National Institute for Public Health
and the Environment
Ministry of Health, Welfare and Sport

EURL-*Salmonella* Proficiency Test food-feed 2019

Detection of *Salmonella* in flaxseed

RIVM Report 2019-0134

R.E. Diddens | K.A. Mooijman

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Colophon

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Synopsis

EURL-*Salmonella* Proficiency Test food-feed 2019
Detection of *Salmonella* in flaxseed

In March 2019, the EURL-*Salmonella* organised a Proficiency Test on the detection of *Salmonella* in flaxseed. All participating National Reference Laboratories (NRLs) for *Salmonella* were able to detect both low- and high-level concentrations of *Salmonella*. All laboratories, except one, scored good performance. The one laboratory swapped the results of the control samples when reporting their results and scored a moderate performance.

All NRLs from EU Member States responsible for the analysis of *Salmonella* in food samples were obliged to participate in this Proficiency Test. For the NRLs-*Salmonella* which analyse animal feed products, participation was voluntarily. In total, 42 NRLs for *Salmonella* participated in this Proficiency Test: 37 NRLs from 28 EU Member States and five NRLs from third countries.

The laboratories used an internationally accepted method to detect the presence of *Salmonella* in flaxseed samples. Each laboratory received a package containing flaxseed samples, which were artificially contaminated with two different concentrations of *Salmonella* Typhimurium or did not contain *Salmonella*. The flaxseed samples were artificially contaminated with *Salmonella* at the EURL-*Salmonella* laboratory.

The EURL-*Salmonella* is part of the Dutch National Institute for Public Health and the Environment (RIVM).

Keywords: *Salmonella*, EURL, NRL, Proficiency Test, *Salmonella* detection method, flaxseed

Publiekssamenvatting

EURL-*Salmonella* ringonderzoek voedsel-diervoeder 2019
Detectie van *Salmonella* in lijnzaad

In maart 2019 organiseerde het EURL-*Salmonella* een ringonderzoek om *Salmonella* in lijnzaad aan te tonen. Alle deelnemende Nationale Referentie Laboratoria (NRL's) voor *Salmonella* waren in staat om lage en hoge concentraties van *Salmonella* aan te tonen. Op één na hebben alle laboratoria een goede score behaald. Dat ene laboratorium had de resultaten van de controlemonsters verwisseld toen ze hun resultaten invoerden en hebben daarom een matige score behaald.

Alle NRL's van Europese lidstaten die verantwoordelijk zijn om *Salmonella* in voedsel voor mensen op te sporen, zijn verplicht om aan het ringonderzoek deel te nemen. Voor de NRL's die *Salmonella* opsporen in diervoeder was de deelname vrijwillig. In totaal namen 42 NRL's-*Salmonella* deel aan dit ringonderzoek: 37 NRL's van 28 Europese lidstaten en 5 NRL's van andere Europese landen.

De laboratoria hebben een internationaal erkende analysemethode gebruikt om *Salmonella* in de lijnzaadmonsters aan te tonen. Elk laboratorium kreeg een pakket toegestuurd met lijnzaadmonsters die ofwel besmet waren met twee verschillende concentraties *Salmonella* Typhimurium, of geen *Salmonella* bevatten. De monsters zijn op het laboratorium van het EURL-*Salmonella* kunstmatig besmet met *Salmonella*.

Het EURL-*Salmonella* is gevestigd bij het Nederlandse Rijksinstituut voor Volksgezondheid en Milieu (RIVM).

Kernwoorden: *Salmonella,* EURL, NRL, ringonderzoek, *Salmonella*-detectiemethode, lijnzaad

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Summary

In March 2019, an EURL-*Salmonella* Proficiency Test for the detection of *Salmonella* in a food-feed matrix was organised for the NRLs-*Salmonella.* The matrix under analysis was flaxseed. Flaxseed is used as a food product and as an ingredient in animal feed. Participation was obligatory for the NRLs from EU Member States, which are responsible for the analysis of *Salmonella* in food samples. For the NRLs-*Salmonella,* which analyse animal feed products, participation was optional.
In total, 42 NRLs-*Salmonella* participated in this study: 37 NRLs from 28 EU Member States (MS) and five NRLs from third countries (EU candidate MS and members of the European Free Trade Association (EFTA)).

The most important objective was to test the performance of the participating laboratories in their detection of different concentrations of *Salmonella* Typhimurium in the flaxseed samples. The prescribed method for the detection of *Salmonella* spp. was EN ISO 6579-1:2017. The participants were asked to report *Salmonella* 'detected' or 'not detected' for each sample (after confirmation).

Prior to the start of the Proficiency Test, pre-tests were conducted to make sure that the samples were fit for use, especially with respect to the choice of the *Salmonella* serovar and the stability of the artificially contaminated samples at different storage temperatures (5 °C and 10 °C). Additionally, the concentration of the natural background flora (aerobic count and *Enterobacteriaceae*) was monitored under the different conditions. The aim was to prepare stable flaxseed samples with a low level of *Salmonella* Typhimurium (STm) of 5-10 cfu/g and with a high level of *Salmonella* Typhimurium with approximately a 10 times higher concentration.
The results of the pre-test showed that the aerobic count in the flaxseed was between 10^6 and 10^7 cfu/g and the concentration of *Enterobacteriaceae* was between 10^5 and 10^7 cfu/g during the two to three weeks of pre-tests, independent of the storage temperature.

Each laboratory received 18 samples, each containing 25 g of flaxseed. These samples consisted of six negative samples (no *Salmonella* added), six samples with a low level of STm (inoculum 10 cfu/samples) and six samples with a high level of STm (inoculum 105 cfu/sample). The laboratories also had to test two control samples: a procedure control and a positive control with *Salmonella*. The flaxseed samples were artificially contaminated with a diluted culture of *Salmonella* Typhimurium at the laboratory of the EURL-*Salmonella*.

