieve the desired inoculum levels and homogeneity. These materials were tested extensively prior to the pre-trial and collaborative trial to ensure that they could be maintained in a stable and homogeneous state for the duration of the distribution and the trial period. Data from the collaborative trials were used to calculate:

1. the repeatability (r) and reproducibility (R) of the quantitative methods in relation to food type and inoculum level;
2. the performance characteristics relating to an 'equivalent value' of r and R appropriate to qualitative data (using model statistical systems which is developed as one of the objectives of this project [7]).

The project started in December 1996 and was finished in December 2000. The results are intended for publication by ISO in the corresponding ISO standards and subsequently by CEN in the CEN standards via the Vienna Agreement. The results of the validation of 4 methods have already been reported [1, 2, 3, 4, 5].

This report describes the validation of the method for the enumeration of *Clostridium perfringens* according to ISO 7937 (1997) [8]. The validation study was organised by RIVM-MGB. In this second edition of ISO 7937, lactose-sulfite (LS) medium is prescribed to confirm the characteristic colonies found on tryptose-sulfite-cycloserine (TSC) agar. However, in the first edition of ISO 7937 (1985), a combination of motility-nitrate (MN) medium and lactose-gelatine (LG) medium was prescribed instead. In the European standard for the enumeration of *Clostridium perfringens* (EN 13401 (1999) [9]) a choice is allowed between confirmation using lactose-sulfite medium and confirmation using motility-nitrate & lactose-gelatine (MN&LG) medium (see Figure 1). In this validation study both confirmation methods have been included in order to provide data for comparison testing.

To establish the precision of ISO 7937, the method was challenged with samples of fresh cheese, minced beef, dried animal feed and a reference material (RM). Cheese samples were prepared by Centre d'Etude et de Controle des Analyses en Industrie Laitiere (CECALAIT, Poligny, France), meat samples by MAFF-CSL and dried potato samples by RIVM-MGB. The RM, a capsule containing milk powder that is inoculated with *C. perfringens* spores was supplied by the Foundation for the Advancement of Public Health and Environmental Protection (SVM, Bilthoven, The Netherlands). The collaborative trial was carried out during January/February 2000.

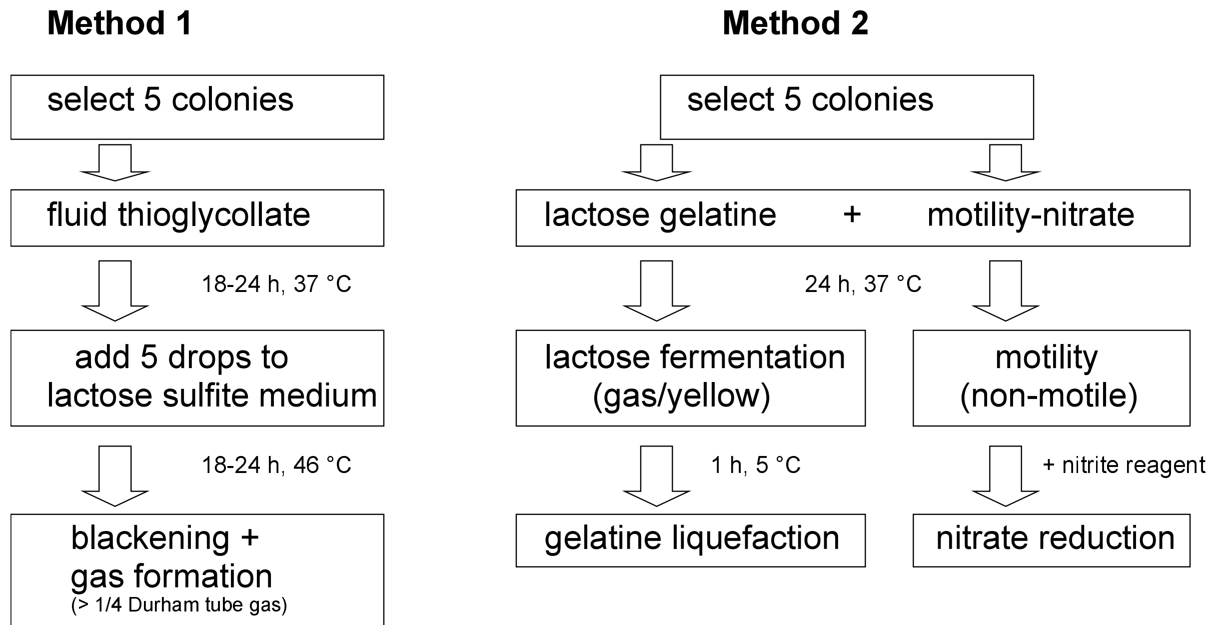

Figure 1 Two methods for confirmation of colonies as Clostridium perfringens

2. Materials and methods

2.1 Preparation of test materials

2.1.1 Introduction

For each food type, 4 inoculum levels were prepared in a range that can be found in natural contaminated samples:

- Blank level: containing only background flora
- Low level: 10^2 - 10^3 cfp/g[1] *C. perfringens* plus background flora
- Medium level: 10^4 cfp/g *C. perfringens* plus background flora
- High level: 10^5 cfp/g *C. perfringens* plus background flora

Background flora differed for each food type, simulating the autochthonous flora.
To achieve test materials containing a stable concentration of *C. perfringens*, samples needed to be inoculated with *C. perfringens* spores. Different strains were tested, however only two strains produced a sufficient amount of spores to achieve inoculum levels up to 10^5 cfp/g: *C. perfringens* TQA049, originating from spaghetti bolognese (AFSSA-LERPAC, Maisons-Alfort, France) and *C. perfringens* D10, originated form patient material (RIVM, Bilthoven, The Netherlands). Cheese samples were inoculating with the TQA049 strain. The meat, dried animal feed and the RM were inoculated with the D10 strain.
Acceptance of all production batches for use in the trials was made on the basis of achieving satisfactory homogeneity and stability (see 2.2).

2.1.2 Cheese samples

Cheese samples were prepared by CECALAIT (Poligny, France). All batches were prepared according to the following procedure:

Preparation of C. perfringens *spore suspension*
C. perfringens TQA049 was cultivated into de-aerated thioglycollate (FTG) broth (see Appendix 3) for 18 – 24 h at (37 ± 1) °C. One ml of this culture was added to each of 300 tubes containing 10 ml Duncan & Strong (D&S) medium (see Appendix 10), supplemented with 300 µl 10 % sodium bicarbonate solution, and incubated at (37 ±1) °C. After 10 days of incubation, the cultures were centrifuged for 20 min at 1700 g and rinsed with sterile distilled water. The pellet was resuspended into sterile distilled water, making a total of 250 ml spore suspension. This spore suspension was heated for 10 minutes at 70 °C to kill all vegetative cells. The spore suspension was preserved by freezing at –80 °C overnight, followed by freezing at –20 °C for 18 days. Before and after freezing, the spore level was verified on Tryptose Sulfite Cycloserine agar (TSC, see Appendix 3), incubated anaerobically at

[1] The term colony-forming units (cfu) is commonly used in microbiology. However the word 'unit' in metrology is reserved for SI units; therefore the term colony forming particles (cfp) will be used instead [10].

(37 ± 1) °C for (24 ± 2) hours. Final spore level was ca. 5 x 10^6 spores / ml. The strain showed positive reactions for all confirmation tests used in this trial. Results from API 20A: 99.9% *C. perfringens*.

Background flora

Together with *C. perfringens* spores, also 4 other strains, isolated from cheese by the INRA laboratory, were added to the cheese samples, to simulate natural background flora:

- *Lactococcus lactis lactis*
- *Enterococcus faecalis*
- *Lactobacillus paracasei*
- *Lactobacillus plantarum*

L. lactis lactis and *E. faecalis* were isolated on M17 agar (see Appendix 10), and the two *Lactobacillus* strains on MRS agar (see Appendix 10). The bacteria were inoculated in M17 or MRS broth (see Appendix 10) respectively during 18 hours at 30°C. After that, cultures were mixed: *L. lactis* with *E. faecalis*, and the *Lactobacillus* strains together. The inoculum levels for the target organisms were verified using a spiral plating system.

Preparation of cheese samples

Cheese samples were prepared in a liquid form. The cultures and the spore solution were diluted in ¼ Ringer's solution (see appendix 10) and then added to the cheese. Final levels of *C. perfringens* were ca 0, 5 x 10^2, 5 x 10^3 and 5 x 10^4 cfp/g. Background flora was added at the same level as *C. perfringens*, except for the blank samples: 5 x 10^3 cfp/g background flora. A bacteriostatic mixture (CECALAIT formulation) was added to stabilise the microflora. The bacteriostatic effect is negated when the sample is diluted during examination. Subsequently, the inoculated cheese was clotted after dispensing aliquots of 50 - 100 ml into individual vials by the addition of rennet (Figure 2). Samples were stored at 1 - 5 °C until use. Batches of cheese samples to be used in the collaborative trial were prepared 1 day prior to dispatch to participants due to their relatively short stability.

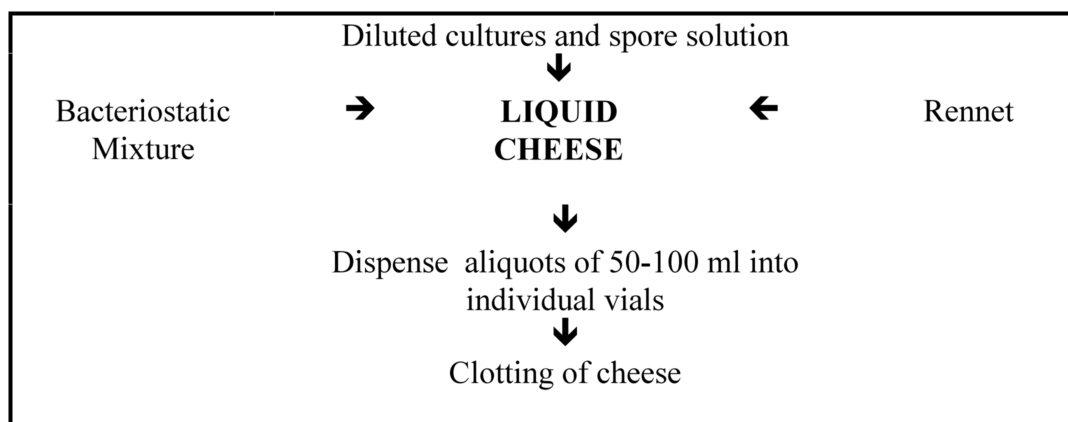

Figure 2 Preparation of cheese samples

2.1.3 Meat samples

Meat samples were prepared by the MAFF Central Science Laboratory (York, United Kingdom).

Preparation of C. perfringens *spore suspension*

C. perfringens strain D10 was cultivated in cooked meat medium (Appendix 10) for (72 ± 2) h at (37 ± 1) °C under anaerobic conditions. The culture was then added to D&S medium (see appendix 10), and incubated for (24 ± 2) h at (37 ±1) °C (anaerobic conditions).

Background flora

All samples were additionally inoculated with a simulated autochthonous meat flora, comprising *Lactobacillus plantarum* NCDO 1752 (CLS, York), *Micrococcus luteus* NCTC 8340 (PHLS, London) and *Pseudomonas aeruginosa* NCDO 1369 (CSL, York), at a level of *ca.* 1 x 10^5 cfp/g. This background flora was cultivated into Nutrient Broth (appendix 10), incubated at (30 ± 1) °C for (72 ± 2) h, and again for (24 ± 2) h in a fresh tube.

Preparation of meat samples

Batches of test materials were prepared using retail fresh minced beef which was freeze dried in bulk, sub-sampled into vials (portions of 10 g equivalents) and irradiated at 25 kGy. The test strain and background flora were diluted to the appropriate level and added as a 'cocktail' to each vial. Finally, the vials were freeze dried a second time to stabilise the samples and stored at 1 – 5 °C until use. It was necessary to prepare different batches for the pre-trial and trial to achieve the desired stability and homogeneity.

2.1.4 Dried animal feed samples

Dried animal feed samples were prepared by the RIVM-MGB (Bilthoven, The Netherlands). Due to the stickiness of the dried animal feed it was not possible to contaminate the dried animal feed directly with spores of the D10 strain. Therefore it was decided to prepare a milk powder contaminated with *C. perfringens* spores, which would then be added to the dried animal feed.

Preparation of C. perfringens *spore suspensions*

C. perfringens D10 was used to inoculated the dried animal feed. The strain was cultivated into 10 ml deaerated FTG (Appendix 3) for (24 ± 2) h at (37 ± 1) °C at anaerobic conditions. Two spore suspensions were prepared: for the medium and low level samples the complete 10 ml FTG culture was added to 100 ml D&S medium (appendix 10) and incubated anaerobically at (37 ± 1) °C for (24 ± 2) °C. For the high level samples 0.5 ml of the FTG culture was added to each of 20 tubes containing 10 ml D&S medium (appendix 10). These 20 tubes have been incubated under anaerobic conditions for (48 ± 2) h at (37 ± 1) °C (extension of the incubation period and the use of more D&S medium, increases the level of

spores formed). To kill vegetative cells, the D&S cultures were heated for 10 minutes at 70 °C. The cultures were centrifuged for 15 minutes at 2000 g. The pellets were resuspended in 10 ml peptone salt solution (Appendix 3) and centrifuged again for 15 minutes at 2000 g.

Preparation of highly contaminated milk powders (HCMP)

Each pellet of spores was dissolved in *ca.* 50 ml condensed sterile milk (Friesche Vlag Goudband, The Netherlands) directly after centrifugation. Then *ca.* 10 ml of each suspension was dried onto 500 g of sterile milk powder (Carnation, Nestlé; irradiated at 25 kGy) by means of fluid bed spray granulation (STREA-1, Niro-Aeromatic). This resulted in two highly contaminated milk powders (HCMP), a high level HCMP (batch 080799I, containing approx. 10^6 cfp/g), and a medium level HCMP (batch 240699, containing approx. 10^4 cfp/g). For the low level, part of the medium level HCMP was mixed 1:10 with sterile milk powder (Carnation, Nestlé). The inoculum level of this HCMP (batch 110100M) was approx. 10^3 cfp/g. Contamination levels have been verified on TSC (Appendix 3), incubated anaerobically at (37 ± 1) °C for (20 ± 2) h. The D10 strain showed positive reactions for all confirmation tests used in this trial (see SOP, Appendix 3). The HCMPs were stored at –20 °C until use.

Background flora

The natural contamination of the animal feed was used as background flora. The dried animal feed powder was obtained in bulk from Cehave n.v. (Veghel, The Netherlands). The level of bacteria which grow under anaerobic conditions on Plate Count agar (PCA, appendix 10) was about 1 x 10^3 cfp/g. The level of *C. perfringens*, counted on TSC agar, was about 20 cfp/g. As this *C. perfringens* level is negligible to the levels of artificial contamination (10^3 to 10^5 cfp/g), this batch of animal feed was approved for use in the trial.

Preparation of dried animal feed samples

Final samples were obtained by adding a certain amount of contaminated milk powder (depending on the inoculum level of the milk powder batches, see 3.1.3.1) to a vial containing dried animal feed, making a total of 10 g of test sample (Table 1). As the contents of the vials were not homogenised, the vials needed to be analysed completely. The final samples were stored at -20 °C until use.

Table 1 Preparation of dried animal feed samples

Level	Milk powder	Animal feed	Test sample
Blank	1.0 g sterile milk powder *	9.0 g	10.0 g
Low	0.6 g milk powder batch 110100M	9.4 g	10.0 g
Medium	1.0 g HCMP batch 240699	9.0 g	10.0 g
High	1.0 g. HCMP batch 080799I	9.0 g	10.0 g

HCMP = highly contaminated milk powder.
Weight tolerance milk powders 2%, animal feed powder 5%.
* = Nestlé , Carnation milk powder, irradiated at 25 kGy.

2.1.5 Reference material

For reference material (RM), capsules containing *C. perfringens* D10, were obtained from the Foundation for the Advancement of Public Health and Environmental Protection (Bilthoven, The Netherlands):
- batch number: R08002B, exp. date: May 2002.
- mean level (95% confidence limits) of *C. perfringens* per capsule was
  5.08×10^3 cfp ($3.88 \times 10^3 - 6.63 \times 10^3$ cfp).

The capsules were stored at –20 °C until use.

2.2 Homogeneity and stability of test materials

2.2.1 Cheese samples

2.2.1.1 Homogeneity

Ten samples of the low level batch were examined. The samples were examined in duplicate, 3 days after preparation, which was the day of examination at the trial. For each examination, 10 g of cheese was diluted in 90 ml K_2HPO_4 solution [11] and blended during 3 minutes. This solution was inoculated on 2 plates of TSC agar (Appendix 3), incubated anaerobically 18-24 hours at (37 ± 1) °C.

The CECALAIT computer program was used for statistical analysis. The χ^2 (Chi square test, α=0.05) was calculated on the total count of *C. perfringens* colonies per sample. In this way the dispersion of the contamination was compared to the dispersion obtained in a Poisson series.

2.2.1.2 Stability

Freshly prepared cheese samples are stable for a few days only. For the trial the stability of the low level batch was tested by analysing 3 samples each day in duplicate on TSC agar (Appendix 3), up to 10 days after preparation of these samples. The same examination procedure was used as for the homogeneity testing (2.2.1.1).

2.2.2 Meat samples

2.2.2.1 Homogeneity

Ten samples of the low, medium and high level batches were examined in duplicate on TSC agar (Appendix 3) at the day of preparation and at the test day in trial (12 days after preparation). The examination procedure is as written in the SOP (see Appendix 3). Homogeneity was determined by an F-test (α=0.05) on the counts.

2.2.2.2 Stability

The stability of the contamination levels of the meat samples was monitored over a period of 30 days. At several intervals, 10 samples of the low, medium and high level pre-trial batch were examined in duplicate on TSC agar (Appendix 3). Examination was done according to the SOP (see Appendix 3).

2.2.3 Dried animal feed samples

2.2.3.1 Homogeneity

For testing the homogeneity of the contaminated milk powder batches (and thus the final samples, as each sample contains the same amount of contaminated milk powder), 10 x 1 g. milk powder was diluted in 10 ml peptone salt solution (appendix 10) and mixed for 10 seconds on a whirl-mixer. One ml of this –1 dilution and subsequent dilutions were analysed onto TSC agar (Appendix 3). The results of the duplicate counts per sample on TSC agar were analysed for the variation between duplicate counts (T_1-test) [14] and between the average of duplicate counts between samples (T_2-test) [14]. A value for T_2 / (I-1) ≤ 2, where I = the number of samples analysed, is regarded as indication of good homogeneity.

2.2.3.2 Stability

The stability of *C. perfringens* spores in each batch of animal feed was monitored by analysing the inoculum levels in the milk powder batches (both animal feed samples and milk powder batches were stored at –20 °C). For the high level (batch 080799-I) and medium level (batch 240699) stability was monitored over a period of 30 weeks (from preparation until trial). For the low level batch (110100M) stability was only monitored over a period of 9 weeks, as this batch was prepared shortly before the trial. At several intervals 5 to 10 samples of 1 gram milk powder were examined at each interval. The examination procedure was the same as for homogeneity testing.