Forty-one laboratories detected *Salmonella* in all contaminated flaxseed samples with a low level of STm. One laboratory detected *Salmonella* in five out of six contaminated flaxseed samples with a low level of STm, which is still above the set criteria of at least three positive samples for a good performance. All laboratories did not detect *Salmonella* in the negative samples and detected *Salmonella* in all contaminated samples with a high level of STm.

The specificity rate for the negative samples was 100% and the accuracy rate of all artificially contaminated flaxseed samples was 99,9%.

Laboratory 13 swapped the results of the control samples when reporting their results. This laboratory scored a moderate performance. All other laboratories scored a good performance.

In addition to the prescribed method (EN ISO 6579-1:2017), the NRLs-*Salmonella* were given the opportunity to analyse the flaxseed samples with a second detection method, if this method was (routinely) used in their laboratories.
Thirteen laboratories also used a second detection method for analysing the flaxseed samples. The methods used were PCR, qPCR and mini VIDAS. The results of the second detection methods were all similar to the reported results obtained with EN ISO 6579-1:2017.

1 Introduction

An important task of the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*), as laid down in Commission Regulation EC No. 882/2004 (EC, 2004) and its successor No 2017/625 (EC, 2017), is the organisation of Proficiency Tests (PTs) to evaluate the performance of the National Reference Laboratories for *Salmonella* (NRLs-*Salmonella*). The history of the Proficiency Tests on the detection of *Salmonella*, as organised by EURL-*Salmonella* from 1995, is summarised on the EURL-*Salmonella* website (EURL-*Salmonella*, 2017).

The objective of the current study, organised by EURL-*Salmonella* in March 2019, was to test whether the participating laboratories could detect different contamination levels of *Salmonella* in flaxseed. This is important in order to verify that the examination of samples is carried out uniformly in all EU Member States (MS) and that comparable results are obtained by all NRLs-*Salmonella*.

The method prescribed for the detection of *Salmonella* spp. is set out in EN ISO 6579-1:2017.

The set-up of this study on the detection of *Salmonella* in food and feed matrix was comparable to former EURL-*Salmonella* Proficiency Tests. For the current PT, the flaxseed samples were artificially contaminated with a diluted culture of *Salmonella* Typhimurium (STm) at the EURL-*Salmonella* laboratory.

Flaxseed is used as a food product and as an ingredient in animal feed. For this reason, NRLs-*Salmonella*, which analyse food (products), and NRLs-*Salmonella*, which analyse animal feed, were invited to participate in this Proficiency Test. Participation was obligatory for NRLs-*Salmonella* of the EU Member States that analyse food. For NRLs-*Salmonella* that analyse animal feed, participation was optional.

In total, 18 flaxseed samples were tested by each NRL-*Salmonella*: six samples per contamination level (low level and high level) containing *Salmonella* Typhimurium and six negative samples. Additionally, two control samples (procedure control and positive control with *Salmonella*) were tested. The number and contamination level of samples tested were in accordance with EN ISO 22117:2019.

2 Participants

Country	City	Product(s) under analysis at the NRL-*Salmonella*	Institute / NRL-*Salmonella*
Austria	Graz	Food	AGES - Institute for Medical Microbiology and Hygiene, NRC *Salmonella* Austria
Austria	Linz	Animal feed	AGES - Österreichische Agentur für Gesundheit und Ernährungsicherheit GmbH, Institute for Animal Nutrition and Feed, Abteilung Kartoffelprüfung, Mikro- & Molekularbiologie
Belgium	Elsene	Food & Animal feed	Sciensano, Foodborne pathogens
Bulgaria	Sofia	Food	National Diagnostic and Research Veterinary Institute, NRL "*Salmonella*, *Campylobacter*, *Staphylococci* and AMR"
Croatia	Zagreb	Food	Croatian Veterinary Institute (CVI) Zagreb, Department for Veterinary Public Health, Laboratory for Food Microbiology
Croatia	Zagreb	Animal feed	Croatian Veterinary Institute (CVI), Department for Veterinary Public Health
Cyprus	Nicosia	Food & Animal feed	Cyprus Veterinary Services, Laboratory for the Control of Food of Animal Origin
Czech Republic	Prague	Food & Animal feed	State Veterinary Institute Prague, Bacteriology
Denmark	Ringsted	Food & Animal feed	Danish Veterinary and Food Administration, Department of Microbiology
Estonia	Tartu	Food & Animal feed	Estonian Veterinary and Food Laboratory, Food Microbiology Department
Finland	Helsinki	Food & Animal feed	Finnish Food Authority, Microbiology Unit
France	Ploufragan	Food	Anses, Unité HQPAP
France	Ploufragan	Animal feed	Anses, Unité HQPAP
Germany	Berlin	Food & Animal feed	German Federal Institute for Risk Assessment, Biological Safety
Greece	Chalkida	Food & Animal feed	Veterinary Laboratory of Chalkis, Hellenic Ministry of Rural Development and Food

Country	City	Product(s) under analysis at the NRL-*Salmonella*	Institute / NRL-*Salmonella*
Hungary	Budapest	Food & Animal feed	National Food Chain Safety Office, Food Chain Safety Laboratory Directorate, Microbiological NRL
Iceland	Reykjavík	Food & Animal feed	Matís, Analysis and Infrastructure
Ireland	Celbridge	Food & Animal feed	Central Veterinary Research Laboratory (CVRL), DAFM Laboratories, Department of Agriculture
Italy	Legnaro (PD)	Food & Animal feed	Istituto Zooprofilattico Sperimentale delle Venezie, SCS1- Centro di Referenza Nazionale per le Salmonellosi
Latvia	Riga	Food & Animal feed	Institute of Food Safety, Animal Health and Environment BIOR, Microbiology
Lithuania	Vilnius	Food & Animal feed	National Food and Veterinary Risk Assessment Institute, Bacteriology Unit
Luxembourg	Dudelange	Food & Animal feed	Laboratoire National de Santé, Surveillance Alimentaire
Malta	Valletta	Food & Animal feed	Public Health Laboratory, Environmental Health
Netherlands, the	Bilthoven	Food & Animal feed	National Institute for Public Health and the Environment (RIVM), Centre for Zoonoses and Environmental Microbiology (cZ&O)
Netherlands, the	Wageningen	Food & Animal feed	Wageningen Food Safety Research
Norway	Oslo	Food & Animal feed	Norwegian Veterinary Institute, Microbiology
Poland	Puławy	Animal feed	National Veterinary Research Institute, Department of Hygiene of Animal Feeding Stuffs
Poland	Puławy	Food	National Veterinary Research Institute (NVRI), Department of Hygiene of Food of Animal Origin
Portugal	Vairão	Food & Animal feed	Instituto Nacional de Investigação Agrária e Veterinária, I.P., Food Microbiology
Republic of North Macedonia	Skopje	Food & Animal feed	Food Institute, Faculty of Veterinary Medicine, Laboratory of Food and Feed Microbiology
Romania	Bucharest	Food & Animal feed	Hygiene and Veterinary Public Health Institute, Microbiology