2.3 Design of the collaborative trial

2.3.1 Dispatch of test materials

In the collaborative trial per food type 8 samples were analysed: blind duplicates of each of the four inoculation levels: blank, low, medium and high. In addition 2 reference capsules were examined. The sample codes were C1-C8 for cheese samples, M1-M8 for meat samples, D1-D8 for dried animal feed samples and R1-R2 for the reference materials. The sample codes were randomised for each participant.
Meat, dried animal feed and reference capsules were dispatched to the participants by RIVM on 17 January 2000. Cheese samples were prepared by CECALAIT on 7 February 2000 and dispatched on 8 February 2000 to participants. All samples were shipped by international express carriers in polystyrene isolated boxes. Several ice packs (frozen to -20 °C) were included in the parcel to prevent the samples from being subjected to high temperatures. To detect possible temperature abuse during transport, two time-temperature control devices (WarmMark; Blanken Controls, Loenen, The Netherlands) were included in each parcel with response temperatures of 10 °C and 20 °C. When the temperature in the parcel exceeds the response temperature for more than 2 °C, the device turns red. The longer the temperature abuse or the higher the temperature in the box, the more compartments (brief, moderate, or prolonged exposure) of the device will turn red. Participants were asked to check the devices at arrival and, in case of severe temperature abuse, to contact the organising laboratory.

2.3.2 Standard Operating Procedure

Prior to the trial participants received general information regarding the objectives of the trial (Appendix 2). Analysis of the samples had to take place within a specified time schedule: the examination of the meat samples had to start between 19 and 24 January 2000. Dried animal feed samples had to be analysed between 27 and 31 January 2000. Cheese samples had to be examined on a specific day, 10 February 2000, due to the limited stability of these samples. The examination of the reference samples had to take place concurrently with one of the food types, chosen by the participant.
Detailed instructions for the analysis of the test materials is written in a standard operating procedure (SOP) (Appendix 3). This SOP was in concordance with ISO 7937, supplemented with extra information on how to handle the specific sample types. Media, needed for the examinations were not supplied by the organising laboratory, but was the responsibility of each participant. Media compositions and instructions on preparation were also included in the SOP.

In the SOP (Appendix 3) the analysis of the test samples is given in 6 steps:
1) Preparation of the initial suspensions with detailed instructions for the different sample types.
2) Pour plating in duplicate of 1 ml of the initial suspension and three further decimal dilutions with 10-15 ml Egg-Yolk-Free-Tryptose-Sulfite-Cycloserine (TSC) agar and adding an overlayer of *ca.* 10 ml of the same agar.
3) Anaerobic incubation of the plates at (37 ± 1) °C for (20 ± 2) h.
4) Enumeration of the characteristic (black) colonies.
5) Confirmation of max. 5 characteristic colonies of each plate containing less than 150 characteristic colonies at all dilutions:
   – using lactose-sulfite medium
   – using motility-nitrate medium and lactose-gelatine medium.
6) Calculation of the final number of *C. perfringens* per gram of sample using all plates with colony counts < 150 of two successive dilutions. Calculations are made on both confirmation techniques.

Participants were asked to complete the test report (Appendix 4), that they received together with the SOP. The test report contained the following subjects to be filled in:
- shipment conditions
- media ingredients (manufacturer, code numbers, batch numbers, expiry dates)
- temperature in laboratory room during examination
- the kind of pipette that was used
- incubation conditions
- test results as number of typical colonies per plate
- confirmation results per colony tested
- all deviations from SOP

This detailed information enabled deviant or aberrant results to be correlated with technical errors (like incubator failure, incorrect composition of media). The test report had to be returned to the trial leader for analysis before 21 February 2000.

2.4 Data analysis trial

2.4.1 Screening test results

All test reports have been studied carefully whether results were obtained correctly: were the media used, incubation temperatures, periods etc. according to the SOP? When the SOP was not strictly followed and the deviation could have influenced the test results (this was decided after discussion with the group of participating laboratories), the concerning data were excluded from further analysis (Appendix 7).

A Duncan's Multiple Range test was performed on all test results to check for outliers (Appendix 6). In this test the mean $\log_{10}$ values of the blind duplicate samples for each inoculation level and each sample type are compared between all laboratories. Means that are not significantly different ($\alpha = 0.05$) from each other are indicated with the same character. A single mean, significant different from all the other means, is indicating for an outlying result. The results that were regarded as suspected outliers in the Duncan's Multiple Range test were checked for errors during analysis. When a reason could be appointed, explaining that these results were not obtained correctly, the results were excluded from further analysis (Appendix 7). When no explanation could be found, the results were regarded as natural occurring variation and thus not excluded from further analysis.
The blank samples were only studied for false positives. Data from blank samples were excluded from further analysis.
All remaining data were analysed using the statistical computer program SAS for Windows, version 6.12 (SAS Institute Inc., Cary, USA). The protocol is given in Appendix 8.

2.4.2 Performance within each laboratory

To check the performance within each laboratory two tests were carried out, based on the T_1 test and T_2 test for microbiological reference materials [14]. The adjusted versions are called $T_{(repl)}$ and $T_{(dupl)}$ and were calculated for each sample type separately. $T_{(repl)}$ represents the variation between the replicate counts within each dilution i.e. whether the inoculation of plates was performed correctly. It was expected that the variation between replicate counts from the same dilution is Poisson distributed, which means that the $T_{(repl)}$ measure would follow a χ^2-distribution with I degrees of freedom ($\alpha = 0.05$). A high $T_{(repl)}$ value means large differences between the results from replicate counts.

$$T_{(repl)} = \sum_{g=1}^{G} \sum_{i=1}^{I} \sum_{j=1}^{J} \frac{\left(z_{gij} - \frac{z_{gi+}}{J} \right)^2}{\frac{z_{gi+}}{J}}$$

where:

z_{gij}	=	count of replicate j of sample dilution i of sample g
z_{gi+}	=	sum of counts of all replicates of dilution i of sample g
J	=	number of replicates examined per sample dilution i of sample g
I	=	number of dilutions examined per sample g
G	=	number of samples examined for each sample type

$T_{(dupl)}$ represents the variation between the blind duplicate samples within each inoculum level. In ideal case the sum of the counts per dilution is Poisson distributed, which means that the $T_{(dupl)}$ measure would follow a χ^2-distribution with I degrees of freedom ($\alpha = 0.05$). In

practise, a variation of 2 x Poisson is still considered as acceptable variation. A high $T_{(dupl)}$ value means that a large difference between blind duplicate samples was found.

$$T_{(dupl)} = \sum_{h=1}^{H} \sum_{i=1}^{I} \sum_{k=1}^{K} \frac{\left(z_{hik} - \frac{z_{hi+}}{K} \right)^2}{\frac{z_{hi+}}{K}}$$

where:
- z_{hik} = count of duplicate sample *k* (= sum of counts of replicates *j*) of dilution *i* of contamination level *h* (= Z_{gi+} in $T_{(repl)}$)
- z_{hi+} = sum of counts of all duplicate samples *k* of dilution *i* of contamination level *h*
- K = number of duplicate samples examined per contamination level *h*
- I = number of dilutions examined per contamination level *h*
- H = number of contamination levels examined per sample type

2.4.3 Comparison of confirmation techniques

The results obtained using lactose-sulfite medium and using both motility-nitrate & lactose-gelatin medium as confirmation technique, were compared using an F-test ($\alpha = 0.05$).

2.4.4 Precision data from trial

The precision characteristics, in terms of repeatability and reproducibility, were calculated in two ways:

1) According to ISO 5725 (1994) [12]. This is the traditional approach for determination of precision characteristics (repeatability r and reproducibility R), originally developed for analysing data from chemical analyses. Calculations have been performed by RIVM using analysis of variance / SAS System, release 6.12 [15]. The SAS protocol is given in Appendix 8.
2) According to the draft EN ISO 16140 (2000) [13]. This is the new approach, derived from the Microval project [16] and currently standardised by CEN TC 275/WG6: Task Committee 275 (Food Analysis–Horizontal Methods), Working Group 6 (Microbial Contamination). This standard is specially developed for data from microbiological analyses. The precision data are calculated with robust statistics, using the median in stead of the mean values. In this way outlying results will not influence the calculations. Calculations have been performed by AFSSA (Maisons-Alfort, France) using Excel 97.

3. Results and discussion

3.1 Homogeneity and stability of test materials

3.1.1 Cheese samples

3.1.1.1 Homogeneity

The χ^2-value on the total count of colonies of *C. perfringens* per sample of the 10 samples tested from the low level trial batch was 5.5 with a 95 % interval of 2.7 - 19.0. For this value, agreement with a Poisson series was not rejected at the 95 % probability level, and therefore the test batch was regarded as homogeneous. Homogeneity of the other batches was assumed to be similar to the low level batch.

3.1.1.2 Stability

Stability results of the low level batch are presented in Figure 3. The level of *C. perfringens* spores was stable (less than 5 % decline over the period tested) for at least up to 10 days after preparation. Stability of the other levels was assumed to be similar to the low level batch. During the trial, samples were analysed within 3 or 4 days after preparation (except for laboratory 9, see 3.2.2).

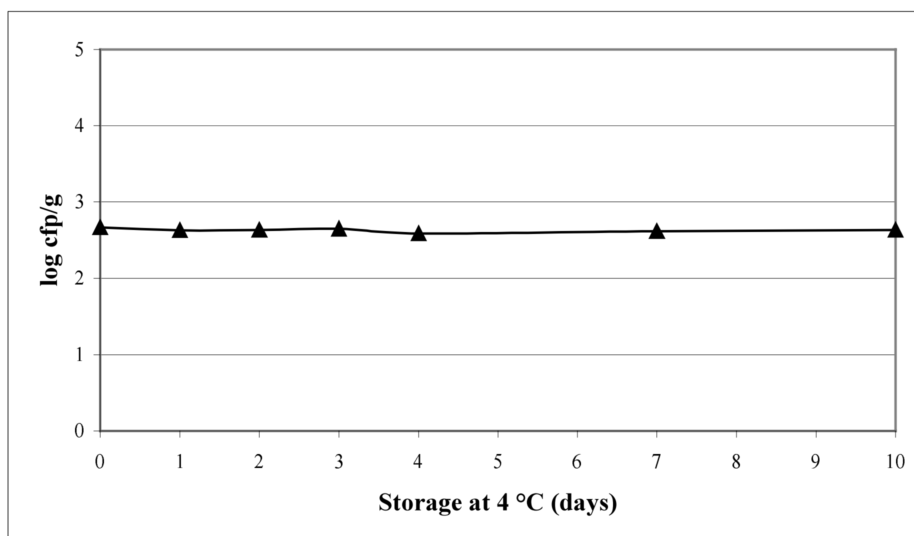

Figure 3 Stability of the low level of C. perfringens TQA049 spores in fresh cheese samples, stored at 4 °C.

3.1.2 Meat samples

3.1.2.1 Homogeneity

Homogeneity data for the different batches that were used in the trial are given in Table 2. An F-test ($\alpha = 0.05$) was performed on the counts on the plates. At the day of preparation, the low level and medium level were homogeneous, the high level showed more variability than can be explained by Poisson distribution. At day of examination in the trial (day 12), only the low level showed heterogeneity.

Table 2 Homogeneity of meat samples

Inoculum level	Test day	I	mean $\log_{10}$ cfp/g	T_2 / (I-1)	F-test (P)
low	Day 0	10	3.03	1.54	Not significant (0.591)
	Day 12	10	2.87	12.37 *	Significant (0.004) *
Medium	Day 0	10	3.93	1.90	Not significant (0.249)
	Day 12	10	3.92	0.78	Not significant (0.775)
High	Day 0	10	4.97	23.77 *	Significant (0.030) *
	Day 12	10	4.88	1.79	Not significant (0.075)

I = number of samples analysed on TSC agar.

T_2/(I-1) = dispersion factor. Criterion: ≤ 2

* = evidence of heterogeneity between vials tested

3.1.2.2 Stability

Stability test results are presented in Figure 4. The level of *C. perfringens* spores was stable (less than 5 % decline) for at least up to 30 days after preparation. In the collaborative trial samples were analysed within 30 days.

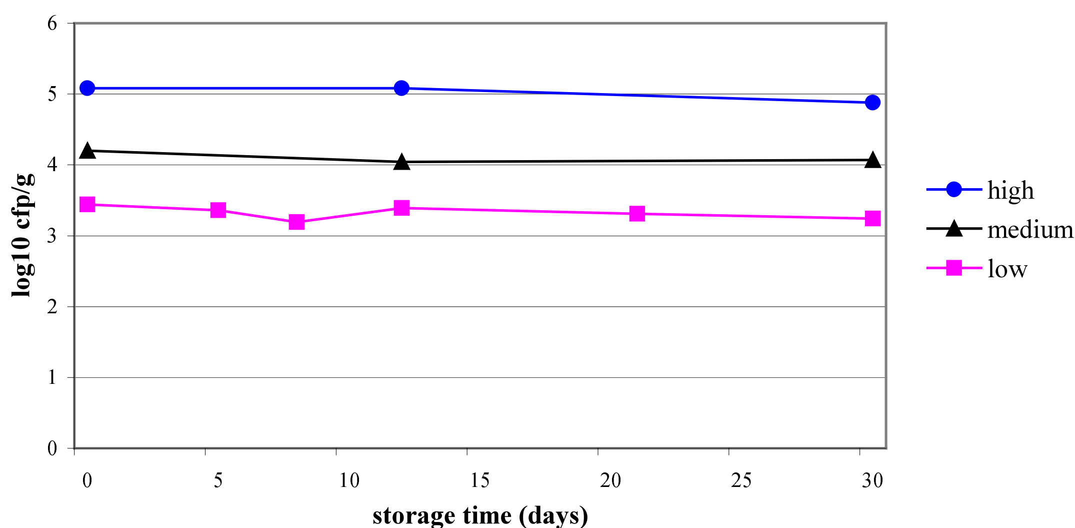

Figure 4 Stability of the level of C. perfringens D10 spores in meat samples, stored at 4 °C.

3.1.3 Dried animal feed samples

3.1.3.1 Homogeneity

The homogeneity of the contamination in the HCMP batches is given in Table 3. Homogeneity was satisfactory for all batches as $T_2/(I-1) \leq 2$ in all cases (low level batch was just satisfactory). Note that all inoculum levels given are the contamination levels in the HCMP, thus a 10-fold higher than in the final dried animal feed samples. The final inoculum levels in the animal feed samples (see also Table 1) were: < 10^2 cfp/g (natural contamination), 6.3 x 10^2 cfp/g, 5.5 x 10^3 cfp/g and 4.6 x 10^4 cfp/g for blank, low, medium and high level samples respectively.

Table 3 Homogeneity of the HCMPs containing C. perfringens D10

HCMP batch	I	Mean (cfp/g) *	mean $\log_{10}$ cfp/g **	T_2 / (I - 1)
080799-I (high)	10	4.6 x 10^5	5.67	0.94
240699 (medium)	10	5.5 x 10^4	4.74	0.91
110100/M (low)	10	9.8 x 10^3	3.99	2.13

I = number of samples analysed (1 g powder);

T_2 /(I-1) = dispersion factor. Criterion: ≤ 2

* Final levels in animal feed samples (see also Table 1): 6.3 x 10^2 (low), 5.5 x 10^3 (medium); 4.6 x 10^4 (high)

* * Final levels in animal feed samples (see also Table 1): 2.78 (low), 3.74 (medium), 4.67 (high)

3.1.3.2 Stability at -20°C

Stability results are presented in Figure 5. As milk powder batches were tested, in stead of the animal feed samples, the mean levels as shown in Figure 5 are about 10-fold higher than in the actual samples. All batches were stable over the period tested (no significant decrease).

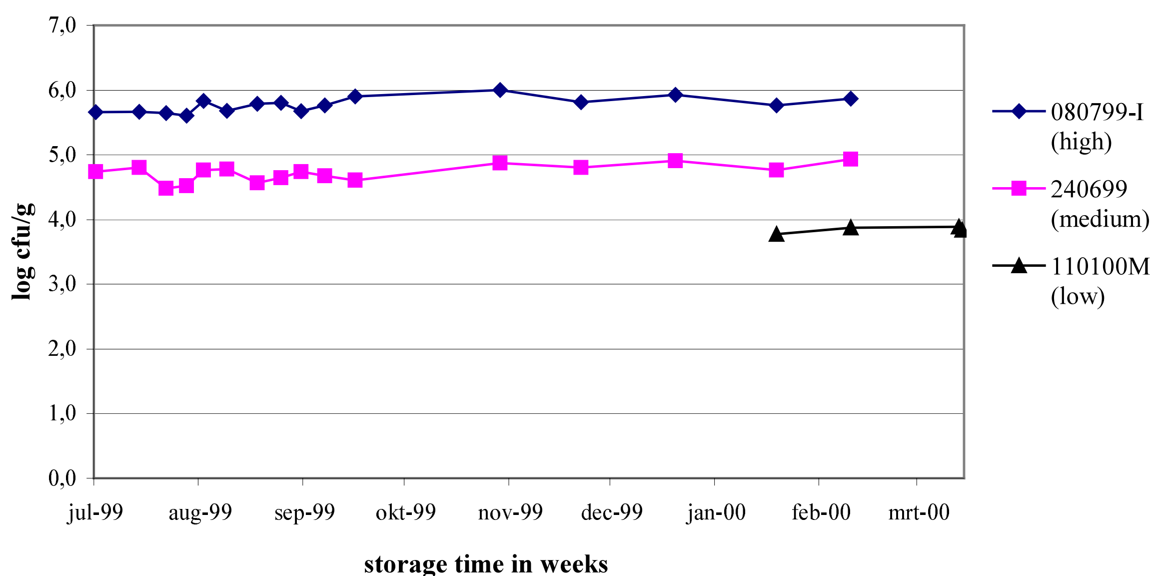

Figure 5 Stability of the level of C. perfringens spores in HCMP batches, stored at -20 °C.

3.2 General results of the trial

3.2.1 Participants

A total of 17 laboratories from 13 European countries participated in the collaborative trial. Twelve laboratories examined all sample types and used both confirmation techniques. Two laboratories (12, 13) did not perform the lactose-sulfite confirmation, and three laboratories (2, 9 and 11) did not perform the motility-nitrate / lactose-gelatine confirmation. Therefore a total of 15 laboratories analysed all samples using lactose sulfite technique (ISO 7937), and 14 laboratories examined the samples using the motility-nitrate and lactose-gelatine technique (EN 13401).

3.2.2 Dispatch of test materials

Meat, dried animal feed and RMs were shipped on 17 January 2000 from RIVM (The Netherlands) and were delivered at 9 laboratories within 24 hours, at 5 laboratories between 24 and 48 hours and at three laboratories between 48 and 72 hours after dispatch. Only three laboratories (laboratories 2, 6 and 9) received samples that had been exposed to temperatures above 10 °C (only brief to moderate exposure).
Cheese samples were shipped from CECALAIT (France) to the participating laboratories on 8 February 2000. Twelve laboratories received the parcel within 24 hours and three laboratories within 48 hours. These 15 laboratories commenced examinations on the prescribed date of 10 February 2000. However two laboratories (9 and 17) received the samples on 11 February 2000 (72 hours after shipment). Only samples dispatched to laboratories 9 and 14 were exposed to temperatures above 10 °C (only brief exposure). None of the samples have been exposed to temperatures over 20 °C.
Laboratory 17 was able to start the examination on 11 February 2000, however laboratory 9 started examination on 15 February (after the weekend). Based on the results of the stability tests of the cheese samples (3.1.1.2) it was agreed that the results from laboratory 9, who analysed the samples 8 days after preparation, were not excluded from statistical analysis (Appendix 7).

3.2.3 Media

Each participant was allowed to choose whether to use commercially available dehydrated media or use media prepared from individual ingredients, as long as the ingredients and concentrations were identical to the prescription in the SOP. In case of using individual ingredients, all batch numbers and concentrations used had to be recorded on the test report.
Table 4 summarises the number of laboratories using either commercial available media and home prepared media for each medium used in the trial.
Complete dehydrated TSC agar base was obtained from MERCK (8x), Difco (2x), Biokar (1x) and AES Laboratories (1x). Laboratory 8 used TSC agar base from Oxoid. This

dehydrated formula contains additional to the composition described in ISO 7937, 5.0 g lab-lemco powder[2]. Apart from that also egg yolk was added to the base (not to the base used for analysing the cheese samples), which was not according to the SOP. This resulted in an agar which is more enriched and subsequently this might have resulted in enhanced growth of *C. perfringens* on the TSC plates. As the objective of this trial was to validate the ISO 7937 (1997) as written, it was decided between the partners of this project to exclude the results from laboratory 8.

Commercial Fluid thioglycollate (FTG) was obtained from Oxoid (8x), MERCK (4x), Diagnostics Pasteur (1x), and Lab M (1x).

Complete lactose-sulfite medium was used from Sanofi Diagnostics Pasteur (5x) and SCHARLAN (1x) and 1 laboratory used lactose gelatine medium from AES Laboratoires.

Table 4 Number of laboratories using commercially available media and home prepared media

Medium	Complete (dehydrated) media	Individual ingredients
TSC agar base	13	4
FTG	14	1
Lactose-sulfite	6	9
Motility-nitrate	0	14
Lactose-gelatine	1	13

All media, prepared from individual ingredients, have been checked and confirmed being made according to the formulation in the SOP. The pH values were nearly all according to the criteria, only for a small number of media the pH differed for max. 0.3 pH units outside the given range. This small deviation was considered as of minor influence in the final results. It was agreed that all media that were used in the collaborative trial were approved, except for the TSC agar base from Oxoid, used by laboratory 8.

It was suggested by a participant to include in ISO 7937 (1997) a maximum storage time for TSC agar plates in case they are not freshly prepared before use. This laboratory experienced that *C. perfringens* colonies were white on TSC plates, which were prepared *ca* 30 days before use.

3.2.4 Incubation conditions of media

The incubation of the lactose sulfite medium varied between participants, due to indistinctness of the description in ISO 7937. It was not clear from the procedure in this ISO whether the tubes needed to be incubated under aerobic or anaerobic conditions. Part of the participants incubated the tubes in a water bath as prescribed in the ISO, while others incubated the tubes in an anaerobic jar in an incubator. It is not clear whether a waterbath is

[2] Note: Oxoid changed the composition of the TSC base since Summer 2000 in accordance with ISO 7937 (lab lemco powder is not in the formula anymore).

obligatory or that anaerobic incubation in a jar is also allowed, in the case that the specified temperature range of 46 ± 0.5 °C can be achieved in the incubator used. Furthermore, some laboratories had difficulties with reading of the gas formation as in some cases only a small amount of gas was formed. Using tubes or bottles may have effected this.
Incubation periods and incubator temperatures were nearly all according to the criteria in the SOP. Deviations were regarded as of minimal influence on the final results and therefore no results have been excluded on this basis.

3.2.5 Laboratory temperature

Temperature in the laboratory rooms varied from 17 °C to 25 °C, with no more than 4 °C deviation over the different days of analysis within one laboratory.

3.2.6 Use of pipettes

Each laboratory was allowed to use the kind of pipette they normally use, although it was stated in the SOP that a micropipette was preferred. For preparation of the dilutions 13 laboratories used graduated pipettes made of glass or plastic and 4 laboratories used a micropipette with fixed or variable volume. For inoculating the plates this was 12 and 5 laboratories respectively.

3.3 Statistical analysis of trial data

3.3.1 Screening data

The results from the collaborative trial are summarised in Appendix 5 for each food type and for both confirmation techniques separately.
Blank samples were checked on false positive test results. In none of the blank cheese samples *C. perfringens* was isolated. Only 1 colony at the –1 dilution was isolated in a blank meat sample (lab 13, M8). This was not regarded as a false positive test result or indicating an exchange of two samples. For the dried animal feed, in 16 of the 34 blank samples 1 to 5 colonies of the –1 dilution were confirmed as *C. perfringens*. As this level corresponds to the natural level of contamination in the dried animal feed (ca 20 cfp/g) no samples have been exchanged and results cannot be seen as ‘false positive test results’. However in laboratory 4, sample D5 (blank) and sample D2 (low) the levels found were 4.9 x 10^2 cfp/g in D5 and 15 cfp/g in D2. This can be due to exchanging these samples during analysis or a mistake in coding the samples. However, as both possible explanations cannot be confirmed by laboratory 4 or by the RIVM, it was decided to exclude the results of these two samples from further analysis. Except for samples D2 and D5 in laboratory 4, all other results correspond to the sample levels, meaning that no other samples have been exchanged or that false positive test results were obtained. Blank samples were excluded from statistical analyses.

Deviations from the protocol are listed in Appendix 7. If the deviations could have had a significant influence on the results obtained, then the corresponding data were excluded from further analysis. Decisions on this were agreed upon in a meeting with all participants.
In laboratory 7 many false negative test results were found using lactose-sulfite medium. The laboratory indicated to have only little experience with this medium. Using the MN&LG media, mostly positive confirmation results were obtained. It was agreed upon with laboratory 7 and with the partners of this project that the results from laboratory 7 using LS medium were not reflecting normal performance of this medium but were a result of their lack of experience with this medium. Therefore the results of laboratory 7 using LS medium were excluded from further analysis (their results using MN&LG confirmation were not rejected).
Results of the Duncan's Multiple Range test are summarised in Appendix 6. Some results came out as suspected outliers. However, as analyses of these test results have been performed according to the SOP, these results were regarded as naturally occurring variation.

3.3.2 Performance within each laboratory

Statistical analysis (data not published) showed that the variation between replicate counts at the same dilution ($T_{(repl)}$) was generally not significantly different from Poisson distribution (except for laboratory 12 regarding cheese). Statistical analysis on the variation between the duplicate samples ($T_{(dupl)}$) gave more deviations from Poisson distribution. For some laboratories this was even more than 2x Poisson.

3.3.3 Comparison of confirmation techniques

All confirmation results obtained by all participants together (except from the non-valid results mentioned in Appendix 7) have been summarised for each sample type in Table 5 (lactose-sulfite medium) and Table 6 (motility-nitrate & lactose-gelatine medium).
All media gave acceptable test results. Generally, 98.1 % of the colonies confirmed using LS medium was identified as *C. perfringens.* Using the MN&LG technique this percentage was 96.1 %. This was mainly due to the motility-nitrate medium as this medium showed a number of false negative results, while lactose-gelatine medium gave nearly 100% positive test results. An F-test (data not published) showed no significant difference between the two confirmation techniques. Both methods are currently used in laboratories, and therefore it is desirable to recommend to ISO to include both confirmation techniques (LS as well as MN&LG) in ISO 7937, like in EN 13401. In this way the user can choose the technique they prefer and it will give an harmonisation between ISO 7937 and EN 13401. However, it has to be kept in mind that during this trial only two strains have been tested. For acceptance by ISO that both confirmation methods perform equally, it might be necessary to test more strains, including naturally contaminated samples.

Table 5 Confirmation of C. perfringens *colonies using lactose-sulfite medium.*

Lactose-sulfite technique	Cheese	Meat	Dried animal feed	RM	Totals
No. of colonies tested in LS medium	1316	1363	1484	260	4423
Formation of gas (+)	1290	1360	1452	249	4351
(%)	98.0	99.8	97.8	95.8	98.4
Blackening (+)	1304	1358	1476	260	4398
(%)	99.1	99.6	99.5	100	99.4
No. of colonies confirmed as *C. perfringens* (%)	1283 (97.5)	1358 (99.6)	1447 (97.5)	249 (95.8)	4337 (98.1)

Table 6 Confirmation of C. perfringens *colonies using MN&LG medium.*

Motility-nitrate & Lactose-gelatine technique (MN & LG)	Cheese	Meat	Dried animal feed	RM	Totals
No. of colonies tested in MN&LG tubes	1297	1345	1487	260	4389
Motility * (+)	1263	1342	1453	258	4316
(%)	97.4	99.8	97.7	99.2	98.3
Nitrate reduction (+)	1248	1304	1436	257	4245
(%)	96.2	97.0	96.6	98.8	96.7
Lactose fermentation (+)	1294	1324	1486	260	4364
(%)	99.8	98.4	99.9	100	99.4
Gelatine liquefaction (+)	1297	1343	1481	260	4381
(%)	100	99.9	99.6	100	99.8
No. of colonies confirmed as *C. perfringens* (%)	1244 (95.9)	1285 (95.5)	1433 (96.4)	255 (98.1)	4217 (96.1)

* positive reaction means positive for *C. perfringens* i.e. non-motile

3.3.4 Precision data from trial

The precision parameters, in terms of repeatability (r) and reproducibility (R), were calculated using both ISO 5725 [12] and pr EN ISO 16140 [13] and for each confirmation technique separately. Repeatability and reproducibility values, calculated according to ISO 5725, were very similar to those calculated according to pr EN ISO 16140. The pr EN ISO 16140 is especially developed for microbiological analysis, and will be the new standard for calculating precision characteristics on microbiological data. Therefore the values calculated with the pr EN ISO 16140 standard will be adopted as the precision characteristics of the ISO 7937 method, resulting from this collaborative study. The results using pr EN ISO 16140 (2000) are presented in Table 7, the results of ISO 5725 are given in Appendix 9.

The contamination levels as found in this trial corresponded to the levels found in the homogeneity studies (see 3.1). The repeatability values ranged from 0.11 - 0.35 on $\log_{10}$ scale or 1.3 - 2.2 on normal scale, which means that the method performs very well under repeatability conditions. Under reproducibility conditions, more variation was found. Reproducibility values varied from 0.21 - 0.85 on $\log_{10}$ scale or 1.6 - 7.1 on normal scale.

The method showed the best performance when analysing the RMs. This was already expected as this material was very homogeneous and contained no disturbing background flora. Furthermore the procedure of analyzing the RMs is very strict. The variation in precision found between the three food types might be explained by the difference in matrix, but can also be caused by the difference in the way the samples were artificially contaminated. The highest reproducibility value was found for the low level dried animal feed samples. The homogeneity of this batch of samples was just acceptable (see 3.1.3), however homogeneity was tested only on the batch of HCMP and not on the final egg powder samples. An explanation might be that adding the HCMP to the egg powder may have caused some extra variation between the samples or that the background flora in the final samples had some effect on the *C. perfringens* counts found.

The values RSD_r and RSD_R (Table 7) can be used to observe a trend between contamination level and precision: the values express the repeatability and reproducibility relative to the contamination level. The method was challenged with contamination levels over a range of 10^2 - 10^5 cfp/g. For cheese and dried animal feed the repeatability and reproducibility were the highest for the low level contamination. For the meat samples however no trend could be observed between precision and contamination level.

The precision data obtained using LS confirmation were very similar to the precision data obtained with the MN&LG confirmation. Together with the results from Table 5 and 6, it can be concluded that both techniques show equal performance. It will therefore be suggested to

include both methods in the ISO 7937 standard, leaving the choice at the user which one to perform.
No conclusions can be made towards the effect of strain type on the precision of the method, as only two strains were used (meat, dried and RM were inoculated with D10, cheese with TQA049). To do so, many more strains should have been taken into consideration.

Table 7 Precision values of ISO 7937 (1997) and EN 13401 (1999), calculated according to pr EN ISO 16140 (2000) for each sample type, contamination level and confirmation technique.

CONFIRMED COUNTS USING LACTOSE-SULFITE MEDIUM

Sample type	Level	No. of Labs	Median (log cfp/g)	r (log) [a]	r [b]	R (log) [c]	R [d]	RSD_r [e]	RSD_R [f]
Cheese	Low	13	2.52	0.28	1.9	0.34	2.2	3.95	4.80
	Medium	13	3.53	0.19	1.5	0.21	1.6	1.89	2.14
	High	13	4.54	0.25	1.8	0.22	1.7	1.99	1.73
Meat	Low	13	2.72	0.23	1.7	0.34	2.2	3.07	4.48
	Medium	13	3.61	0.21	1.6	0.60	4.0	2.08	5.93
	High	13	4.54	0.35	2.2	0.70	5.0	2.78	5.49
Dried	Low	13	2.61	0.28	1.9	0.75	5.6	3.90	10.30
	Medium	13	3.82	0.11	1.3	0.69	4.9	1.07	6.50
	High	13	4.79	0.17	1.5	0.52	3.3	1.25	3.84
RM		13	3.72	0.19	1.5	0.27	1.9	1.87	2.60

CONFIRMED COUNTS USING MOTILITY-NITRATE & LACTOSE-GELATINE MEDIUM

Sample type	Level	No. of Labs	Median (log cfp/g)	r (log) [a]	r [b]	R (log) [c]	R [d]	RSD_r [e]	RSD_R [f]
Cheese	Low	13	2.49	0.30	2.0	0.35	2.2	4.31	5.04
	Medium	13	3.55	0.20	1.6	0.27	1.9	2.02	2.69
	High	13	4.49	0.24	1.7	0.32	2.1	1.88	2.54
Meat	Low	13	2.71	0.33	2.1	0.47	3.0	4.32	6.23
	Medium	13	3.61	0.26	1.8	0.61	4.1	2.55	6.02
	High	13	4.62	0.27	1.9	0.47	3.0	2.10	3.65
Dried	Low	13	2.61	0.15	1.4	0.85	7.1	2.05	11.67
	Medium	13	3.87	0.13	1.3	0.74	5.5	1.22	6.86
	High	13	4.83	0.14	1.4	0.58	3.8	1.07	4.26
RM		13	3.71	0.12	1.3	0.29	1.9	1.15	2.75

a) r (log) = repeatability limit: the value less than or equal to which the absolute difference between two test results, expressed in log_{10} units and obtained under repeatability conditions, may be expected to be with a probability of 95%.
b) r = repeatability limit: the value less than or equal to which the absolute ratio between two test results, expressed on normal scale and obtained under repeatability conditions, may be expected to be with a probability of 95%.
c) R (log) = reproducibility limit: the value less than or equal to which the absolute difference between two test results, expressed in log_{10} units and obtained under reproducibility conditions, may be expected to be with a probability of 95%.
d) R = reproducibility limit: the value less than or equal to which the absolute ratio between two test results, expressed on normal scale and obtained under reproducibility conditions, may be expected to be with a probability of 95%.
e) RSD_r = 100 x S_r/median, expressing the value of r relative to the contamination level
f) RSD_R = 100 x S_R/median, expressing the value of R relative to the contamination level

Average repeatability and reproducibility values for each food type were calculated as the arithmetic mean of the values obtained in the three levels (Table 8).

Table 8 Precision values per sample type, calculated using pr EN ISO 16140 (2000).

	LS confirmation technique				MN&LG confirmation technique			
Sample type	r (log) [a]	r [b]	R (log) [c]	R [d]	r (log) [a]	r [b]	R (log) [c]	R [d]
Cheese	0.24	1.7	0.26	1.8	0.25	1.8	0.31	2.1
Meat	0.26	1.8	0.55	3.5	0.29	1.9	0.52	3.3
Dried animal feed	0.19	1.5	0.65	4.5	0.14	1.4	0.72	5.3
RM	0.19	1.5	0.27	1.9	0.12	1.3	0.29	1.9

a) r (log) = repeatability limit: the value less than or equal to which the absolute difference between two test results, expressed in $\log_{10}$ units and obtained under repeatability conditions, may be expected to be with a probability of 95%.

b) r = repeatability limit: the value less than or equal to which the absolute ratio between two test results, expressed on normal scale and obtained under repeatability conditions, may be expected to be with a probability of 95%.

c) R (log) = reproducibility limit: the value less than or equal to which the absolute difference between two test results, expressed in $\log_{10}$ units and obtained under reproducibility conditions, may be expected to be with a probability of 95%.

d) R = reproducibility limit: the value less than or equal to which the absolute ratio between two test results, expressed on normal scale and obtained under reproducibility conditions, may be expected to be with a probability of 95%.

4. Conclusions and recommendations to ISO/CEN

The conclusions from this collaborative trial and recommendations to ISO and CEN are:

- The method for the enumeration of *Clostridium perfringens* in foods and animal feeding stuffs as currently written in ISO 7937 (1997) and EN 13401 (1999) is satisfactory.

- To include the precision data as found in this trial for both confirmation techniques (LS and MN&LG) into the ISO 7937 (1997) standard and the EN 13401 (1999) standard for the enumeration of *Clostridium perfringens* in foods and animal feeding stuffs.

- To include the precision data calculated using pr EN ISO 16140 (2000).

- To include confirmation using motility-nitrate medium and lactose-gelatine medium in ISO 7937 (1997) as an alternative confirmation to the confirmation using lactose-sulfite medium. In this collaborative trial the two methods have been given equal performance in both accuracy and precision and both methods are currently used in the laboratories. It is recommended to harmonise the ISO 7937 (1997) standard with the EN 13401 (1999) standard in which both confirmation techniques are allowed.

- To improve the text of ISO 7937 (1997) and EN 13401 (1999) in the following paragraphs at the next revision:
  - § 5.3 Egg-yolk-free tryptose-sulfite-cycloserine agar: it is suggested to include in ISO 7937 a maximum storage time for the TSC plates in case they are prepared in advance;
  - § 5.5 Lactose-sulfite medium: it is suggested that this medium should only be dispensed into test tubes, as bottles do not seem to work;
  - § 9.4 Confirmation technique using the Lactose-sulfite medium:
    - (editorial) NOTE in § 9.4.2 of EN 13401: It is therefore NOT necessary to ensure that the black colonies picked from the agar are pure before inoculation into the thioglycollate broth and subsequently into the lactose sulfite medium;
    - § 9.4.1 of ISO 7937 and § 9.4.2.1 of EN 13401: A clarification whether lactose-sulfite medium needs to be incubated aerobically in a waterbath or whether anaerobic incubation in an incubator, capable of holding the temperature at (46 ± 0.5) °C, is also allowed.