Country	City	Product(s) under analysis at the NRL-*Salmonella*	Institute / NRL-*Salmonella*
Serbia	Belgrade	Food & Animal feed	Institute of Veterinary Medicine of Serbia, Department of Food and Feed Safety
Slovak Republic	Bratislava	Food & Animal feed	State Veterinary and Food Institute
Slovenia	Ljubljana	Food & Animal feed	Institute of Microbiology and Parasitology, Veterinary Faculty (UL, NVI)
Spain	Algete - Madrid	Animal feed	Laboratorio Central de Veterinaria, Bacteriology
Spain	Lugo	Food (Agriculture Primary Production)	Centro Tecnológico Agroalimentario de Lugo (LSA-CETAL), Microbiología
Spain	Majadahonda - Madrid	Food	Centro Nacional de Alimentación - AECOSAN, Microbiology Laboratory
Sweden	Uppsala	Food & Animal feed	National Veterinary Institute, Department of Microbiology
Switzerland	Zürich	Food	ILS Institute for Food Safety and Hygiene, National Centre for Enteropathogenic Bacteria and *Listeria* (NENT)
United Kingdom	Addlestone	Animal feed	Animal and Plant Health Agency (APHA), Bacteriology
United Kingdom	Belfast	Food & Animal feed	Agri-Food and Bioscience Institute (AFBI), Bacteriology
United Kingdom	Wiltshire	Food	Public Health England - Food, Water & Environmental Microbiology Laboratory – Porton Laboratory

3 Materials and methods

3.1 Preparation of artificially contaminated flaxseed samples

3.1.1 *General*

The matrix used for this Proficiency Test (PT) was flaxseed, which was obtained from a mill in the Netherlands. A batch of 35,5 kg was bought in November 2018 for pre-tests and for the Proficiency Test. The batch of flaxseed was checked for the absence of *Salmonella*. Ten randomly taken samples of 25 g each were checked in accordance with EN ISO 6579-1:2017.

For this purpose, 225 ml of Buffered Peptone Water (BPW) was added to each of the 25 g samples and left to stand for 20 to 30 min at laboratory ambient temperature (18 °C to 27 °C) in order to assist resuscitation of damaged organisms. Then the sample was mixed for 60 s with a homogeniser (EN ISO 6887-1 and -4:2017).
After pre-enrichment at 37 °C ± 1 °C for 18 h ± 2 h, selective enrichment was carried out in Muller-Kauffmann TetraThionate-novobiocin broth (MKTTn) and on Modified Semi-solid Rappaport Vassilliadis agar (MSRV) agar. The MKTTn tubes and the suspect growth on MSRV plates were then plated out on Xylose Lysine Deoxycholate (XLD) agar and Brilliance *Salmonella* Agar (BSA). Suspected colonies were then confirmed biochemically and serologically.
After verifying the absence of *Salmonella*, the flaxseed was repacked in portions of 25 g in Whirl-Pak plastic filter bags, after which the samples were artificially contaminated with a low and high level of *Salmonella* Typhimurium (STm) and stored at 5 °C.

3.1.2 *Pre-tests for the preparation of flaxseed samples*

Salmonella Typhimurium (STm) from the American Type Culture Collection (ATCC 14028, Manassas, USA) was chosen to artificially contaminate the flaxseed samples. The *Salmonella* strain was inoculated in Brain Heart Infusion broth (BHI) and incubated at 37 °C ± 1 °C for 18 h ± 2 h.
Next, tenfold dilutions were prepared from each culture in peptone saline solution in order to inoculate the flaxseed samples with approximately 5 cfu/25 g, 10 cfu/25 g and 20 cfu/25 g. For the enumeration of the contamination level, 0,1 ml of the diluted culture was spread on XLD agar and incubated at 37 °C ± 1 °C for 24 h ± 3 h.

In addition to the artificially contaminated samples, negative samples were prepared without the addition of *Salmonella*.

To test the stability of *Salmonella* in the flaxseed samples during storage and transport, samples were stored at 5 °C for 21 days and stored at 10 °C for 14 days.

After storage of 0, 7, 14 and 21 days, six artificially contaminated samples were tested for the presence of *Salmonella* following EN ISO 6579-1:2017 (see 3.1.1). This was done for every inoculation level and two storage temperatures.

Negative flaxseed samples (no *Salmonella* added) were also stored at 5 °C and 10 °C. On the same sampling days (t = 0, 7, 14 and 21 days), the level of the natural background flora was determined in these samples by analysing the number of aerobic bacteria and *Enterobacteriaceae* (see 3.1.4).

3.1.3 Preparation of flaxseed samples for the Proficiency Test

Approximately two weeks prior to the PT, samples were prepared for 43 participating laboratories. Per laboratory, 18 flaxseed samples were prepared. The Whirl-Pak filter bags were first labelled and then 25 g of flaxseed was added to 774 filter sample bags. The flaxseed samples were individually, artificially contaminated with a diluted overnight culture of STm or no *Salmonella* at all (negative samples).