Participants and acknowledgements

Responsible person of EU Project SMT4-CT96-2098

- I. de Froidment-Görtz, European Commission, Directorate-Générale XII / C-5 (responsible commission scientific officer)	Brussels	B
- C. Lahellec, AFSSA Direction Générale (project coordinator)	Paris	F
- L. Cox, MAFF-CSL Food Science Laboratory (partner)	York	UK
- K.A. Mooijman, RIVM-MGB (partner)	Bilthoven	NL

Organisation *C. perfringens* trial

- K.A. Mooijman, S.M. Schulten, RIVM-MGB; N.J.D. Nagelkerke, RIVM-IMA (statistical analysis)	Bilthoven	NL

Preparation of test material

- Cheese: P. Rollier, CECALAIT INRA SRTAL	Poligny	F
- Meat: K. Capps, MAFF-CSL Food Science Laboratory	York	UK
- Dried animal feed: E. Benschop, RIVM-MGB	Bilthoven	NL

Participants pre-trial

- H. Bergis, S. Rudelle, AFSSA LERPAC	Maisons Alfort	F
- L. Cox, K. Capps, MAFF-CSL, Food Science Laboratory	York	UK
- S.M. Schulten, E. Benschop, RIVM-MGB	Bilthoven	NL

Participants collaborative trial

- H. Asperger, Veterinärmedizinische Universität Wien, Institut für Milchhygiene und Milchtechnologie	Wien	A
- K. de Reu, Dairy Research Station, Microbiology Laboratory	Melle	B
- E. Heck, Nestlé Research Center Lausanne, Quality and Safety Assurance Department	Lausanne	CH
- E. Bartelt, Federal Institute for Health Protection of Consumers and Veterinary Medicine, Food Microbiology	Berlin	D
- S. Qvist, Institute of Food safety and Toxicology	Soeborg	DK
- C. Allaert, Lab. of Microbiology of the Food Technology Department	Lleida	E
- H. Bergis/S. Rudelle, AFSSA-LERPAC	Maisons Alfort	F
- T. Johansson, National Veterinary and Food Research Institut, Department of Food Microbiology	Helsinki	FIN
- M. Lambiri, National School of Public Health, Bacteriology Department	Athens	GR
- Dr. M. Knodlseder/Dr. K. Friedrich, Milchwirtschaftliche Untersuchungs- und Versuchsanstalt Kempt	Kempten	D
- M.L. Stecchini, Dipartimento di Scienze Degli Alimenti	Udine	I
- V. Young, EHB Public Analysts Laboratory	Dublin	IRE
- H. Stegeman, RIKILT-DLO	Wageningen	NL
- S.M. Schulten, RIVM-MGB	Bilthoven	NL

- D. Roberts, Central Public Health Laboratory, Food Hygiene Laboratory	London	UK
- S. Hall, Unilever Research, Colworth Laboratory	Bedford	UK
- H. Becker, Lehrstuhl für Hygiene und Technologie der Milch Ludwig-Maximilians-Universität-München	München	D

Acknowledgements

The collaborative trial demanded a lot of time and effort from each participant. Also the expenses for the media and carrying out the examinations could unfortunately not be covered. The RIVM and all other partners in this project are therefore extremely grateful to each participant for all the work they have done in producing precision data for the ISO 7937 method and for having an active role during the discussion of the results in the final meeting!

Thank you very much!

References

[1] Schulten, S.M., B.E.B. van de Lustgraaf, N.J.D. Nagelkerke, P.H. in 't Veld (1998) "Validation of Microbiological Methods. Enumeration of Bacillus cereus according to ISO 7932 (second edition, 1993)". National Institute of Public Health and the Environment (RIVM), Bilthoven, The Netherlands. Report no. 286555001

[2] Schulten, S.M., P.H. in 't Veld, N.J.D. Nagelkerke, S. Scotter, M.L. de Buyser, P. Rollier, C. Lahellec (2000) "Evaluation of the ISO 7932 standard for the enumeration of Bacillus cereus in foods". International Journal of Food Microbiology, 57, p. 53-61.

[3] Scotter, S. and Langton, S. (1999) "EU project SMT4-CT96-2098. Validation of Microbiological Methods. Detection of *Listeria monocytogenes* by EN ISO 11290 Part 1:1997 Final report". Ministry of Agriculture, Fisheries and Food, Central Science Laboratory, York, England. Report no. FD 98/15.

[4] Scotter, S. and Langton, S. (1999) "EU project SMT4-CT96-2098. Validation of Microbiological Methods. Enumeration of *Listeria monocytogenes* by EN ISO 11290 Part 2:1997 Final report". Ministry of Agriculture, Fisheries and Food, Central Science Laboratory, York, England. Report no. FD 98/68.

[5] Buyser, M.L. de, B. Lombard (1999) "EU project SMT4-CT96-2098 Validation of Microbiological Methods. Enumeration of coagulase-positive staphylococci (S. aureus and other species) by EN ISO 6888 Part 1 and Part 2 (1999). Draft Final Report". Agence Française de Sécurité Sanitaire des Aliments, Laboratoire Central d'Hygiène Alimentaire.

[6] Horwitz, W. (1995) "International Harmonised protocol for the Design, Conduct and Interpretation of Method-Performance Studies (Technical report)". IUPAC/AOAC/ISO. Pure and Applied Chemistry, 67, no. 2, p. 331-343.

[7] Langton, S. (to be published) (Title article not yet known). Ministry of Agriculture, Fisheries and Food, Central Science Laboratory, York, England.

[8] Anonymous (1997). ISO 7937, second edition. Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of *Clostridium perfringens* – Colony-count technique. International Organisation for Standardization, Geneva, Switzerland.

[9] Anonymous (1999). EN 13401. Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of *Clostridium perfringens* – Colony-count technique (ISO 7937:1997 modified). European Committee for Standardization, Brussels, Belgium.

[10] In 't Veld, P.H., N.G.W.M. van Strijp-Lockefeer, A.H. Havelaar and E.A. Maier (1996). The certification of a reference material for the evaluation of the ISO method for the detection of *Salmonella*. J. Appl. Bacteriol. 80, 496-504.

[11] Anonymous (1996) FIL Bulletin 122C. Milk and Milk products – Preparation of test samples and dilutions for microbiological examination. Federation Internationale de Laiteriere / International Dairy Federation (FIL/IDF).

[12] Anonymous (1994) ISO 5725. Application of statistics – Accuracy (truness and precision) of measurement methods and results – part 2: basic method for the determination of repeatability and reproducibility of a standard measurement method. International Organisation for Standardization, Geneva, Switzerland.

[13] Anonymous (2000) pr EN ISO 16140 Microbiology of food and animal feeding stuffs – Protocol for the validation of alternative methods. European Committee for Standardization, Brussels, Belgium.

[14] Heisterkamp, S.H., J.A. Hoekstra, N.G.W.M. van Strijp-Lockefeer, A.H. Havelaar, K.A. Mooijman, P.H. in 't Veld, S.H.W. Notermans, E.A. Mayer, B. Griepink. 1993. Statistical analysis of certification trials for microbiological reference materials. Commision of the European Communities, Community Bureau of Reference (BCR), Brussels, Belgium. Report no. EUR 15008 EN.

[15] SAS/STAT 1989. User's guide. Version 6, 4th edition, volume 2. Cary, N.C.: SAS Institute Inc. 846 p.

[16] Rentenaar, I.M.F. 1996. MicroVal, a challenging Eureka project. Food Control, vol. 7 (1), p. 31-36

Appendix 1 Mailing list

1 Director-General Public Health, the Netherlands (VWS)
2 Mrs. I. de Froidment-Görtz, European Commission, EU DG XII SM&T, Brussels
3 President of the Council of Health, the Netherlands
4 Mrs. C. Lahellec, AFSSA Paris, coördinator EU-project SMT4-CT96-2098
5 Mrs. P. Rollier, CECALAIT, Poligny, France
6 Mr. R. Chevennement, ENILBIO, Poligny, France
7 Mrs. R. Leuschner, MAFF-CSL, York, UK
8 Mr. S. Langton, MAFF-CSL, York, UK
9 Mr. W. Andrews, AOAC, Arlington, USA
10 Mrs. C. Guissé, Secretariat ISO/TC34/SC9, AFNOR, Paris, France
11 Mr. P. in 't Veld, Inspectorate for Health Protection, Den Bosch, the Netherlands
12-27 Participants collaborative trial
28 Dutch National Library for Publications and Bibliography
29 Mr. H. Pont, Board of Directors RIVM
30 Mr. D. Kromhout, Director Sector Public Health Research
31 Mr. A.M. Henken, head Microbiological Laboratory for Health Protection, RIVM
32 Mr. F. van Leusden, Microbiological Laboratory for Health Protection, RIVM
33-36 Authors
37 SBD/Information and Public Relations
38 Registration agency for Scientific Reports
39 Library RIVM
40-49 Sales department of RIVM reports
50-60 Spare copies

Appendix 2 General information

Evaluation of the horizontal method for the enumeration of *Clostridium perfringens* - EU, SM&T project no. SMT4-CT96-2098

INTRODUCTION

This general information in combination with the standard operating procedure (SOP) and test report describes the procedure for the evaluation of the method for the enumeration of *Clostridium perfringens* as described in the International Organization for Standardization (ISO) method 7937 (1997) and in the (draft) European Standard prEN 13401 (1998). The application of the time schedule, SOP and test report is limited to the evaluation of the *C. perfringens* method carried out in the framework of a Standards, Measurement and Testing project (EU, SM&T project no SMT4-CT96-2098). The results are intended ultimately for publication by the Centre Europienne de Normalization (CEN) in the corresponding CEN methods.

OBJECTIVES

1) To determine the precision, in terms of repeatability (r) and reproducibility (R), of the ISO method in relation to the type of food and/or the concentration of *C. perfringens*.
2) To evaluate differences between the confirmation procedures as decsribed in ISO 7937 (1997) and in prEN 13401 (1998).

OUTLINE OF THE TRIAL

Within a prescribed period (see time schedule), the various types of food samples must be examined. Both reference materials must be examined concurrently with one of the food types. The samples will be enumerated after reconstitution and serial dilution onto egg-yolk-free tryptose-sulfite-cycloserine agar (SC). Typical colonies will be confirmed as *C. perfringens* using the confirmation procedure according to ISO 7937 (1997) and prEN 13401 (1998). For details see the Standard Operating Procedure (SOP). All data must be reported to the organising laboratory on the test report supplied. Fax the report and send the original test report by mail. Retain a copy to yourself!
The practical work may be divided among more than one technician. **However for reason of repeatability conditions different technicians may examine different food types, but it is NOT allowed to have more than one technician working on the same food type!**

TEST MATERIALS

Three types of (simulated) food samples (cheese samples, meat samples and dried animal feed samples) and a reference material (RM) will be examined. For each type of food 8 samples will be examined as well as two reference materials (26 samples in total).
Cheese samples are prepared by the CECALAIT (Poligny, France), meat samples by the Ministry of Agriculture, Fisheries and Foods, Central Science Laboratory (MAFF-CSL, York, United Kingdom), dried animal feed samples by the National Institute of Public Health and the Environment (RIVM, Bilthoven, The Netherlands) and the RMs by the Foundation for the Advancement of Public Health and Enviromental Protection (SVM, Bilthoven, The Netherlands).
The cheese samples are sent by CECALAIT by special delivery, and must be examined within two days after shipment (see time schedule). The other samples (meat, dried animal feed and RM) will be sent by

the RIVM by DHL delivery services. At receival check the parcels for completeness, check the temperature control devices and report this on the test report.

CHRONOLOGICAL DESCRIPTION OF THE TRIAL

Date	Action
16 December 1999	Mailing of SOP and test report to participants
17 January 2000	Meat samples, dried animal feed samples and references capsules dispatched by RIVM. Upon arrival note date, temperature, condition and record on test report. **Store meat samples at 1 °C – 5 °C until examination.** **Store dried animal feed samples and references capsules at –20 °C ± 5 °C until examination**
19 - 24 January 2000	Commence examination of meat samples *
27 - 31 January 2000	Commence examination of dried animal feed samples *
8 February 2000	Cheese samples dispatched by CECALAIT. Upon arrival note date, temperature, condition and record on test report. **Store cheese samples at 1°C – 5°C until examination**
10 February 2000	Commence examination of cheese samples *
21 February 2000	Closing date for receipt results by RIVM for analysis. Fax the report and send the original test report by mail. Retain a copy to yourself!
Oct/Nov 2000	Discussion of the results with contractors and participants.
* Note : the references capsules must be examined concurrently with any of the food types and is at the discretion of the participant.	

For any information concerning the study, contact:

Saskia Schulten (Telephone: +31 30 2742090, E-mail: Saskia.Schulten@rivm.nl)
Kirsten Mooijman (Telephone : +31 30 2743537, E-mail: Kirsten.Mooijman@svm.rivm.nl)
National Institute of Public Health and the Environment (RIVM)
Microbiological Laboratory for Health Protection
(pb 63)
P.O. Box 1
3720 BA Bilthoven
The Netherlands
Fax: +31 30 2744434

Appendix 3 Standard Operating Procedure (SOP)

Evaluation of the horizontal method for the enumeration *of Clostridium perfringens* – colony count technique.

1. SCOPE AND FIELD OF APPLICATION

This part of the standard operating procedure (SOP) describes the procedure for the evaluation of the method for the enumeration of *Clostridium perfringens* as described in the International Organization for Standardization (ISO) method ISO 7937:1997, second edition. For confirmation of suspected colonies, both Lactose sulfite (LS) medium (ISO 7937:1997) as well as a combination of motility-nitrate and lactose-gelatine medium (ISO 7937:1985) will be evaluated. This because in the new European Standard (prEN 13401:1998) a choice between both methods is allowed. Three types of (simulated) food samples and a reference material will be examined to determine the precision of the method.

2. NORMATIVE REFERENCES

- ISO 7937:1997. Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of *Clostridium perfringens* - Colony count technique.
- prEN 13401:1998 (final draft). Microbiology of food and animal feeding stuffs - Horizontal method for enumeration of *Clostridium perfringens* - Colony count technique (ISO 7937:1997 modified).
- ISO 6887:1983. Microbiology - General guidance for the preparation of dilutions for microbiological examination.
- ISO 7218:1996. Microbiology of food and animal feeding stuffs - General rules for microbiological examinations.

3. DEFINITIONS

For the purpose of this SOP the following definitions apply.

- *Clostridium perfringens*: bacteria that form typical characteristic colonies (surrounded by a black halo) in the specified selective medium and which give positive confirmatory reactions when the test is carried out by the method specified in this SOP.
- Enumeration of *Clostridium perfringens*: determination of the number of viable and confirmed *Clostridium perfringens* bacteria per millilitre or per gram of sample when the test is carried out by the method specified in this SOP.
- Reference material (RM): a gelatin capsule containing ca. 0.3 g of artificially contaminated spray-dried milk.

4. PRINCIPLE

Three types of (simulated) food samples (cheese, meat and dried animal feed) and RMs will be pre-treated according to a standardized procedure to obtain an initial suspension. Petri dishes will be inoculated with 1 ml of the initial suspensions and serial dilutions and mixed with egg-yolk-free tryptose-sulfite-cycloserine (SC) agar (pour-plate technique). After adding an overlay of the same medium, plates are incubated anaerobically for 20 ± 2 h at 37 ± 1 °C. Typical colonies will be submitted to the confirmation tests. For each type of food, 8 samples will be examined in addition to 2 RMs.

NOTE: For reason of repeatability conditions different technicians may examine different food types, but it is NOT allowed to have more than one technician working on the same food type!

5. DILUENTS, CULTURE MEDIA AND REAGENTS

It is recommended that commercially available dehydrated media are used whenever possible. For the preparation of these media, the manufacturer's instructions should be followed carefully. Note the manufacturer's name, code number and batch number of the media used on the test report. If the media are prepared from individual ingredients, note the ingredients and their concentration in an annex on the test report

5.1 Peptone-salt solution

Peptone	1.0 g
Sodium chloride (NaCl)	8.5 g
Water	1000 ml

Weigh sufficient ingredients to prepare ca 3 l. solution. Dissolve the components in the water, if necessary by heating, with frequent stirring. Adjust the pH, if necessary, so that after sterilization it is 7.0 ± 0.5. Transfer the solution to screw capped bottles (6.7). Sterilize by autoclaving at (121±3)°C for 15 minutes. Cool and store at (5 ± 3)°C for at max. 6 months. The peptone-salt solution is used to prepare the initial suspensions of the meat and animal feed samples (at least 16 portions of 90 ml are needed), for the reconstitution of the RMs (two times 10 ml is needed) and for the preparation of the serial dilutions. For the serial dilutions transfer the peptone-salt solution in quantities of 9 ml ± 0.2 ml to sterile tubes (6.7), at least 98 tubes are needed.

5.2 Sodium-citrate solution

Trisodium-citrate ($Na_3C_6H_5O_7.2H_2O$)	20.0 g
Water	1000 ml

Weigh sufficient trisodium-citrate to prepare ca 800 ml solution. Dissolve the component in the water by heating at 45-50 °C, with frequent stirring. Adjust the pH, if necessary, so that after sterilization it is 7.5 ± 0.2. Transfer the medium in 200 ml volumes to screw capped bottles (6.7). Sterilize by autoclaving at (121 ± 3)°C for 15 minutes. Cool and store at (5 ± 3)°C for at max.. 6 months. Use this solution to prepare the initial suspension of the cheese samples. At least 8 portions of 90 ml is needed.

5.3 Buffered Peptone water

Peptone	10.0 g
Sodium chloride (NaCl)	5.0 g
Di-sodium phosphate dodecahydrate ($Na_2HPO_4.12H_2O$)	9.0 g
Potassium di-hydrogen phosphate (KH_2PO_4)	1.5 g
Water	1000 ml

Weigh sufficient medium to prepare ca 100 ml solution. Dissolve the component in the water by heating at 45 - 50 °C, with frequent stirring. Adjust the pH, if necessary, so that after sterilization it

is 7.0 ± 0.2. Transfer the medium in 200 ml volumes to screw capped bottles (6.7). Sterilize by autoclaving at (121 ± 3)°C for 15 minutes. Cool and store at (5 ± 3)°C for at max. 6 months. Use this solution to rehydrate the meat samples. At least 8 portions of 10 ml is needed.

5.4 Egg-yolk-free tryptose-sulfite-cycloserine agar (SC)

5.4.1 Base medium

Component	Amount
Tryptose [a]	15.0 g
Soytone [a]	5.0 g
Yeast extract	5.0 g
Disodium disulfite ($Na_2S_2O_5$), anhydrous	1.0 g
Ammonium iron (III) citrate [b]	1.0 g
Agar	9.0 g to 18.0 g [c]
Water	1 000 ml

a) The names tryptose and soytone are used at present only by certain producers of media. Any other pancreatic casein or soybean digest giving comparable results may be used.
b)This reagent should contain at least 15 % (*m/m*) of iron.
c) Depending on the gel strengh of the agar.