For each participant, the following set of samples were prepared:

- 6 negative samples, each containing 25 g of flaxseed (no *Salmonella* added);
- 6 samples, each containing 25 g of flaxseed with a low level of *Salmonella* Typhimurium (STm), aimed at 5-10 cfu/25 g;
- 6 samples, each containing 25 g of flaxseed with a high level of *Salmonella* Typhimurium (STm), aimed at 50-100 cfu/25 g;
- 2 control samples consisting of empty filter sample bags for the procedure control (only BPW) and own positive control.

After artificial contamination, the samples were mixed by hand and stored at 5 °C until transport to the NRLs-*Salmonella* on 18 March 2019.

3.1.4 Determination of level of background flora in flaxseed

The total number of aerobic bacteria and the number of *Enterobacteriaceae* in flaxseed were investigated by following, respectively, EN ISO 4833-1:2013 and EN ISO 21528-2:2017.
For this purpose, an initial suspension was prepared by adding 225 ml of peptone saline solution to 25 g of flaxseed (EN ISO 6887-1:2017). This suspension was left to stand for 20 to 30 min at laboratory ambient temperature (18 °C to 27 °C) and then mixed for 60 s with a homogeniser. Finally, tenfold dilutions of the initial suspension were analysed on Plate Count Agar (PCA) and on Violet Red Bile Glucose (VRBG) Agar.

3.1.5 Determination of the number of Salmonella in flaxseed samples by MPN

The number of *Salmonella* was determined in the final flaxseed samples at the start of the PT. This was determined using a five-tube, Most Probable Number (MPN) technique. For this purpose, tenfold dilution of five artificially contaminated flaxseed samples of each contamination level were tested, representing 25 g, 2,5 g and 0,25 g of the original sample. The presence of *Salmonella* was determined in each dilution by following EN ISO 6579-1:2017. From the number of confirmed positive dilutions, the MPN of *Salmonella* in the original sample was calculated using freely available Excel-based MPN software (Jarvis *et al*., 2010).

3.2 Design of the Proficiency Test

3.2.1 Number and type of samples

On 18 March 2019, the flaxseed samples were prepared for shipment and sent to the participants by door-to-door courier service. After arrival at the laboratories, the flaxseed samples were stored at 5 °C until the start of the PT.

Eighteen samples (numbered B1–B18) and two control samples (numbered C1 and C2) were tested by each participating laboratory. Table 1 gives an overview of the number and type of samples tested by each participant.

For the control samples, the laboratories used their own positive *Salmonella* control strain, which was normally used when analysing routine samples for the detection of *Salmonella*. In addition to this positive control (C2), a procedure control (C1) consisting only of Buffered Peptone Water (BPW) was analysed.

Table 1. Overview of the number and type of samples tested per laboratory in the Proficiency Test food-feed 2019

Contamination level	**Test samples with flaxseed (n=18)**
Negative sample (no *Salmonella* added)	6
Low level of *S*. Typhimurium (low level STm)	6
High level of *S*. Typhimurium (high level STm)	6
	Control samples (n=2)
Procedure control (only BPW)	1
Positive control with *Salmonella*	1

3.2.2 Shipment of parcels and temperature recording during shipment

Twenty sample bags were sent to each NRL-*Salmonella* containing the flaxseed samples that were artificially contaminated with *Salmonella*, negative flaxseed samples and the control samples (empty filter sample bags). The 20 sample bags were packed in one large plastic safety bag. The safety bag was placed in one large shipping box, together with three frozen cooling devices. Each parcel was sent to the participants as ‘biological substances category B (UN3373)’ using a door-to-door courier service.

To monitor exposure to excessive temperatures during shipment and storage, temperature buttons were used to record the temperature. These buttons are tiny units sealed in a stainless-steel case, 16 mm in diameter and 6 mm deep.
Each parcel contained one button packed together with the flaxseed samples in a large safety bag. The loggers were programmed by the EURL-*Salmonella* to measure the temperature every hour. Each NRL-*Salmonella* had to return the temperature recorder to the EURL-*Salmonella* on the day the laboratory started the PT. At the EURL-*Salmonella*, the loggers were read using a computer program and all

recorded temperatures from transport and storage were transferred to an Excel sheet.

Further details about the shipping and handling of the samples and the reporting of the test results can be found in the protocol (EURL-*Salmonella*, 2019a) and in (a printout from) the result form (EURL-*Salmonella,* 2019b).

3.3 Methods

The prescribed method was EN ISO 6579-1:2017 and the underlying EN ISO documents, e.g. the EN ISO 6887 series for preparation of test samples.

EN ISO 6579-1:2017 describes the technical steps for the detection of *Salmonella* in food, animal feed and samples from the primary production stage.

The laboratories were asked to prepare the test samples in this PT as follows:

- add the BPW to the 25 gram test sample (instead of weighing accurately the sample into a pre-dispensed volume of BPW, as prescribed in EN ISO 6887-4:2017);
- resuscitate the sample for 20 to 30 minutes at 18 °C to 27 °C (room temperature);
- mix for 60 s ± 5 s with a homogeniser.

It was stipulated that these three steps should be done observing the practical aspect of this combined food-feed PT. In this way, the laboratories could leave the artificially contaminated flaxseed samples inside the sample bags.

The prescribed method in summary:

- pre-enrichment in:
  - Buffered Peptone Water (BPW);
- selective enrichment in/on:
  - Muller-Kauffmann TetraThionate-novobiocin (MKTTn) broth;
  - Modified Semi-solid Rappaport Vassilliadis (MSRV) agar and/or;
  - Rappaport Vassilliadis with Soya (RVS);
- plating-out on two isolation media:
  - first isolation medium: Xylose Lysine Deoxycholate agar (XLD);
  - second isolation medium (obligatory): medium of choice;
- confirmation by means of:
  - appropriate biochemical and serological tests (EN ISO 6579-1:2017) or reliable, commercially available identification kits.