Weigh out sufficient amounts of base medium to prepare at least 5 l. of complete medium (enough to prepare ca 200 plates, overlayer included). Dissolve the components in the water by boiling. Adjust the pH so that after sterilization it will be 7.6 ± 0.2 at 25 °C. Dispense the base into flasks or bottles of appropriate capacity. Sterilize for 15 min at (121 ± 3)°C. Store at (5 ± 3)°C for not more than 2 weeks.

N.B. For confirmation with the motility-nitrate medium and the lactose-gelatine medium, it may be necessary to prepare dishes of SC agar base medium. For this purpose, transfer portions of about 15 ml of the base, melted and cooled to approximately 47 °C (waterbath, 6.11) into Petri dishes and allow to solidify. Dry the plates immediately before use.

5.4.2 D-Cycloserine solution

Component	Amount
D-cycloserine [a]	4.0 g
Water	100 ml

a) Use white crystalline powder only

Dissolve the D-cycloserine in the water and sterilize the solution by filtration through a filter with 0.2 µm pore size. Store at (5 ± 3)°C. Discard unused solutions 4 weeks after preparation.

5.4.3 Complete medium

Immediately before use in the pour-plate method (see 7.2) add, to each 100 ml of sterile molten base (5.4.1) cooled to (47 ± 2)°C, 1 ml of D-cycloserine solution (5.4.2).

5.5 Fluid thioglycollate medium (FTG)

Enzymatic digest of casein	15.0 g
L-Cysteine	0.5 g
D-Glucose	5.5 g
Yeast extract	5.0 g
Sodium chloride	2.5 g
Sodium thioglycollate (mercaptoacetate)	0.5 g
Agar	0.5 g to 2.0 g a)
Resazurin	0.001 g
Water	1 000 ml
a) Depending on the gel strength of the agar.	

Weigh sufficient medium to prepare at least 4 l. broth (enough to prepare ca 400 tubes). Dissolve the components in the water by boiling. Adjust the pH so that after sterilization it is 7.1 ± 0.2 at 25 °C. Dispense 10 ml portions into tubes and sterilize at (121 ± 3)°C for 15 min. Before use, this medium shall be de-aerated.

5.6 Lactose sulfite (LS) medium (if necessary)

5.6.1 Base medium

Enzymatic digest of casein	5.0 g
Yeast extract	2.5 g
Sodium chloride	2.5 g
Lactose	10.0 g
L-cysteine hydrochloride	0.3 g
Water	1 000 ml

Prepare a sufficient amount of base medium to prepare at least 4 l. of complete medium (enough to prepare ca 400 tubes). Dissolve the components in the water by boiling (if necessary). Adjust the pH so that after sterilization it will be 7.1 ± 0.2 at 25 °C. Dispense 8 ml portions into test tubes or bottles with inverted Durham tubes (6.7) and sterilize at (121 ± 3)°C for 15 min. The medium may be stored at (5 ± 3)°C for at max. 4 weeks.

5.6.2 Disodium disulfite, anhydrous solution

Disodium disulfite ($Na_2S_2O_5$), anhydrous	1.2 g
Water	100 ml

Dissolve the disodium disulfite in the water and sterilize the solution by filtration through a filter with 0.2 µm pore size. Use the solution within one day.

5.6.3 Ammonium iron(III) citrate solution

Ammonium iron(III) citrate	1.0 g
Water	100 ml

Dissolve the ammonium iron(III) citrate in the water and sterilize the solution by filtration through a filter with 0.2 µm pore size. Use the solution within one day.

5.6.4 Complete medium

If the base medium is not used on the day of the preparation, then de-aerate the medium before adding the supplements (5.6.2 and 5.6.3) to the base medium. If the base medium is in screw-cap bottles, loosen the caps before heating and tighten them before cooling.
After cooling to 46 °C, add 0.5 ml of the disodium disulfite solution (5.6.2) and 0.5 ml of the ammonium iron(III) citrate solution (5.6.3) to each 8 ml of base (5.6.1)

5.7 Motility-nitrate medium (if necessary)

Petone	5.0 g
Beef extract	3.0 g
Galactose	5.0 g
Glycerol	5.0 g
Potassium nitrate (KNO_3)	1.0 g
Disodium hydrogenorthophosphate (Na_2HPO_4)	2.5 g
Agar	1.0 g to 5.0 g a)
Water	1 000 ml
a) Depending on the gel strength of the agar.	

Weigh sufficient amounts of each ingrediënt to prepare at least 4 l. of medium (enough to prepare ca 400 tubes). Dissolve the components in the water by boiling. Adjust the pH so that after sterilization it will be 7.3 ± 0.2 at 25 °C. Transfer the medium to culture tubes in 10 ml quantities and sterilize at (121 ± 3)°C for 15 min. If not used the same day, store at (5 ± 3)°C. Discard unused medium 4 weeks after preparation.Prior to use, heat in boiling water or flowing steam for 15 min. Cool rapidly to the incubation temperature (37 °C).

5.8 Nitrite-detection reagent (if necessary)

5.8.1 5-amino-2-naphthalenesulfonic acid (5-2 ANSA) solution

5-2 ANSA	0.1 g
15% (v/v) acetic acid solution	100 ml

Dissolve the 5-2 ANSA in the 15 % acetic acid solution. Filter through a filter paper. Store the filtrate in a well-stoppered brown bottle (preferably with a bulbtype dropper) at (5 ± 3)°C.

5.8.2 Sulfanilic acid solution

sulfanilic acid	0.4 g
15% (v/v) acetic acid solution	100 ml

Dissolve the sulfanilic acid in the 15 % acetic acid solution. Filter through a filter paper. Store the filtrate in a well-stoppered brown bottle (preferably with a bulbtype dropper) at (5 ± 3)°C.

5.8.3 Complete reagent
Mix equal volumes of the two solutions (5.8.1 and 5.8.2) just before use. Discard unused reagent immediately.

5.9 Zinc dust (if necessary)

5.10 Lactose-gelatine medium (if necessary)

Tryptose [a)]	15.0 g
Yeast extract	10.0 g
Lactose	10.0 g
Gelatine	120.0 g
Phenol red	0.05 g
Water	1 000 ml
a) The name tryptose is used at present only by certain producers of media. Any other pancreatic casein digest giving comparable results may be used.	

Weigh sufficient medium to prepare at least 4 l. of medium (enough to prepare ca 400 tubes). Dissolve the components, except the lactose and phenol red, in the water. Adjust the pH so that after sterilization it will be 7.5 at 25 °C. Add the lactose and phenol red, dispense 10 ml portions into test-tubes and sterilize at (121 ± 3)°C for 15 min. If not used the same day, store at (5 ± 3)°C. Just prior to use, heat in boiling water or flowing steam for 15 min. Cool rapidly to the incubation temperature (37 °C). Discard unused medium 3 weeks after preparation.

6. APPARATUS AND GLASSWARE
Usual microbiological laboratory equipment (see ISO 7218) and in particular:

6.1 **Apparatus for dry sterilization (oven)** and **wet sterilization (autoclave)**, see ISO 7218.
6.2 **Incubator**, capable of being maintained at (37 °C ± 1)°C.
6.3 **Anaerobic jars** or any other apparatus appropriate for anaerobic culture, and anaerobic systems (e.g. bags or gas)
6.4 **pH meter**, capable of being read to the nearest ± 0.01 pH unit at 25 °C, enabling measurements to be made which are accurate to 0.1 pH unit.
6.5 **Loops,** of platinum-iridium or nickel-chromium, of diameter approximately 3 mm, and **stab-inoculation needle** of the same material.
6.6 **Filtration apparatus,** for sterilization of solutions.
6.7 **Test tubes, bottles** or **flasks** of appropriate capacity, in particular 16 mm x 160 mm test tubes with inverted Durham tubes, for example of length 35 mm and of diameter 7 mm.
6.8 **Test tubes**, with caps (sterile), length 190, diameter 25 mm, for reconstitution of RMs.
(Two tubes will be included in the parcel containing the RMs)

6.9 **Total-delivery graduated pipettes**, of nominal capacities 1 ml and 10 ml, graduated in 0.1 ml and 0,5 ml respectively **or micropipettes with capacity of 1 ml and filter tips.**
6.10 **Petri dishes**, made of glass or plastic material, of diameter 90 mm to 100 mm.
6.11 **Water bath**, or similiar apparatus, capable of being maintained at (47 ± 2)°C.
6.12 **Water bath**, or similiar apparatus, capable of being maintained at (46 ± 0.5)°C.
6.13 **Water bath**, or similiar apparatus, capable of being maintained at (38.5 ± 0.5)°C.
6.14 **Rubber bulbs**, for use with the graduated pipettes for distributing the components of the nitrite detection reagent.

7. PROCEDURE

7.1 Pretreatment and preparation of initial suspension

7.1.1 Cheese samples (samples marked C1 - C8)

Label 8 sterile stomacher bags with the sample code (C1 - C8). Weigh out 10 g ± 0.2 g of each sample in the stomacher bag with corresponding sample code and add 90 ml ± 2 ml sodium citrate solution (5.2) at room temperature. Mix the contents for 3 minutes at normal speed using a stomacher. Repeat for the other samples. Leave the stomacher bags standing for 5 - 10 minutes so that the large particles can settle before preparing further dilutions as described in 7.2.
Note the time of the start of the preparation of the initial suspensions.

7.1.2 Meat samples (samples marked M1 - M8)

Carefully open a vial, add 10 ml Buffered Peptone water (5.3) at room temperature and close the lid. Shake the vial vigorously for a few seconds and allow to stand at room temperature for 30 minutes. This rehydrated meat sample is equivalent to 10 g of meat and should be regarded as the **meat sample**. Label 8 sterile stomacher bags with the sample code (M1 - M8). Tip the reconstituted meat into the stomacher bag with corresponding sample code. Take 90 ml ± 2 ml peptone-salt solution (5.1) at room temperature. Add ca 20 ml to the empty vial, replace the lid and invert several times to rinse thoroughly and add the rinse to the stomacher bag with corresponding sample code. Repeat the rinsing of the vial one more time. Pour the remaining of the peptone-salt solution into the stomacher bag. Mix the contents for 2 minutes at normal speed using the stomacher. Repeat for the other samples. *Note the time of the start of the preparation of the initial suspensions.*

7.1.3 Dried animal feed samples (samples marked D1 - D8)

Each vial contains exactly 10 g of dried animal feed. Label 8 sterile stomacher bags with the sample code (D1 - D8). Empty the vials into the stomacher bag with corresponding sample code. Take 90 ml ± 2 ml peptone-salt solution (5.1) at room temperature. Add ca 20 ml to the empty vial, replace the lid and invert several times to rinse thoroughly and add to the stomacher bag with corresponding sample code. Repeat the rinsing of the vial one more time. Pour the remaining of the peptone-salt solution into the stomacher bag. Mix the contents for 1 minute at normal speed using a stomacher. Repeat for the other samples. Leave the stomacher bags standing for 5 - 10 minutes so that the large particles can settle before preparing further dilutions as described in 7.2.
Note the time of the start of the preparation of the initial suspensions.

7.1.4 Reference materials (samples marked R1 - R2)

To the large test tubes (6.8) add 10 ml ± 0.2 ml of peptone-salt solution (5.1). Place the tubes in the waterbath at (38.5 ± 0.5)°C (6.13) for at least 30 min. Take the capsules out of the freezer 1 hour before reconstitution. Aseptically add one gelatin capsule to each tube. Replace the tubes in the waterbath. After 10 minutes place the first tube on the whirlmixer and mix well for 5-10 seconds (mixing speed about 1500 rpm, not faster than 2000 rpm). Replace the tube immediately in the waterbath *). Repeat for the other tube. After 20 and 30 minutes of reconstitution mix again as described above (each capsule therefore receives 3 mixings, after 10, 20 and 30 minutes of reconstitution, total duration of reconstitution is 30 minutes). Place the tubes after the last mixing immediately in melting ice. Use the reconstituted capsule solution within 2 hours.
Note the time of the start and end of the reconstitution procedure on the test report.

*): In order to ensure good dispersion of the gelatin capsule it is critically important that the tubes are kept out of the waterbath (during mixing) for the minimum length of time.

7.2 Serial dilution and inoculation of plates

7.2.1 Food samples (samples C1 - C8, M1 - M8 and D1 - D8)

The suspensions obtained from samples C1 - C8, M1 - M8 and D1 - D8 are the initial suspensions, corresponding to the 10^{-1} dilution. For preparing serial dilutions, 1 ml ± 0.02 ml of the initial suspension is transferred to a tube containing 9 ml ± 0.2 ml peptone-salt solution (5.1) and is mixed thoroughly on a whirlmixer for 5-10 seconds to obtain the 10^{-2} dilution. Repeat this procedure to obtain a dilution series up till 10^{-4} dilution for each sample. It is recommended to use a micropipette with filter tips and to rinse the 'pipettip' before use.
Label empty Petri dishes with sample identification number (for example C1), replicate (a and b) and dilution of the sample (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}). Transfer, by means of a sterile pipette, 1 ml ± 0.02 ml of each of the dilutions 10^{-1} till 10^{-4} to each of two petri dishes. It is recommended to use a micropipette with filtertip and to rinse the 'pipettip' before use. Pour 10 ml to 15 ml of the SC agar (5.4.3) maintained at (47 ± 2)°C in the water bath (6.11), into each dish and mix well with the inoculum by gently rotating each dish. Proceed in the same way for the other samples. When the medium has solidified, add an overlayer of 10 ml of the same SC agar. Allow to solidify.
Note the kind of pipette used on the test report. Note the time of the end of the inoculation procedure. The time between the preparation of the initial suspensions and adding the agar to the inoculum should not exceed 45 minutes.

7.2.2 Reference materials (samples R1-R2)

The reconstituted reference material is regarded as the 10^{-1} dilution. Prepare a 10^{-2} dilution by transferring 1 ml ± 0.02 ml of the initial suspension to a tube containing 9 ml ± 0.2 ml peptone-salt solution (5.1) and mix thoroughly on a whirlmixer for 5-10 seconds. Label empty Petri dishes with sample identification number (R1, R2), and replicate (a and b). Transfer, by means of a sterile pipette, 1 ml of the 10^{-2} dilution to each of two dishes. It is recommended to use a micropipette with filter tip and to rinse the 'pipettip' before use. Pour 10 ml to 15 ml of the SC agar (5.4.3) maintained at (47 ± 2)°C in the water bath (6.11), into each dish and mix well with the inoculum by gently rotating each dish. When the medium has solidified, add an overlayer of 10 ml of the same SC agar. Allow to solidify.
Note the kind of pipette used on the test report. Note the time between preparation of serial dilution and end of the inoculation procedure on the test report. This period should not exceed 45 minutes.

7.3 Incubation of the plates

Place the plates in anaerobic jars or other suitable containers (6.3) and incubate at (37 ± 1)°C (6.2) for 20 h ± 2 h anaerobically. Longer incubation may result in excess blackening of the plates.
Note the date/time and temperature at the start of the incubation period on the test report. If the incubator is connected to a continuous temperature reading system, it is preferred to send a print-out of the specific incubation period

7.4 Counting of the plates after incubation

Record on the test report the date/time and temperature at the end of the incubation period and whether all jars used were anaerobic during the full incubation period.
Count on each plate the black colonies of presumptive *C. perfringens. Note the number of typical colonies on tables 1 to 4 in the column marked "number of presumptive colonies".* Keep the plates containing less than 150 colonies at all dilutions and, if possible at two subsequent dilutions.

7.5 Selection of colonies for confirmation

Select 5 characteristic colonies from each plate retained for the enumeration. If less than 5 colonies are available on the plates counted, select all the characteristic colonies present. Confirm these colonies as described in 7.6. *Note the number of colonies to be confirmed on tables 1 to 4 in the column marked "number of colonies submitted to confirmation".*

.

7.6 Biochemical confirmation

Two confirmation methods are described in this SOP: using lactose sulfite medium (7.6.1) or using both motility-nitrate reduction medium and lactose-gelatine medium (7.6.2).
NOTE: If both procedures are carried out, use the same colony to inoculate all three media to be able to compare both methods.

7.6.1 Lactose sulfite (LS) medium

NOTE: The reaction obtained in lactose sulfite medium (5.6) when incubated at 46 °C is very specific for *C. perfringens* and some strains of *C. paraperfringens* and *C. absonum*. It is therefore not necessary to ensure that the black colonies picked from the agar are pure before inoculation into the thioglycollatebroth and subsequently into the lactose sulfite medium.

Inoculate each selected colony into fluid thioglycollate medium (5.5). Incubate under anaerobic conditions at 37 ± 1 °C for 18 h tot 24 h. After incubation, transfer with no delay 5 drops of the thioglycollate culture to the LS medium by means of a sterile pipette. Incubate at (46 ± 0.5)°C for 18 h to 24 h in the waterbath (6.12)
Tubes of LS medium are read for the production of gas and the presence of a black colour (iron sulfide precipitate). Durham tubes being more than one-quarter full of gas and tubes having a black precipitate are considered positive.
In case of doubt, when the Durham tube in a blackened medium is less than one-quarter full of gas, transfer with no delay, using a sterile pipette, 5 drops of the previous groth on LS medium to another tube of LS medium. Incubate in the water bath at (46 ± 0.5)°C for 18 h to 24 h. Read this tube as described above. In all other cases, the tubes will be considered as negative.
Record on Tables 1-4 on the test report, the results for each colony seperately (see example on page 9 of the test report).

7.6.2 Motility-nitrate reduction medium and lactose-gelatine medium

7.6.2.1 *General information*

This confirmation technique necessitates well isolated characteristic colonies. If it is not the case (the surface area of the plates is overgrown and it is not possible to select well-isolated characteristic colonies), inoculate 5 characteristic colonies into pre-deaerated fluid thioglycollate medium (5.5). Incubate under anaerobic conditions at (37 ± 1)°C for 18 h to 24 h. Streak the colonies on SC base agar plates (see 5.4.1) and add an overlayer of 10 ml of the SC base agar. Allow to solidify and incubate anaerobically at (37 ± 1)°C for 18 h to 24 h. Select from each plate at least one characteristic and well separated colony. If necessary, repeat the streaking and inoculation on SC base agar plates until well isolated characteristic black colonies are obtained. **Use the same colony to inoculate both the motility-nitrate and the lactose-gelatine medium.**

7.6.2.2 *Motility-nitrate medium*

Stab-inoculate each selected colony into pre-deaerated motility-nitrate medium (5.7). Incubate under anaerobic conditions at (37 ± 1)°C for 24 h ± 2 h.
Examine the tube of motility-nitrate medium for the type of growth along the stab line. Motility is evident from diffuse growth out into the medium away from the stab line.
Test for the presence of nitrite by adding with the rubber bulb (6.14) 0.2 ml to 0.5 ml of the nitrite-detection reagent (5.8) to each tube of motility-nitrate medium. *For health reasons this test should be carried out under a fume hood.* The formation of a red colour confirms the reduction of nitrate to nitrite. If no red colour is formed within 15 min., add a small amount of zinc dust (5.9) and allow to stand for 10 min. If a red colour is formed, after the addition of zinc dust, no reduction of nitrate has taken place. *Record on Tables 1-4 on the test report, the results for each colony seperately (see example on page 9 of the test report).*

7.6.2.3 *Lactose-gelatine medium*

Use the same colonies as used in 7.6.2.2 to inoculate the pre-deaerated lactose-gelatine medium tubes (5.10). Incubate under anaerobic conditions at (37 ± 1)°C for 24 h ± 2 h.
Examine the tubes of lactose-gelatine medium for the presence of gas and of a yellow colour (due to acid) indicating fermentation of lactose. Chill the tubes for 1 h at (5 ± 3)°C and check for gelatine liquefaction. If the medium has solidified, reincubate for an additional 24 h and again check for gelatine liquefaction. *Record on Tables 1-4 on the test report, the results for each colony seperately (see example on page 9 of the test report).*

7.7 Interpretation of confirmation test results

Record on Tables 1-4 on the test report, the number of colonies that has been confirmed as C. perfringens *for both methods separately in the columns marked "number of colonies confirmed as* C. perfringens*"*

Biochemical results for the identification of *Clostridium perfringens*	
lactose-sulfite medium	blackening, gas formation (Durham tubes at least one quarter full of gas)
motility-nitrate medium	non-motile, reduction of nitrate [a)]
lactose-gelatine medium	production of acid and gas from lactose (gas/yellow), gelatine liquefaction

[a)] Cultures that show a faint reaction for nitrite (i,e, a pink colour) should be eliminated, since *C. perfringens* consistently gives an intense and immediate reaction.

8 TEST REPORT

The test report should contain all information on operational details not mentioned or specified in this SOP that might influence the test result. Any incidents or deviations from the specifications should also be recorded. *Please record also the temperature in the laboratory room during the examinations.* The test report must state the names of the persons carrying out the work and must be signed by them and by the person in charge. If the examination is carried out by more than one technician, please write down (if possible) in which way the tasks were devided.
NOTE: For reason of repeatability conditions different technicians may examine different food types, but it is NOT allowed to have more than one technician working on the same food type!

Appendix 4 Test report

Evaluation of the horizontal method for the enumeration of *Clostridium perfringens* – colony count technique.

Laboratory code: ....

Laboratory name: ....

Shipment meat, animal feed and reference samples

Date of arrival parcel: .... - .... - 2000

Parcel damaged: ☐ yes ☐ no

Colour of compartments of temperature device at time of arrival:

Brief:	☐ white	☐ partly coloured (red)	☐ completely coloured (red)
Moderate:	☐ white	☐ partly coloured (red)	☐ completely coloured (red)
Prolonged:	☐ white	☐ partly coloured (red)	☐ completely coloured (red)

Shipment cheese samples

Date of arrival parcel: .... - .... - 2000

Parcel damaged: ☐ yes ☐ no

Colour of compartments of temperature device at time of arrival

Brief:	☐ white	☐ partly coloured (red)	☐ completely coloured (red)
Moderate:	☐ white	☐ partly coloured (red)	☐ completely coloured (red)
Prolonged:	☐ white	☐ partly coloured (red)	☐ completely coloured (red)

MEDIA USED

Peptone-salt solution

Medium prepared from:

☐ individual ingredients (please record ingredients and concentration used on annex)

☐ commercially available medium

Manufacturer: ....

Code number: ....

Batch number(s) : ....

Expiry date(s) : ....

pH of medium measured at …….. °C: ....……….

Sodium-citrate solution

Medium prepared from:

☐ individual ingredients (please record ingredients and concentration used on annex)

☐ commercially available medium

Manufacturer: ....

Code number: ....

Batch number(s) : ....

Expiry date(s) : ....

pH of medium measured at …….. °C: ....……….

Buffered Peptone water

Medium prepared from:

☐ individual ingredients (please record ingredients and concentration used on annex)

☐ commercially available medium

Manufacturer: ....

Code number: ....

Batch number(s) : ....

Expiry date(s) : ....

pH of medium measured at …….. °C: ....……….

SC-agar

<u>SC base prepared from:</u>

☐ individual ingredients (please record ingredients and concentration used on annex)

☐ commercially available medium:

Manufacturer: ....

Code number: ....

Batch number(s) : ....

Expiry date(s) : ....

pH of base measured at …….. °C: ....……….

<u>D-cycloserine:</u>

Manufacturer: ....

Code number: ....

Batch number(s) : ....

Expiry date(s) : ....

Fluid thioglycollate medium (FTG)

Medium prepared from:

☐ individual ingredients (please record ingredients and concentration used on annex)

☐ commercially available medium

Manufacturer: ....

Code number: ....

Batch number(s) : ....

Expiry date(s) : ....

pH of medium measured at …….. °C: ....……….

Lactose-sulfite medium

<u>Medium prepared from:</u>

☐ individual ingredients (please record ingredients and concentration used on annex)

☐ commercially available medium:

Manufacturer: ....

Code number: ....

Batch number(s) : ....

Expiry date(s) : ....

pH of base measured at …….. °C: ....……….

Motility-nitrate medium

Medium prepared from:

☐ individual ingredients (please record ingredients and concentration used on annex)

☐ commercially available medium

Manufacturer: ....

Code number: ....
Batch number(s) : ....
Expiry date(s) : ....
pH of medium measured at …….. °C: ....……….

Nitrite-detection reagent

Reagent prepared from:

☐ individual ingredients (please record ingredients and concentration used on annex)

☐ commercially available solutions:

5-2 ANSA solution:
Manufacturer: ....
Code number: ....
Batch number(s) : ....
Expiry date(s) : ....

Sulfanilic acid solution:
Manufacturer: ....
Code number: ....
Batch number(s) : ....
Expiry date(s) : ....

Lactose-gelatine medium

Medium prepared from:

☐ individual ingredients (please record ingredients and concentration used on annex)

☐ commercially available medium

Manufacturer: ....
Code number: ....
Batch number(s) : ....
Expiry date(s) : ....
pH of medium measured at …….. °C: ....……….

LAB TEMPERATURE DURING TRIAL

Date	Temperature	Remarks

EXAMINATION OF SAMPLES

Cheese samples:

Examination of cheese samples started at: ...... - .... - 2000

Time between start of preparation of initial suspensions and pouring the agar in the dishes:
Was this for each sample less than 45 minutes? ☐ yes ☐ no, ...
....

Type of pipette used:
☐ micro pipette Manufacturer: ....
Accuracy: .... Volume range: ....
tips used: ☐ with filter ☐ without filter
pipette used for: ☐ preparing serial dilutions ☐ inoculation of plates
☐ other pipette, type: ☐ graduated ☐ other, namely: ....
Accuracy: .... Volume range: ....
Disposable: ☐ yes ☐ no Made of: ☐ glass ☐ plastic
pipette used for: ☐ preparing serial dilutions ☐ inoculation of plates

INCUBATION CONDITIONS

CHEESE	Date	Time	Temp. incubator	Anaerobic conditions?
SC plates (37 °C)				
start of incubation			°C	
end of incubation			°C	YES / NO
FTG tubes (37 °C)				
start of incubation			°C	
end of incubation			°C	YES / NO
Lactose-sulfite tubes (46 °C)				
start of incubation			°C	
end of incubation			°C	YES / NO
Motility-nitrate tubes (37 °C)				
start of incubation			°C	
end of incubation			°C	YES / NO
Lactose-gelatine tubes (37 °C)				
start of incubation			°C	
end of incubation			°C	YES / NO

Meat samples:

Examination of meat samples started at: ...... - .... - 2000

Time between start preparation of initial suspensions and pouring the agar in the dishes:
Was this for each sample less than 45 minutes? ☐ yes ☐ no, ...
....

Type of pipette used:
same as for .... samples: ☐ yes ☐ no (fill in questions below)
☐ micro pipette Manufacturer: ....
Accuracy: .... Volume range: ....
tips used: ☐ with filter ☐ without filter
pipette used for: ☐ preparing serial dilutions ☐ inoculation of plates
☐ other pipette, type: ☐ graduated ☐ other, namely: ....
Accuracy: .... Volume range: ....
Disposable: ☐ yes ☐ no Made of: ☐ glass ☐ plastic
pipette used for: ☐ preparing serial dilutions ☐ inoculation of plates

MEAT	Date	Time	Temp. incubator	Anaerobic conditions?
SC plates (37 °C)				
start of incubation			°C	
end of incubation			°C	YES / NO
FTG tubes (37 °C)				
start of incubation			°C	
end of incubation			°C	YES / NO
Lactose-sulfite tubes (46 °C)				
start of incubation			°C	
end of incubation			°C	YES / NO
Motility-nitrate tubes (37 °C)				
start of incubation			°C	
end of incubation			°C	YES / NO
Lactose-gelatine tubes (37 °C)				
start of incubation			°C	
end of incubation			°C	YES / NO

Dried animal feed samples (D1-D8)

Examination of the dried animal feed samples started at: ...... - .... - 2000

Time between start preparation of initial suspensions and pouring the agar in the dishes:
Was this for each sample less than 45 minutes? ☐ yes ☐ no, ...
....

Type of pipette used:
same as for .... samples: ☐ yes ☐ no (fill in questions below)
☐ micro pipette Manufacturer: ....
Accuracy: .... Volume range: ....
tips used: ☐ with filter ☐ without filter
pipette used for: ☐ preparing serial dilutions ☐ inoculation of plates
☐ other pipette, type: ☐ graduated ☐ other, namely: ....
Accuracy: .... Volume range: ....
Disposable: ☐ yes ☐ no Made of: ☐ glass ☐ plastic
pipette used for: ☐ preparing serial dilutions ☐ inoculation of plates

DRIED ANIMAL FEED	Date	Time	Temp. incubator	Anaerobic conditions?
SC plates (37 °C)				
start of incubation			°C	
end of incubation			°C	YES / NO
FTG tubes (37 °C)				
start of incubation			°C	
end of incubation			°C	YES / NO
Lactose-sulfite tubes (46 °C)				
start of incubation			°C	
end of incubation			°C	YES / NO
Motility-nitrate tubes (37 °C)				
start of incubation			°C	
end of incubation			°C	YES / NO
Lactose-gelatine tubes (37 °C)				
start of incubation			°C	
end of incubation			°C	YES / NO

Reference materials (R1-R2)

Examination of reference materials started at: ...... - .... - 2000

Reconstitution of reference materials:

- Were the capsules taken out of the freezer 1 hour before reconstitution? ☐ yes ☐ no
- Reconstitution period: start: ……….. end: .... - Mixing speed: .... rpm
- Temp. of waterbath during reconstitution: at start: ....°C end : ....°C
- Were all gelatin capsules dissolved after reconstitution? ☐ yes ☐ no, ...……………..
- Were the tubes placed in melting ice immediately after the last mixing? ☐ yes ☐ no
- Time of start preparation of serial dilution: ……… - End (adding agar to inoculum): …………

Type of pipette used:

same as for .... samples: ☐ yes ☐ no (fill in questions below)

☐ micro pipette Manufacturer: ....

Accuracy: .... Volume range: ....

tips used: ☐ with filter ☐ without filter

pipette used for: ☐ preparing serial dilutions ☐ inoculation of plates

☐ other pipette, type: ☐ graduated ☐ other, namely: ....

Accuracy: .... Volume range: ....

Disposable: ☐ yes ☐ no Made of: ☐ glass ☐ plastic

pipette used for: ☐ preparing serial dilutions ☐ inoculation of plates

RM	Date	Time	Temp. incubator	Anaer. conditions?
SC plates (37 °C)				
start of incubation			°C	
end of incubation			°C	YES / NO
FTG tubes (37 °C)				
start of incubation			°C	
end of incubation			°C	YES / NO
Lactose-sulfite tubes (46 °C)				
start of incubation			°C	
end of incubation			°C	YES / NO
Motility-nitrate tubes (37 °C)				
start of incubation			°C	
end of incubation			°C	YES / NO
Lactose-gelatine tubes (37 °C)				
start of incubation			°C	
end of incubation			°C	YES / NO

Tables for recording the test results (example)

Sample X												
dilution	replicate	number of presumptive colonies	number of colonies submitted to confirmation	lactose-sulfite medium		number of colonies confirmed as *C. perfringens*	motility-nitrate medium		lactose-gelatine medium		number of colonies confirmed as *C. perfringens*
				gas formation	blackening		motility	nitrate reduction	lactose fermentation (gas/yellow)	gelatine liquefaction	
10^{-1}	a	50	5	+ + - + +	+ + + + +	4	- - - - -	+ + - + +	+ + + + +	+ + - + +	4
	b	48	5	+ + + + +	+ + + + +	5	- - - - -	+ + + + +	+ + + + +	+ + + + +	5
10^{-2}	a	6	5	+ - + + +	+ + + + +	4	- - - - -	+ + + + +	+ - + + +	+ + + + +	4
	b	4	4	+ + - +	+ + + +	3	- - - -	+ + + +	+ + + +	+ + + +	4
10^{-3}	a	0	0								
	b	0	0								
10^{-4}	a	0	0								
	b	0	0								

Remark(s) on operational details that might have influenced the test results:

Name(s) of technican(s) who carried out the trial:.....…..

Signature(s): ....………..…....

Name of person in charge: ....……………………………………….

Signature: ....…………….…. Date: .... - .... - 2000

Appendix 5 Results collaborative trial

CHEESE - USING LACTOSE-SULFITE CONFIRMATION

labcode	repl.	Blank		Low		Medium		High	
		N	Log N	N	Log N	N	Log N	N	Log N
1	a	<10		223	2.35	2636	3.42	23636	4.37
1	b	<10		277	2.44	3727	3.57	17727	4.25
2	a	<10		355	2.55	4182	3.62	35909	4.56
2	b	<10		473	2.67	3955	3.60	33636	4.53
3	a	<10		159	2.20	1864	3.27	18636	4.27
3	b	<10		236	2.37	2591	3.41	23636	4.37
4	a	<10		373	2.57	3136	3.50	32273	4.51
4	b	<10		386	2.59	3409	3.53	26818	4.43
5	a	<10		245	2.39	3409	3.53	30909	4.49
5	b	<10		305	2.48	4182	3.62	29091	4.46
6	a	<10		373	2.57	3455	3.54	17273	4.24
6	b	<10		364	2.56	4000	3.60	27273	4.44
7 *	a	< 10		305	2.48	3818	3.58	34546	4.50
7 *	b	< 10		373	2.57	3545	3.55	31364	4.43
8 *	a	<10		327	2.51	3409	3.53	25455	4.41
8 *	b	<10		282	2.45	3409	3.53	31818	4.50
9	a	<10		377	2.58	3455	3.54	41364	4.62
9	b	<10		327	2.51	4091	3.61	32727	4.51
10	a	<10		355	2.55	3591	3.56	40455	4.61
10	b	<10		282	2.45	3182	3.50	33182	4.52
11	a	<10		341	2.53	3136	3.50	37273	4.57
11	b	<10		400	2.60	3318	3.52	33182	4.52
12	a								
12	b								
13	a								
13	b								
14	a	<10		359	2.56	3455	3.54	25455	4.41
14	b	<10		141	2.15	3318	3.52	48636	4.69
15	a	<10		345	2.54	3864	3.59	35909	4.56
15	b	<10		323	2.51	2909	3.46	34091	4.53
17	a	<10		286	2.46	3682	3.57	30000	4.48
17	b	<10		200	2.30	3545	3.55	35909	4.56
18	a	<10		482	2.68	3955	3.60	35000	4.54
18	b	<10		309	2.49	4682	3.67	34091	4.53

N = number of *C. perfringens* per gram sample (cfp/g).
* = results excluded from analysis (see appendix 7)

CHEESE - USING MN&LG[1)] CONFIRMATION

labcode	repl.	Blank		Low		Medium		High	
		N	Log N	N	Log N	N	Log N	N	Log N
1	a	<10		223	2.35	2682	3.43	23636	4.37
1	b	<10		277	2.44	3727	3.57	21818	4.34
2	a								
2	b								
3	a	<10		123	2.09	1591	3.20	10000	4.00
3	b	<10		236	2.37	2591	3.41	23636	4.37
4	a	<10		373	2.57	3136	3.50	32273	4.51
4	b	<10		386	2.59	3409	3.53	26818	4.43
5	a	<10		245	2.39	3409	3.53	30909	4.49
5	b	<10		305	2.48	4182	3.62	29091	4.46
6	a	<10		373	2.57	3455	3.54	17273	4.24
6	b	<10		364	2.56	4000	3.60	27273	4.44
7	a	<10		295	2.47	1364	3.13	35000	4.54
7	b	<10		259	2.41	1364	3.13	26818	4.43
8 *	a	<10		327	2.51	3455	3.54	25455	4.41
8 *	b	<10		282	2.45	3409	3.53	31818	4.50
9	a	<10							
9	b	<10							
10	a	<10		391	2.59	3955	3.60	40455	4.61
10	b	<10		309	2.49	3545	3.55	35455	4.55
11	a								
11	b								
12	a	<10		405	2.61	5273	3.72	45909	4.66
12	b	<10		309	2.49	4409	3.64	28182	4.45
13	a	<10		264	2.42	2773	3.44	24091	4.38
13	b	<10		359	2.56	3727	3.57	25909	4.41
14	a	<10		450	2.65	4409	3.64	35909	4.56
14	b	<10		200	2.30	4182	3.62	48636	4.69
15	a	<10		345	2.54	3864	3.59	35909	4.56
15	b	<10		323	2.51	2909	3.46	34091	4.53
17	a	<10		286	2.46	3682	3.57	30000	4.48
17	b	<10		200	2.30	3455	3.54	35909	4.56
18	a	<10		459	2.66	4000	3.60	35000	4.54
18	b	<10		282	2.45	4682	3.67	30455	4.48

N = number of *C. perfringens* per gram sample (cfp/g).
1) MN&LG = motility-nitrate & lactose-gelatine medium
* = results excluded from analysis (see appendix 7)

MEAT - USING LACTOSE-SULFITE CONFIRMATION

labcode	repl.	Blank		Low		Medium		High	
		N	Log N	N	Log N	N	Log N	N	Log N
1	a	<10		682	2.83	3182	3.50	26818	4.43
1	b	<10		741	2.87	3773	3.58	33182	4.52
2	a	<10		573	2.76	6636	3.82	24545	4.39
2	b	<10		477	2.68	8182	3.91	33636	4.53
3	a	<10		450	2.65	5455	3.74	60000	4.78
3	b	<10		582	2.76	5636	3.75	45455	4.66
4	a	<10		418	2.62	2682	3.43	37273	4.57
4	b	<10		509	2.71	3136	3.50	32273	4.51
5	a	<10		518	2.71	3682	3.57	44545	4.65
5	b	<10		536	2.73	3500	3.54	51818	4.71
6	a	<10		623	2.79	3955	3.60	31818	4.50
6	b	<10		459	2.66	4182	3.62	25909	4.41
7 *	a	<10		<10		<100		<1000	
7 *	b	<10		<10		<100		<1000	
8 *	a	<10		995	3.00	7545	3.88	53182	4.73
8 *	b	<10		741	2.87	9591	3.98	67727	4.83
9	a	<10		668	2.82	5318	3.73	63182	4.80
9	b	<10		650	2.81	4091	3.61	45000	4.65
10	a	<10		768	2.89	6636	3.82	36818	4.57
10	b	<10		1032	3.01	7000	3.85	70909	4.85
11	a	<10		586	2.77	1955	3.29	17273	4.24
11	b	<10		414	2.62	2682	3.43	30909	4.49
12	a								
12	b								
13	a								
13	b								
14	a	<10		441	2.64	2500	3.40	26818	4.43
14	b	<10		445	2.65	3682	3.57	32273	4.51
15	a	<10		645	2.81	5455	3.74	41364	4.62
15	b	<10		559	2.75	4455	3.65	45455	4.66
17	a	<10		823	2.92	5545	3.74	41818	4.62
17	b	<10		900	2.95	5864	3.77	52273	4.72
18	a	<10		327	2.51	2091	3.32	20455	4.31
18	b	<10		245	2.39	1773	3.25	12273	4.09