Additionally, the NRLs-*Salmonella* were given the opportunity to analyse the samples using a second detection method if this method was (routinely) used in their laboratories. These results could also be reported, but only the results obtained with EN ISO 6579-1:2017 were used to assess the performance of the NRL.

3.4 Statistical analysis of the data

The specificity, sensitivity and accuracy rates were calculated for the artificially contaminated flaxseed samples. For the control samples, only the accuracy rates were calculated. The rates were calculated according to the following formulae:

Specificity rate

$$\frac{\text{number of negative results}}{\text{Total number of (negative) samples}} \times 100\%$$

Sensitivity rate

$$\frac{\text{number of positive results}}{\text{Total number of (expected positive) samples}} \times 100\%$$

Accuracy rate

$$\frac{\text{number of correct results (positive and negative)}}{\text{Total number of samples}} \times 100\%$$

3.5 Criteria for good performance

For the determination of 'good performance', the criteria indicated in Table 2 were used. For the determination of good performance per laboratory, the results obtained with all combinations of selective enrichment media and isolation media used by the laboratory were taken into account.

Table 2. Criteria for good performance used in PT food-feed 2019

Contaminated samples	Percentage positive	# pos. samples/ total # samples
Negative samples*	20% max	1/6 max
Low level of *S.* Typhimurium	≥ 50%	≥ 3/6
High level of *S.* Typhimurium	≥ 80%	≥ 5/6
Control samples	**Percentage positive**	**# pos. samples/ total # samples**
Procedure control	0%	0/1
Positive control with *Salmonella*	100%	1/1

*100% *Salmonella*-free matrix cannot be guaranteed, 1 positive out of 6 negative samples is still considered as acceptable (20%).

4 Results and discussion

4.1 Preparation of artificially contaminated flaxseed samples

4.1.1 General

Ten random samples of the batch of 35,5 kg of broken flaxseed were tested for the presence of *Salmonella*. *Salmonella* was not detected in these ten samples.

4.1.2 Pre-tests for the preparation of flaxseed samples

Experiments were performed to test the stability of the flaxseed samples artificially contaminated with *Salmonella* Typhimurium during storage and transport. Samples with different concentrations of *Salmonella* Typhimurium were stored at 5 °C to mimic storage conditions and stored at 10 °C to test the effect of temperature abuse during transport.

The flaxseed samples were inoculated with three different concentrations of *Salmonella* Typhimurium. The actual inoculation level was 6 cfu/25 g of flaxseed, 10 cfu/25 g of flaxseed and 24 cfu/25 g of flaxseed.

The pre-test samples were stored for up to three weeks and analysed for the survival of *Salmonella* using EN ISO 6579-1:2017. The results are presented in Figures 1 and 2.

Figure 1 shows that the flaxseed samples artificially contaminated with different concentrations of *Salmonella* Typhimurium were stable during three weeks of storage at 5 °C.
Figure 2 shows that the same flaxseed samples were also stable when stored at 10 °C for two weeks. Based on these results, the aim was to inoculate the low-level flaxseed samples with *Salmonella* Typhimurium at a level of 5–10 cfu/g.

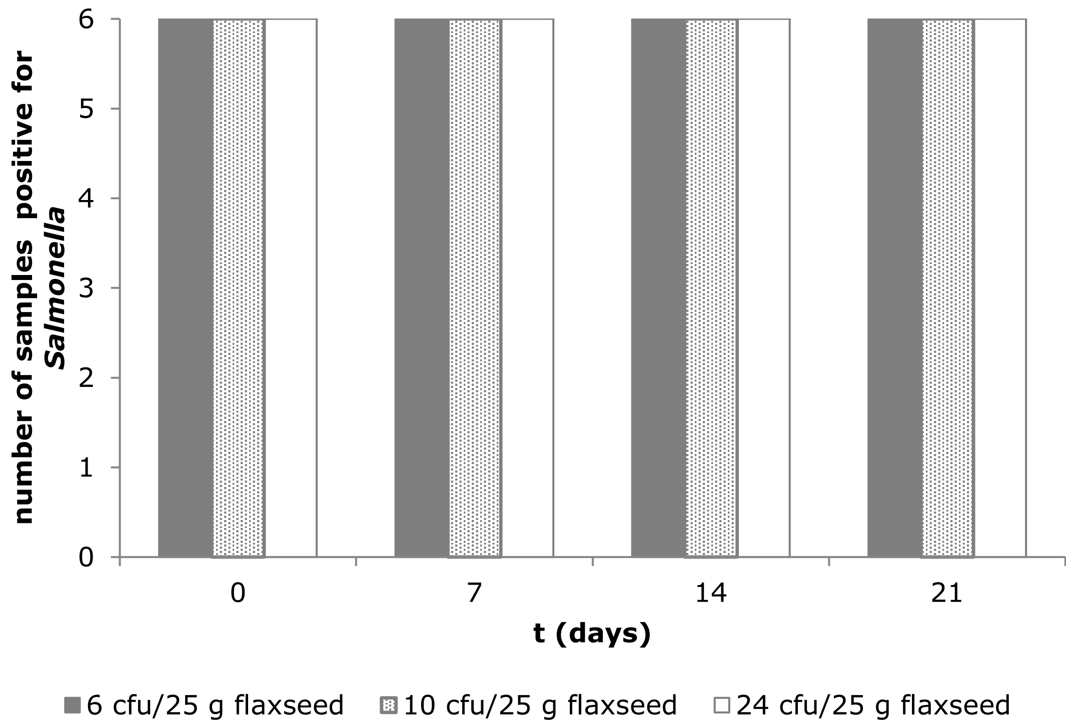

Figure 1. Stability tests of flaxseed samples artificially contaminated with different concentrations of Salmonella *Typhimurium stored at 5 °C*