N = number of *C. perfringens* per gram sample (cfp/g).
* = results excluded from analysis (see appendix 7)

MEAT - USING MN&LG[1)] CONFIRMATION

labcode	repl.	Blank		Low		Medium		High	
		N	Log N	N	Log N	N	Log N	N	Log N
1	a	<10		391	2.59	3182	3.50	26818	4.43
1	b	<10		614	2.79	1955	3.29	33182	4.52
2	a								
2	b								
3	a	<10		450	2.65	5455	3.74	60000	4.78
3	b	<10		582	2.76	5636	3.75	45000	4.65
4	a	<10		418	2.62	2682	3.43	37273	4.57
4	b	<10		509	2.71	3136	3.50	32273	4.51
5	a	<10		518	2.71	3682	3.57	44545	4.65
5	b	<10		536	2.73	3500	3.54	51818	4.71
6	a	<10		623	2.79	3955	3.60	31818	4.50
6	b	<10		459	2.66	4182	3.62	25909	4.41
7	a	<10		277	2.44	3091	3.49	15455	4.19
7	b	<10		459	2.66	1955	3.29	10000	4.00
8 *	a	<10		1100	3.04	7455	3.87	53182	4.73
8 *	b	<10		936	2.97	8091	3.91	60455	4.78
9	a								
9	b								
10	a	<10		768	2.89	6636	3.82	37273	4.57
10	b	<10		1032	3.01	7000	3.85	70909	4.85
11	a								
11	b								
12	a	<10		455	2.66	4682	3.67	35000	4.54
12	b	<10		177	2.25	6364	3.80	55000	4.74
13	a	<10		564	2.75	3273	3.51	41818	4.62
13	b	5	0.70	777	2.89	6455	3.81	42273	4.63
14	a	<10		441	2.64	2500	3.40	26818	4.43
14	b	<10		445	2.65	3682	3.57	32273	4.51
15	a	<10		645	2.81	5455	3.74	41364	4.62
15	b	<10		559	2.75	4455	3.65	45455	4.66
17	a	<10		823	2.92	5545	3.74	41818	4.62
17	b	<10		900	2.95	5864	3.77	52273	4.72
18	a	<10		245	2.39	2273	3.36	14545	4.16
18	b	<10		300	2.48	1773	3.25	12273	4.09

N = number of *C. perfringens* per gram sample (cfp/g).
1) MN&LG = motility-nitrate & lactose-gelatine medium
* = results excluded from analysis (see appendix 7)

DRIED ANIMAL FEED - USING LACTOSE-SULFITE CONFIRMATION

labcode	repl.	Blank		Low		Medium		High	
		N	Log N	N	Log N	N	Log N	N	Log N
1	a	<10		868	2.94	16045	4.21	107273	5.03
1	b	5	0.70	1082	3.03	15000	4.18	119091	5.08
2	a	<10		495	2.69	9000	3.95	91364	4.96
2	b	10	1.00	736	2.87	9500	3.98	64091	4.81
3	a	5	0.70	2000	3.30	19000	4.28	126818	5.10
3	b	<10		1600	3.20	15682	4.20	124091	5.09
4 *	a	5	0.70	15	1.18	5818	3.76	62727	4.80
4 *	b	495	2.69	405	2.61	6364	3.80	55000	4.74
5	a	<10		195	2.29	3318	3.52	57273	4.76
5	b	<10		195	2.29	5727	3.76	60000	4.78
6	a	<10		473	2.67	6773	3.83	48182	4.68
6	b	<10		527	2.72	6409	3.81	46364	4.67
7 *	a	<10		259	2.41	3182	3.50	<1000	
7 *	b	<10		305	2.48	<100		1818	3.26
8 *	a	15	1.18	955	2.98	12273	4.09	110000	5.04
8 *	b	25	1.40	1273	3.10	15318	4.19	104545	5.02
9	a	<10		114	2.06	1500	3.18	35455	4.55
9	b	<10		155	2.19	3000	3.48	40909	4.61
10	a	5	0.70	395	2.60	7818	3.89	89091	4.95
10	b	10	1.00	427	2.63	7500	3.88	73182	4.86
11	a	10	1.00	405	2.61	5045	3.70	50909	4.71
11	b	<10		314	2.50	5045	3.70	45455	4.66
12	a								
12	b								
13	a								
13	b								
14	a	<10		305	2.48	6364	3.80	44545	4.65
14	b	5	0.70	259	2.41	5636	3.75	71818	4.86
15	a	<10		159	2.20	4045	3.61	58182	4.76
15	b	<10		159	2.20	4182	3.62	66818	4.82
17	a	20	1.40	641	2.81	11091	4.04	127727	5.11
17	b	10	1.00	455	2.66	10000	4.00	119091	5.08
18	a	<10		464	2.67	8091	3.91	78636	4.90
18	b	<10		323	2.51	6864	3.84	80455	4.91

N = number of *C. perfringens* per gram sample (cfp/g).
* = results excluded from analysis (see appendix 7)

DRIED ANIMAL FEED - USING MN&LG[1)] CONFIRMATION

labcode	repl.	Blank		Low		Medium		High	
		N	Log N	N	Log N	N	Log N	N	Log N
1	a	<10		877	2.94	16045	4.21	106364	5.03
1	b	5	0.70	982	2.99	15000	4.18	119091	5.08
2	a								
2	b								
3	a	5	0.70	1800	3.26	19000	4.28	126818	5.10
3	b	<10		1600	3.20	14273	4.15	124091	5.09
4 *	a	5	0.70	15	1.18	5818	3.76	62727	4.80
4 *	b	500	2.70	405	2.61	6364	3.80	55000	4.74
5	a	5	0.70	195	2.29	3318	3.52	57273	4.76
5	b	<10		173	2.24	5727	3.76	60000	4.78
6	a	<10		473	2.67	6773	3.83	47727	4.68
6	b	<10		527	2.72	6409	3.81	45909	4.66
7	a	<10		759	2.88	7818	3.89	49091	4.69
7	b	<10		305	2.48	10455	4.02	54091	4.73
8 *	a	10	1.00	386	2.59	11091	4.04	79545	4.90
8 *	b	25	1.40	768	2.89	11000	4.04	75909	4.88
9	a								
9	b								
10	a	5	0.70	395	2.60	7818	3.89	89091	4.95
10	b	10	1.00	427	2.63	7500	3.88	73182	4.86
11	a								
11	b								
12	a	25	1.40	1023	3.01	15636	4.19	132273	5.12
12	b	<10		1095	3.04	25500	4.41	154091	5.19
13	a	20	1.30	227	2.36	3727	3.57	48636	4.69
13	b	<10		159	2.20	3500	3.54	49545	4.69
14	a	<10		305	2.48	6409	3.81	60000	4.78
14	b	5	0.70	273	2.44	5636	3.75	75909	4.88
15	a	<10		159	2.20	4091	3.61	58182	4.76
15	b	<10		159	2.20	4227	3.63	66818	4.82
17	a	20	1.30	641	2.81	11091	4.04	127727	5.11
17	b	10	1.00	455	2.66	10000	4.00	119091	5.08
18	a	<10		464	2.67	8091	3.91	78636	4.90
18	b	<10		323	2.51	6864	3.84	80455	4.91

N = number of *C. perfringens* per gram sample (cfp/g).
1) MN&LG = motility-nitrate & lactose-gelatine medium
* = results excluded from analysis (see appendix 7)

RMs

labcode	repl.	LS		MN&LG	
		N	Log N	N	Log N
1	a	5400	3.73	5400	3.73
1	b	6100	3.79	6100	3.79
2	a	5550	3.74		
2	b	4950	3.69		
3	a	6200	3.79	6200	3.79
3	b	7400	3.87	5900	3.77
4	a	3150	3.50	3950	3.60
4	b	3800	3.58	3800	3.58
5	a	3850	3.59	3850	3.59
5	b	4650	3.67	4650	3.67
6	a	4600	3.66	4600	3.66
6	b	3600	3.56	3600	3.56
7 *	a	<100		5850	3.77
7 *	b	850	2.93	8000	3.90
8 *	a	5100	3.71	5100	3.71
8 *	b	4200	3.62	4200	3.62
9	a	5600	3.75		
9	b	5350	3.73		
10	a	4950	3.69	4950	3.69
10	b	4900	3.69	4900	3.69
11	a	5250	3.72		
11	b	6400	3.81		
12	a			7000	3.85
12	b			6900	3.84
13	a			5300	3.72
13	b			4900	3.69
14	a	3700	3.57	4600	3.66
14	b	4050	3.61	5050	3.70
15	a	4850	3.69	4850	3.69
15	b	5700	3.76	5700	3.76
17	a	6350	3.80	6350	3.80
17	b	6550	3.82	6550	3.82
18	a	4550	3.66	4550	3.66
18	b	5300	3.72	5300	3.72

N = number of *C. perfringens* per gram sample (cfp/g).
LS = lactose-sulfite confirmation
MN&LG = motility-nitrate & lactose-gelatine confirmation
* = results excluded from analysis (see appendix 7)

Appendix 6 Duncan's test results

General Linear Models Procedure
Duncan's Multiple Range Test for variable: LOGCFU
NOTE: This test controls the type I comparisonwise error rate, not the experimentwise error rate

Duncan Grouping: means with the same letter are not significantly different.

Mean:	mean log_{10} values of the blind duplicate samples
N:	number of blind duplicate samples within each laboratory (N=2)
LAB:	laboratory code
*	outlying test result (not excluded from analysis)
Max. difference = 0.29 (0.14)	difference in log_{10} cfp/g between the highest and lowest mean value. The value in brackets is the maximum value without the outlier(s) marked with *.

REFERENCE MATERIALS - LS CONFIRMATION

MEAN LOGCFU = 3.6991 Max. difference = 0.29

Duncan Grouping				Mean	N	LAB
		A		3.83088	2	3
B		A		3.80957	2	17
B		A	C	3.76324	2	11
B		A	C	3.75894	2	1
B		A	C	3.73835	2	9
B	D		C	3.72089	2	15
B	D		C	3.71953	2	2
	D	E	C	3.69249	2	10
	D	E	C	3.69123	2	18
F	D	E		3.62656	2	5
F		E		3.60964	2	6
F		E		3.58794	2	14
F				3.53917	2	4

REFERENCE MATERIALS - MN&LG CONFIRMATION

MEAN LOGCFU = 3.7190 Max. difference = 0.25

Duncan Grouping				Mean	N	LAB
		A		3.84204	2	12
		A		3.83519	2	7
B		A		3.80957	2	17
B		A	C	3.78169	2	3
B		A	C	3.75894	2	1
B	D		C	3.72089	2	15
B	D	E	C	3.70732	2	13
F	D	E	C	3.69249	2	10
F	D	E	C	3.69123	2	18
F	D	E	C	3.68311	2	14
F	D	E		3.62656	2	5
F		E		3.60964	2	6
F				3.58830	2	4

CHEESE SAMPLES – LS CONFIRMATION

LEVEL = LOW MEAN LOGCFU = 2.4885 Max. difference = 0.32

Duncan Grouping		Mean	N	LAB
	A	2.6132	2	2
	A	2.5876	2	18
	A	2.5803	2	4
	A	2.5685	2	11
	A	2.5672	2	6
B	A	2.5470	2	9
B	A	2.5249	2	15
B	A	2.5012	2	10
B	A	2.4384	2	5
B	A	2.3971	2	1
B	A	2.3808	2	17
B	A	2.3542	2	14
B		2.2899	2	3

LEVEL = MEDIUM MEAN LOGCFU = 3.5362 Max. difference = 0.29 (0.14)

Duncan Grouping		Mean	N	LAB	
	A	3.63386	2	18	
	A	3.60934	2	2	
	A	3.57712	2	5	
	A	3.57522	2	9	
	A	3.57034	2	6	
	A	3.55799	2	17	
	A	3.52977	2	14	
	A	3.52907	2	10	
	A	3.52551	2	15	
	A	3.51467	2	4	
	A	3.50880	2	11	
	A	3.49634	2	1	
B		3.34211	2	3	*

LEVEL = HIGH MEAN LOGCFU = 4.4828 Max. difference = 0.25

Duncan Grouping			Mean	N	LAB
	A		4.56578	2	9
	A		4.56395	2	10
	A		4.54638	2	14
	A		4.54616	2	11
	A		4.54393	2	15
	A		4.54102	2	2
	A		4.53837	2	18
B	A		4.51618	2	17
B	A	C	4.47694	2	5
B	A	C	4.46865	2	4
B		C	4.33657	2	6
B		C	4.32199	2	3
		C	4.31113	2	1

CHEESE SAMPLES – MN&LG CONFIRMATION

LEVEL = LOW MEAN LOGCFU = 2.4757 Max. difference = 0.35

Duncan Grouping		Mean	N	LAB
	A	2.5803	2	4
	A	2.5672	2	6
	A	2.5572	2	18
	A	2.5498	2	12
	A	2.5423	2	10
	A	2.5249	2	15
B	A	2.4895	2	13
B	A	2.4787	2	14
B	A	2.4435	2	7
B	A	2.4384	2	5
B	A	2.3971	2	1
B	A	2.3808	2	17
B		2.2339	2	3

LEVEL = MEDIUM MEAN LOGCFU = 3.5166 Max. difference = 0.55 (0.18)

Duncan Grouping		Mean	N	LAB	
	A	3.68328	2	12	
B	A	3.63634	2	18	
B	A	3.63296	2	14	
B	A	3.57712	2	5	
B	A	3.57350	2	10	
B	A	3.57034	2	6	
B	A	3.55235	2	17	
B	A	3.52551	2	15	
B	A	3.51467	2	4	
B		3.50729	2	13	
B		3.50005	2	1	
	C	3.30777	2	3	*
	D	3.13502	2	7	*

LEVEL = HIGH MEAN LOGCFU = 4.4645 Max. difference = 0.43

Duncan Grouping			Mean	N	LAB
	A		4.6211	2	14
B	A		4.5783	2	10
B	A		4.5559	2	12
B	A		4.5439	2	15
B	A		4.5162	2	17
B	A		4.5139	2	18
B	A		4.4863	2	7
B	A		4.4769	2	5
B	A		4.4686	2	4
B	A	C	4.3977	2	13
B		C	4.3562	2	1
B		C	4.3366	2	6
		C	4.1868	2	3

MEAT SAMPLES – LS CONFIRMATION

LEVEL = LOW MEAN LOGCFU = 2.7444 Max. difference = 0.50 (0.30)

Duncan Grouping			Mean	N	LAB	
	A		2.95003	2	10	
	A		2.93525	2	17	
B	A		2.85233	2	1	
B	A	C	2.81956	2	9	
B	D	C	2.77940	2	15	
B	D	C	2.72892	2	6	
B	D	C	2.72279	2	5	
B	D	C	2.71919	2	2	
B	D	C	2.70985	2	3	
	D	C	2.69329	2	11	
	D	C	2.66503	2	4	
	D		2.64756	2	14	
	E		2.45399	2	18	*

LEVEL = MEDIUM MEAN LOGCFU = 3.6044 Max. difference = 0.58

Duncan Grouping				Mean	N	LAB
		A		3.86745	2	2
		A		3.83358	2	10
B		A		3.75613	2	17
B		A	C	3.74396	2	3
B		D	C	3.69287	2	15
B	E	D	C	3.66889	2	9
F	E	D	C	3.60934	2	6
F	E	D	G	3.55519	2	5
F	E		G	3.53979	2	1
F		H	G	3.48215	2	14
		H	G	3.46258	2	4
	I	H		3.35993	2	11
	I			3.28471	2	18

LEVEL = HIGH MEAN LOGCFU = 4.5466 Max. difference = 0.53

Duncan Grouping				Mean	N	LAB
		A		4.7269	2	9
		A		4.7179	2	3
B		A		4.7084	2	10
B		A		4.6817	2	5
B		A		4.6698	2	17
B		A		4.6371	2	15
B		A	C	4.5401	2	4
B		A	C	4.4747	2	1
B		A	C	4.4686	2	14
B			C	4.4584	2	2
B			C	4.4581	2	6
	D		C	4.3637	2	11
	D			4.1999	2	18

MEAT SAMPLES – MN&LG CONFIRMATION

LEVEL = LOW MEAN LOGCFU = 2.6996 Max. difference = 0.51

Duncan Grouping	Mean	N	LAB
A	2.9500	2	10
B A	2.9353	2	17
B A C	2.8215	2	13
B A C	2.7794	2	15
B D A C	2.7289	2	6
B D A C	2.7228	2	5
E B D A C	2.7099	2	3
E B D A C	2.6909	2	1
E B D C	2.6650	2	4
E D C	2.6476	2	14
E D C	2.5537	2	7
E D	2.4548	2	12
E	2.4352	2	18

LEVEL = MEDIUM MEAN LOGCFU = 3.5866 Max. difference = 0.53

Duncan Grouping	Mean	N	LAB
A	3.83358	2	10
B A	3.75613	2	17
B A	3.74396	2	3
B A	3.73714	2	12
B A C	3.69287	2	15
B A C	3.66249	2	13
B D A C	3.60934	2	6
B D C	3.55519	2	5
D E C	3.48215	2	14
D E C	3.46258	2	4
D E	3.39704	2	1
D E	3.39075	2	7
E	3.30281	2	18

LEVEL = HIGH MEAN LOGCFU = 4.5264 Max. difference = 0.62 (0.26)

Duncan Grouping	Mean	N	LAB	
A	4.71569	2	3	
A	4.71106	2	10	
B A	4.68165	2	5	
B A C	4.66983	2	17	
B A C	4.64223	2	12	
B A C	4.63711	2	15	
B A C	4.62372	2	13	
B A C	4.54013	2	4	
B C	4.47468	2	1	
B C	4.46865	2	14	
C	4.45808	2	6	
D	4.12587	2	18	*
D	4.09456	2	7	*

DRIED ANIMAL FEED SAMPLES – LS CONFIRMATION

LEVEL = LOW MEAN LOGCFU = 2.6070 Max. difference = 1.13 (0.66)