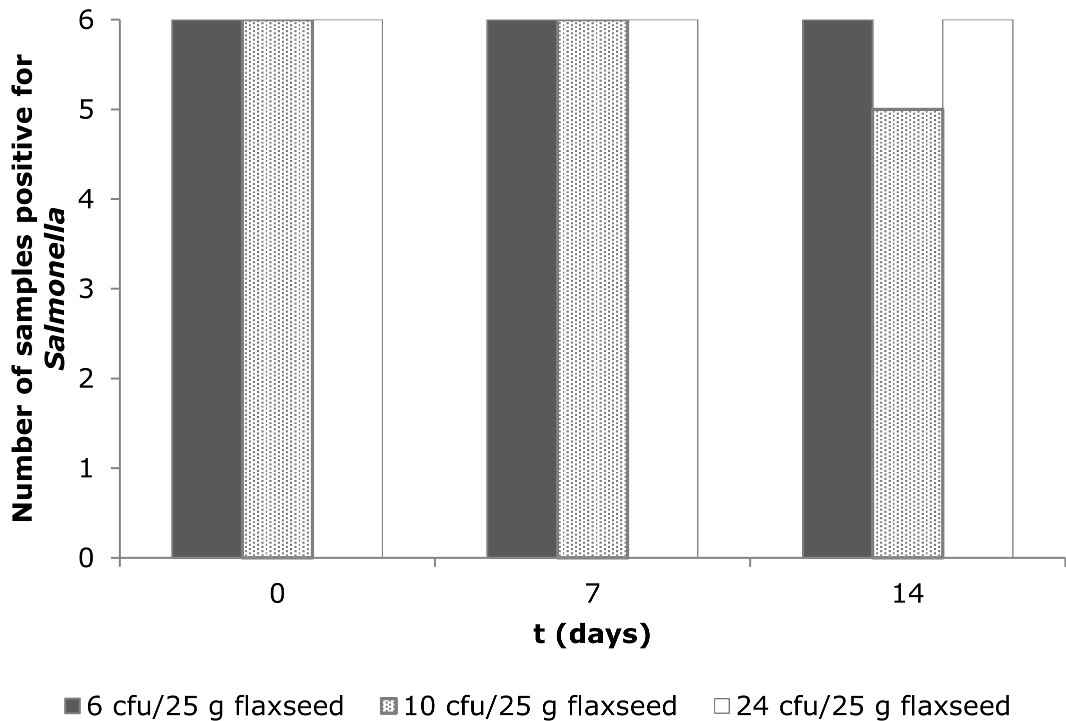

Figure 2. Stability tests of flaxseed samples artificially contaminated with different concentrations of Salmonella *Typhimurium stored at 10 °C*

Figures 3 and 4 show the level of background flora in the flaxseed samples, which remained relatively stable after storage at 5 °C and 10 °C for two to three weeks.

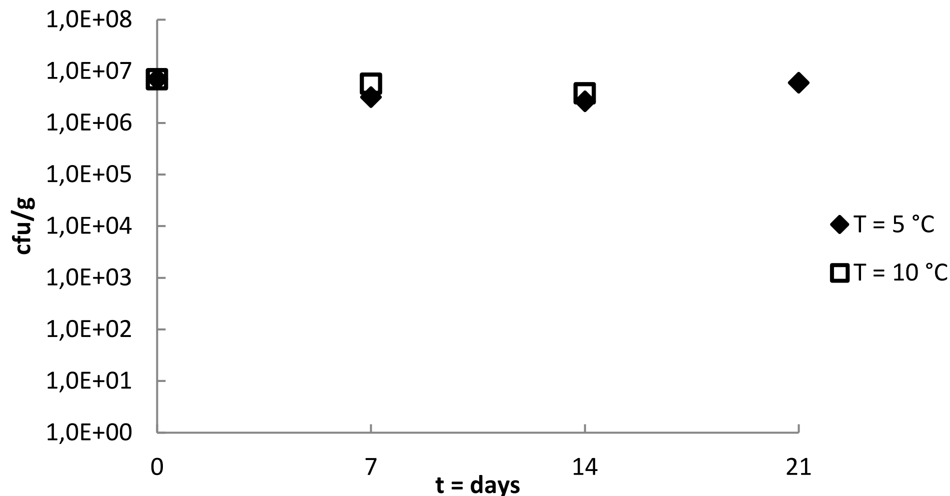

Figure 3. Number of aerobic bacteria per gram of flaxseed (negative for Salmonella*) after storage at 5 °C and 10 °C*

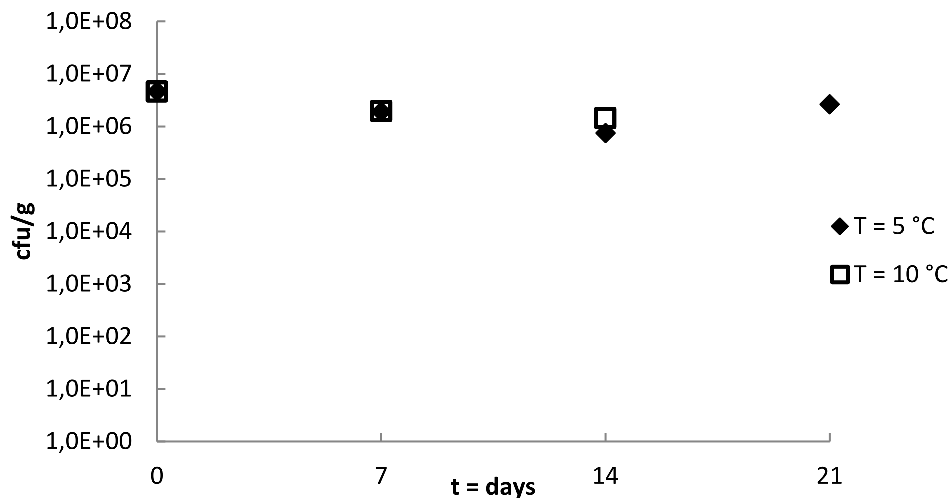

Figure 4. Number of Enterobacteriaceae per gram of flaxseed (negative for Salmonella*) after storage at 5 °C and 10 °C*

The number of aerobic bacteria in the flaxseed varied between 10^6 and 10^7 cfu/g during storage at 5 °C and 10 °C for two to three weeks. The number of *Enterobacteriaceae* in the flaxseed varied between 10^5 and 10^7 cfu/g and was comparable for both storage temperatures during the storage period of two to three weeks.

4.1.3 Natural background flora in flaxseed

The level of natural background flora in the flaxseed was determined after receipt at the EURL-*Salmonella* and at the start of the PT. Table 3 shows the number of aerobic bacteria and *Enterobacteriaceae*.

Table 3. Number of aerobic bacteria and Enterobacteriaceae *per gram of flaxseed*

Date	Aerobic bacteria (cfu/g)	*Enterobacteriaceae* (cfu/g)
21 November 2018	$7,0 \times 10^6$	$4,6 \times 10^6$
25 March 2019[a]	$1,6 \times 10^6$	$4,0 \times 10^5$

a. After storage at room temperature for four months and at 5 °C for two weeks

The concentration of the aerobic bacteria and *Enterobacteriaceae* decreased after storage at room temperature for four months and 5 °C for two weeks.

4.1.4 *Number of* Salmonella *in flaxseed samples*

Table 4 shows the inoculum levels of the diluted culture of *Salmonella* Typhimurium used to artificially contaminate the flaxseed samples. A five-tube Most Probable Number (MPN) test was also performed on the artificially contaminated flaxseed samples with low and high levels of STm at the start of the PT.

Table 4. Number of Salmonella *Typhimurium (STm) in the inoculum and in the contaminated flaxseed samples*

Date of testing	Low level STm cfu/25 g	High level STm cfu/25 g
12 March 2019 Inoculation of flaxseed	10	105
25 March 2019[a] MPN of flaxseed, inoculated with STm (95% confidence limit)	13 (4,5-37,5)	160 (52,5-500)

a. After storage at 5 °C for two weeks

The results show that the intended levels of 5-10 cfu/25 g (low level) and 50-100 cfu/25 g (high level) of *Salmonella* Typhimurium in the flaxseed samples were reached. Additionally, the levels remained stable when stored at 5 °C for two weeks.

4.2 Technical data Proficiency Test

4.2.1 *General*

In total, 42 NRLs-*Salmonella* participated in this PT: 37 NRLs from 28 EU Member States (MS) and 5 NRLs from third countries (EU candidate MS and members of the European Free Trade Association (EFTA)). Of the 42 participants, 28 were NRLs-*Salmonella* for food and animal feed, nine were NRLs-*Salmonella* for food only and six were NRLs-*Salmonella* for animal feed only.

Forty-one laboratories performed the Proficiency Test as requested on 25 March 2019. One participant started the PT, after consulting with the EURL-*Salmonella*, on 26 March 2019.

Originally, 43 laboratories registered to participate in the EURL-*Salmonella* PT food-feed 2019, but due to import problems with the parcel, laboratory 41 did not receive the parcel and for that reason could not participate in this PT.

4.2.2 *Accreditation*

Four laboratories are accredited for EN ISO 6579:2002, 37 laboratories are accredited for EN ISO 6579-1:2017 and one laboratory did not specify the method which they have under accreditation.

Five laboratories also have other *Salmonella* methods under accreditation: NMKL 71, NMKL 187, qPCR method, PCR method and a VIDAS method.

4.2.3 *Transport of samples*

On Monday, 18 March 2019, the flaxseed samples were sent to 43 laboratories. Forty-one parcels were delivered to the NRLs within one to two days and one parcel was held at customs and arrived after seven days (at the start of the PT) at laboratory 31.
The parcel for laboratory 41 could not be delivered.

The temperature during transport and storage was registered using a temperature probe. The temperature of all parcels during transport was below 5 °C. The storage temperature of the samples at 38 laboratories varied between 0 and 7 °C. At three laboratories, a maximum temperature was measured of 9,5 °C and 11,5 °C. No data was received from one laboratory (laboratory 38).
Figure 5 shows the temperature record for the parcel which arrived after seven days at laboratory 31. Initially, the customs stored the parcel in the freezer and the parcel reached a temperature of -12 °C. Next, the parcel seemed to be moved to a refrigerator and was kept at 1,5-2 °C until the start of the PT.