Duncan Grouping			Mean	N	LAB	
	A		3.25282	2	3	*
	B		2.98683	2	1	*
	C		2.78178	2	2	
D	C		2.73300	2	17	
D	C		2.69919	2	6	
D	C	E	2.61496	2	10	
D	C	E	2.60804	1	4	
D		E	2.58865	2	18	
D		E	2.55292	2	11	
	F	E	2.45010	2	14	
G	F		2.29326	2	5	
G			2.20437	2	15	
G			2.12559	2	9	

LEVEL = MEDIUM MEAN LOGCFU = 3.8336 Max. difference = 0.91 (0.62)

Duncan Grouping				Mean	N	LAB	
		A		4.23710	2	3	
B		A		4.19075	2	1	
B	C			4.02252	2	17	
D	C			3.96603	2	2	
D	C		E	3.88414	2	10	
D	C		E	3.87233	2	18	
D		F	E	3.81885	2	6	
D	G	F	E	3.78432	2	4	
D	G	F	E	3.77742	2	14	
	G	F	E	3.70299	2	11	
	G	F		3.63953	2	5	
	G			3.61427	2	15	
		H		3.32682	2	9	*

LEVEL = HIGH MEAN LOGCFU = 4.8428 Max. difference = 0.52

Duncan Grouping			Mean	N	LAB
	A		5.09846	2	3
	A		5.09108	2	17
	A		5.05319	2	1
	B		4.90712	2	10
	B		4.90059	2	18
C	B		4.88379	2	2
C	B	D	4.79485	2	15
C	B	D	4.76892	2	4
C	B	D	4.76806	2	5
C		D	4.75253	2	14
	E	D	4.68220	2	11
	E	D	4.67454	2	6
	E		4.58076	2	9

DRIED ANIMAL FEED SAMPLES – MN&LG CONFIRMATION

LEVEL = LOW MEAN LOGCFU = 2.6448 Max. difference = 1.03

Duncan Grouping		Mean	N	LAB
	A	3.2300	2	3
B	A	3.0251	2	12
B		2.9681	2	1
	C	2.7330	2	17
D	C	2.6992	2	6
D	C	2.6830	2	7
D	C	2.6539	2	4
D	C	2.6150	2	10
D	C	2.5886	2	18
D	E	2.4612	2	14
F	E	2.2814	2	13
F	E	2.2666	2	5
F		2.2044	2	15

LEVEL = MEDIUM MEAN LOGCFU = 3.8955 Max. difference = 0.74

Duncan Grouping		Mean	N	LAB
	A	4.30036	2	12
	A	4.21666	2	3
	A	4.19075	2	1
	B	4.02252	2	17
C	B	3.95625	2	7
C	B	3.88414	2	10
C	B	3.87233	2	18
C		3.81885	2	6
C	D	3.78432	2	4
C	D	3.77897	2	14
E	D	3.63953	2	5
E	D	3.61904	2	15
E		3.55785	2	13

LEVEL = HIGH MEAN LOGCFU = 4.8798 Max. difference = 0.48

Duncan Grouping			Mean	N	LAB
	A		5.15463	2	12
B	A		5.09846	2	3
B	A		5.09108	2	17
B			5.05134	2	1
	C		4.90712	2	10
	C		4.90059	2	18
D	C		4.82923	2	14
D	E		4.79485	2	15
D	E	F	4.76892	2	4
D	E	F	4.76806	2	5
G	E	F	4.71207	2	7
G		F	4.69099	2	13
G			4.67034	2	6

Appendix 7 Deviating test results

lab	Samples	Confirmation technique	Deviation	Excluded?
4	D2 – D5	-	Results indicate a switch between these samples. However this cannot be confirmed by this laboratory.	Yes, test results from D2 and D5 have been excluded from statistical analysis.
7	C, M, D, R	LS	Results LS confirmation not reliable, too many negative test results due to problems using this medium.	Yes
8	All	-	TSC agar prepared from Oxoid base, which is different from the composition as written in ISO 7937 (1997). Furthermore egg yolk was added to the medium (except for cheese).	Yes
9	Cheese	-	Started analysis of samples 5 days later than planned, which was 9 days after preparation of the samples.	No, stability results show that the contamination level did not change significantly during a period of 10 days.

Appendix 8 SAS protocol

Software: SAS release 6.12 Copyright © 1989-1996 by SAS Institute, Inc., Cary, NC, USA
Program files: SASperf7.sas (2/8/00), SASrm2.sas (3/8/00);
Data files: cheese4.sd2, meat4.sd2, dried4.sd2, rm3.sd2 (27/10/00)

```
/*  Clostridium perfringens trial, jan-febr 2000 */

libname saskia  'n:\perfring\trial\results\calcula2';

proc print data=saskia.dried4 (obs=20);
run;

/* arrange data */

proc contents data=saskia.dried4;
run;

data food1;
set saskia.dried4;
drop lsgas lsblack motility nitrate lactose gelatine cfu_g log_cfu_ p q t u v w;
run;

proc print data=food1;
where (lab=1);
run;

/* remove colony counts > 200 */

data food2;
set food1;
if (counts<=200);
run;

/* merge sample codes with test results */

proc print data=saskia.codes; run;

data codering;
set saskia.codes;
if (lab <> .);
run;

proc print data=codering;
run;

proc sort data=food2;
by lab sample;
run;

proc sort data=codering;
by lab sample;
run;

data food3;
merge food2 (IN=ineen) codering (IN =intwee);
by lab sample;
```

```
IF ineen=1 & intwee=1;
run;

proc print data=food3;
run;

/* exclude blanc samples */

data food4;
set food3;
if (level ne "B");
run;

data food3;
set food4;
run;

/* remove empty cells */

data food5;
set food3;
if counts>=0 & counts <=200 ;
run;

data food5;
set food5;
where (
      (level='M'and (dilution= -2 or dilution = -3) ) or
      (level='L'and (dilution= -1 or dilution = -2) ) or
      (level='H'and (dilution= -4 or dilution = -3) ) ) ;
run;

proc freq data=food5;
tables level*dilution;
run;

/* multiply colony counts with confirmation factor */

data food6;
set food5;
if (counts>0.01) then truefac1 = lspos/tested;
if (counts>0.01) then truefac2 = MNlgpos/tested;
if ((counts = 0) and lspos= 0) then truefac1 = 1;
if ((counts = 0) and mnlgpos = 0) then truefac2 = 1;
truefac3=1;
colc1=int(counts*truefac1+0.5);     /* where 'colc1' = confirmation using LS medium  */
colc2=int(counts*truefac2+0.5);     /* where 'colc2' = confirmation using MNLG medium  */
colc3=int(counts*truefac3+0.5);     /* where 'colc3' = no confirmation results included  */
run;

proc print data=food6;
run;

proc sort data=food6;
by lab sample dilution;
run;
```

/* calculate the dilution factor for each plate individually */

```
proc sort data=food6;
by lab sample level dilution;
run;

data food7;
set food6;
dilpower=10**dilution;
dilpowe1=dilpower;
dilpowe2=dilpower;
dilpowe3=dilpower;
if (truefac1=.) then dilpowe1=0;
if (truefac2=.) then dilpowe2=0;
if (truefac3=.) then dilpowe3=0;
run;
```

/* summarising counts and dilution factors for each sample (4 plates together) */

```
proc means data=food7;
by lab sample level;
var colc1 colc2 colc3 dilpowe1 dilpowe2 dilpowe3;
output out=food8
sum(colc1)=cfusom1
sum(colc2)=cfusom2
sum(colc3)=cfusom3
sum(dilpowe1)=dpower1
sum(dilpowe2)=dpower2
sum(dilpowe3)=dpower3;
run;
```

/* calculate N and log N per sample */

```
data food9;
set food8;
cfu1=cfusom1/dpower1;
cfu2=cfusom2/dpower2;
cfu3=cfusom3/dpower3;
logcfu1=log10(cfu1+1);
logcfu2=log10(cfu2+1);
logcfu3=log10(cfu3+1);
run;

proc print data=food9;
where (level= 'L');
run;
```

/* analysis of variance and Duncan's Multiple Range test per level - calculated on log N values */

```
proc sort data=food9;
by level;
run;

proc glm data=food9;
by level;
class lab;
model logcfu1=lab/solution;
random lab;
means lab/duncan;
```

```
run;                                /* → calculation of precision data using LS medium */

proc glm data=food9;
by level;
class lab;
model logcfu2=lab/solution;
random lab;
means lab/duncan;
run;                                /* → calculation of precision data using MN&LG medium *

proc glm data=food9;
by level;
class lab;
model logcfu3=lab/solution;
random lab;
means lab/duncan;
run;                                /* → calculation of precision data on non-confirmed counts */
```

/* F-test on difference between results from both confirmation methods */

```
data food10;
set food9;
deltcfu=logcfu2-logcfu1;
run;

proc glm;
model deltcfu = /solution;
run ;
```

/* calculate T1 & T2 for each laboratory */

```
proc sort data=food5;
by lab sample  dilution;
run;

proc means data = food5;
by lab sample dilution;
var counts;
output out   = food11
sum(counts)  = sumcolc
mean(counts) = meancolc
var(counts)  = varcolc;
run;

proc sort data=food11;
by lab sample;
run;

data food11;
set food11;
if (_freq_=1) then sumcolc=2*meancolc;
run;
```

/* calculation of T2 */

```
proc sort data=codering;
by lab sample;
run;
```

```
data food12;
merge food11 (in=ineen) codering(in=intwee);
by lab sample;
if (ineen=1 & intwee=1);
run;

proc sort data=food12;
by lab level dilution;
run;

proc means data = food12;
by lab level dilution;
var sumcolc;
output out    = food13
mean(sumcolc) = mnscolc
var(sumcolc)  = varscolc;
run;

data food14;
set food13;
t2cont=varscolc/mnscolc;
where ((varscolc <> .) & (mnscolc >0) & (mnscolc <>.)) ;
run;

proc sort data=food14;
by lab;
run;

proc means data=food14;
by lab;
var t2cont;
output out  = food15
sum(t2cont) = t2;
run;

data foodT2;
set food15;
upperlm = _freq_+2*sqrt(2*_freq_);
run;

proc print data=foodT2;
run;

/* calculation of T1  */

data food16;
set food11;
t1cont = varcolc/meancolc;
where ((varcolc <> .) & (meancolc >0) & (meancolc <>.)) ;
run;

proc sort data=food16;
by lab;
run;

proc means data=food16;
by lab;
var t1cont;
```

```
output out  = food17
sum(t1cont) = t1;
run;

data foodT1;
set food17;
upperlm = _freq_+2*sqrt(2*_freq_);
run;

proc print data=foodT1;
run;
```

Appendix 9 Repeatability and reproducibility values

PRECISION OF ISO 7937 METHOD, CALCULATED USING ISO 5725 (1994)

CONFIRMED COUNTS USING LACTOSE-SULFITE MEDIUM

Sample type	Level	Labs	samples	Mean (log cfp/g)	r (log) [a]	r [b]	R (log) [c]	R [d]	RSD_r [e]	RSD_R [f]
Cheese	Low	13	26	2.49	0.30	2.0	0.36	2.3	4.37	5.21
	Medium	13	26	3.54	0.16	1.5	0.23	1.7	1.63	2.32
	High	13	26	4.48	0.23	1.7	0.31	2.1	1.85	2.50
Meat	Low	13	26	2.74	0.18	1.5	0.38	2.4	2.32	5.01
	Medium	13	26	3.60	0.17	1.5	0.51	3.2	1.67	5.07
	High	13	26	4.55	0.29	2.0	0.49	3.1	2.33	3.90
Dried animal feed	Low	13	25	2.61	0.21	1.6	0.88	7.6	2.85	12.21
	Medium	13	26	3.83	0.22	1.7	0.69	4.9	2.09	6.53
	High	13	26	4.84	0.16	1.5	0.47	3.0	1.22	3.50
RM		13	26	3.70	0.13	1.3	0.26	1.8	1.24	2.51

CONFIRMED COUNTS USING MOTILITY-NITRATE & LACTOSE-GELATINE MEDIUM

Sample type	Level	Labs	samples	Mean (log cfp/g)	r (log) [a]	r [b]	R (log) [c]	R [d]	RSD_r [e]	RSD_R [f]
Cheese	Low	13	26	2.48	0.32	2.1	0.35	2.2	4.59	5.11
	Medium	13	26	3.52	0.19	1.6	0.43	2.7	1.97	4.38
	High	13	26	4.46	0.29	1.9	0.39	2.4	2.31	3.11
Meat	Low	13	26	2.70	0.32	2.1	0.49	3.1	4.22	6.54
	Medium	13	26	3.59	0.27	1.9	0.50	3.2	2.70	5.05
	High	13	26	4.53	0.25	1.8	0.60	4.0	2.01	4.76
Dried animal feed	Low	13	25	2.64	0.28	1.9	0.88	7.6	3.79	12.03
	Medium	13	26	3.90	0.21	1.6	0.67	4.7	1.93	6.18
	High	13	26	4.88	0.10	1.3	0.47	3.0	0.75	3.49
RM		13	26	3.72	0.12	1.3	0.25	1.8	1.21	2.39

a) r (log) = repeatability limit: the value less than or equal to which the absolute difference between two test results, expressed in log_{10} units and obtained under repeatability conditions, may be expected to be with a probability of 95%.

b) r = repeatability limit: the value less than or equal to which the absolute ratio between two test results, expressed on normal scale and obtained under repeatability conditions, may be expected to be with a probability of 95%.

c) R (log) = reproducibility limit: the value less than or equal to which the absolute difference between two test results, expressed in log_{10} units and obtained under reproducibility conditions, may be expected to be with a probability of 95%.

d) R = reproducibility limit: the value less than or equal to which the absolute ratio between two test results, expressed on normal scale and obtained under reproducibility conditions, may be expected to be with a probability of 95%.

e) RSD_r = 100 x S_r/mean, expressing the value of r relative to the contamination level

f) RSD_R = 100 x S_R/mean, expressing the value of R relative to the contamination level

PRECISION OF ISO 7937 METHOD, CALCULATED USING prEN ISO 16140 (2000)

CONFIRMED COUNTS USING LACTOSE-SULFITE MEDIUM

Sample type	Level	Labs	samples	Median (log cfp/g)	r (log) [a]	r [b]	R (log) [c]	R [d]	RSD_r [e]	RSD_R [f]
Cheese	Low	13	26	2.52	0.28	1.9	0.34	2.2	3.95	4.80
	Medium	13	26	3.53	0.19	1.5	0.21	1.6	1.89	2.14
	High	13	26	4.54	0.25	1.8	0.22	1.7	1.99	1.73
Meat	Low	13	26	2.72	0.23	1.7	0.34	2.2	3.07	4.48
	Medium	13	26	3.61	0.21	1.6	0.60	4.0	2.08	5.93
	High	13	26	4.54	0.35	2.2	0.70	5.0	2.78	5.49
Dried animal	Low	13	25	2.61	0.28	1.9	0.75	5.6	3.90	10.30
feed	Medium	13	26	3.82	0.11	1.3	0.69	4.9	1.07	6.50
	High	13	26	4.79	0.17	1.5	0.52	3.3	1.25	3.84
RM		13	26	3.72	0.19	1.5	0.27	1.9	1.87	2.60

CONFIRMED COUNTS USING MOTILITY-NITRATE & LACTOSE-GELATINE MEDIUM

Sample type	Level	Labs	samples	Median (log cfp/g)	r (log) [a]	r [b]	R (log) [c]	R [d]	RSD_r [e]	RSD_R [f]
Cheese	Low	13	26	2.49	0.30	2.0	0.35	2.2	4.31	5.04
	Medium	13	26	3.55	0.20	1.6	0.27	1.9	2.02	2.69
	High	13	26	4.49	0.24	1.7	0.32	2.1	1.88	2.54
Meat	Low	13	26	2.71	0.33	2.1	0.47	3.0	4.32	6.23
	Medium	13	26	3.61	0.26	1.8	0.61	4.1	2.55	6.02
	High	13	26	4.62	0.27	1.9	0.47	3.0	2.10	3.65
Dried animal	Low	13	25	2.61	0.15	1.4	0.85	7.1	2.05	11.67
feed	Medium	13	26	3.87	0.13	1.3	0.74	5.5	1.22	6.86
	High	13	26	4.83	0.14	1.4	0.58	3.8	1.07	4.26
RM		13	26	3.71	0.12	1.3	0.29	1.9	1.15	2.75

a) r (log) = repeatability limit: the value less than or equal to which the absolute difference between two test results, expressed in log_{10} units and obtained under repeatability conditions, may be expected to be with a probability of 95%.

b) r = repeatability limit: the value less than or equal to which the absolute ratio between two test results, expressed on normal scale and obtained under repeatability conditions, may be expected to be with a probability of 95%.

c) R (log) = reproducibility limit: the value less than or equal to which the absolute difference between two test results, expressed in log_{10} units and obtained under reproducibility conditions, may be expected to be with a probability of 95%.

d) R = reproducibility limit: the value less than or equal to which the absolute ratio between two test results, expressed on normal scale and obtained under reproducibility conditions, may be expected to be with a probability of 95%.

e) RSD_r = 100 x S_r/median, expressing the value of r relative to the contamination level; S_r = standard deviation reflecting repeatability [13]

f) RSD_R = 100 x S_R/median, expressing the value of R relative to the contamination level; S_R = standard deviation reflecting reproducibility [13]

Appendix 10 Media

(Other media are prescribed in the SOP in Appendix 3)

Duncan & Strong medium
Composition:
Yeast extract..4.0 g.
Proteose peptone ....15.0 g.
Starch ....4.0 g.
Disodium hydrogen phosphate..6.7 g.
Water..1000 ml

Preparation:
Dissolve the components in water by heating. Sterilise by autoclaving at 121 °C for 15 minutes.

Reference: Manavakis, M., J.A. van Dommelen, P.H. in 't Veld. 1995. Enumeration of Clostridium perfringens in reference materials for food microbiology – BCR/FOOD trial 7. National Institute of Public Health and the Environment (RIVM). Report no. 281008007.

M17 broth
Commercially available complete dehydrated medium (Biokar BK012). Prepared according to the manufacturer's instructions.

M17 agar
Commercially available complete dehydrated medium (Biokar BK088). Prepared according to the manufacturer's instructions.

De Man, Rogosa et Sharpe (MRS) broth
Commercially available complete dehydrated medium (Biokar BK070). Prepared according to the manufacturer's instructions.

De Man, Rogosa et Sharpe (MRS) agar
Commercially available complete dehydrated medium (Biokar BK089). Prepared according to the manufacturer's instructions.

1/4 Ringer's solution
Commercially available complete dehydrated tablets (Merck 15525). Prepared according to the manufacturer's instructions.

Cooked meat medium
Commercially available complete dehydrated medium. Prepared according to the manufacturer's instructions.

Nutrient broth
Commercially available complete dehydrated medium. Prepared according to the manufacturer's instructions.

Plate count agar (PCA)
Commercially available complete dehydrated medium (Oxoid CM 325). Prepared according to the manufacturer's instructions.