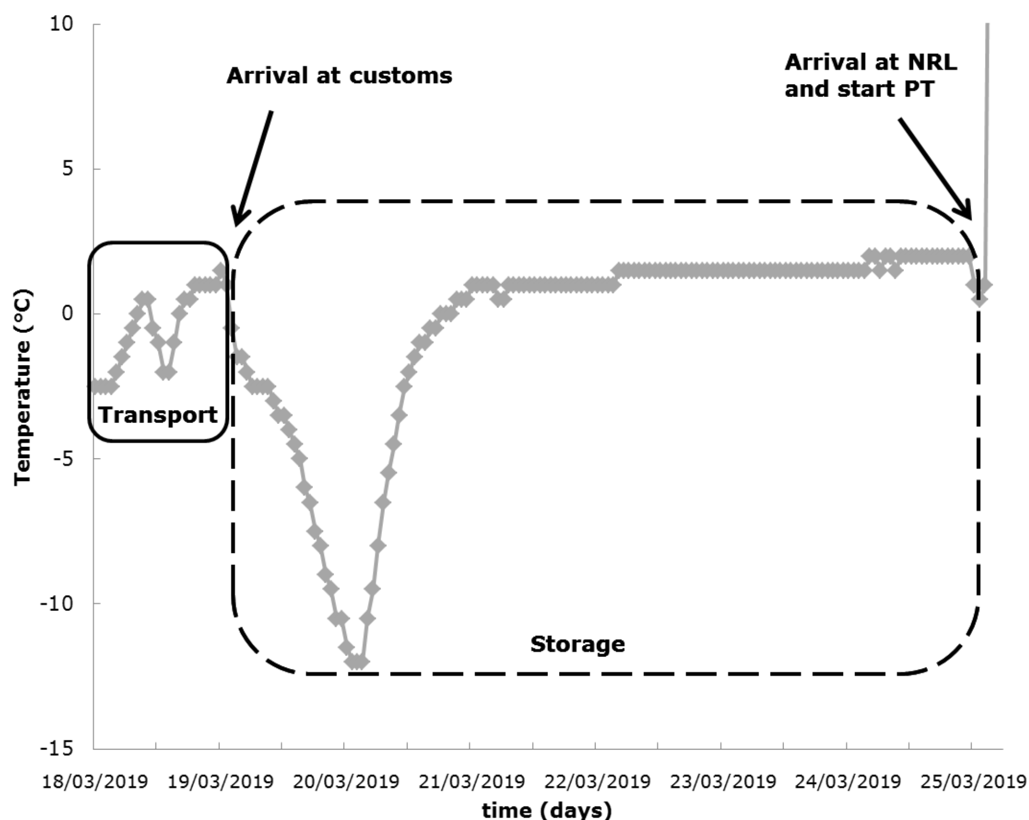

Figure 5. Temperature record of the parcel sent to laboratory 31

4.2.4 *Methods*

For this PT, the prescribed method was EN ISO 6579-1:2017 for the detection of *Salmonella* in flaxseed. EN ISO 6579-1:2017 stipulates that MKTTn and RVS and/or MSRV should be used as selective enrichment media.

Twelve laboratories used all three prescribed selective enrichment media: MKTTn, RVS and MSRV (laboratories 4, 6, 13, 16, 23, 25, 26, 27, 29, 31, 33 and 40).
Fifteen laboratories used MKTTn and RVS as selective enrichment media (laboratories 2, 5, 7, 8, 10, 11, 14, 15, 17, 21, 22, 30, 34, 35 and 38).
Twelve laboratories used MKTTn and MSRV as selective enrichment media (laboratories 3, 12, 18, 19, 20, 28, 32, 36, 37, 39, 42 and 43).
Three laboratories did not use MKTTn as prescribed in EN ISO 6579-1:2017. Laboratories 1 and 24 used only MSRV as the selective enrichment medium. Laboratory 9 used RVS and MSRV as selective enrichment media.

Table 5 shows the reported values of the incubation times, the concentrations of novobiocin, pH and the incubation temperatures of the different media used. Only the laboratories are shown which reported deviating values from EN ISO 6579-1:2017.

Table 5. Reported technical deviations from prescribed method EN ISO 6579-1:2017

		MKTTn			RVS		MSRV		
Laboratory code	**hours incubation BPW**	**concentration novobiocin (mg /L)**	**pH**	**Temperature (°C)**	**pH**	**Temperature (°C)**	**concentration novobiocin (mg/L)**	**pH**	**Temperature (°C)**
EN ISO 6579-1	18 ± 2 hours	40 mg /L	7 - 8,2	37 °C ± 1 °C	5,2 ± 0.2	41,5 °C ± 1 °C	10 mg / L	5,1 - 5,4	41,5 °C ± 1 °C
1	18						10	5,2	41,5
2	18	-	6,9	37	5,3	42			
5	22	39	7,1	37	5,3	41,4			
6	18	40	8	37	5,3	41,5	50	5,3	41,5
9	18				5,3	42	10	5,5	42
12	18	40	8	37			20	5,4	37
13	18	40	8,1	36,9	8,3	36,9		8,1	36,9
15	24	40	not measured	37	not measured	41,5			
17	20	40	6,6	37	5,2	41,5			
20	19	40	8	37			10	5,5	41,5
23	15	40	8,0 +/- 0,2	37	5,2 +/- 0,2	41	10	5,2 +/- 0,2	41
24	20						20	5,3	41,5
29	18	5	8	37	5,2	41,5	10	5,6	41,5
34	19	-	7,8	41,2	5,3	41,2			
36	18	10	8	37			10	5,2	42
37	18	40	8,1	37			10	5,5	41,5
38	18	40	5,2	37	8,1	41,5			
40	20	40	8	37	5,3	41,5	10	5,6	41,5
42	18	40	7,9	37			10	5,6	41,5

Grey cells are deviations from EN ISO 6579-1:2017. -: use not mentioned

Three laboratories (laboratories 5, 15 and 23) had deviating incubation times for the pre-enrichment in BPW.
Two laboratories (laboratory codes 29 and 36) reported a lower concentration of novobiocin in MKTTn and two laboratories (laboratories 2 and 34) did not mention the use of novobiocin in the MKTTn.
According to EN ISO 6579-1:2017, the pH of the base medium of MKTTn broth should be 7,8-8,2. In addition, it indicates that the complete medium should no longer be used if, after storage, the pH is <7. Three laboratories (laboratory codes 2, 17 and 38) used MKTTn with a pH lower than 7. Laboratory 15 did not measure the pH of MKTTn and laboratory 34 incubated the MKTTn at a temperature of 41,2 °C instead of 37 °C ± 1 °C.

For RVS, two laboratories (laboratory codes 13 and 38) reported a higher pH than 5,2 ± 0.2 and one laboratory did not measure the pH of RVS (laboratory code 15). Laboratory 13 incubated the RVS at 36,9 °C.

Three laboratories reported to have used higher concentrations of novobiocin in MSRV than is prescribed (laboratories 6, 12 and 24). Seven laboratories (laboratory codes 9, 13, 20, 29, 37, 40 and 42) reported a higher pH of MSRV than is prescribed and laboratories 12 and 13 incubated MSRV at a lower temperature than is prescribed.

The selective enrichment culture was plated-out on two isolation media: XLD and an obligatory second isolation medium. The choice of the second isolation medium for the different laboratories can be found in Table 6. Most laboratories used Rambach or BGA as a second isolation medium.

Table 6. Second isolation media used by the laboratories

Media	**No. of users**
ASAP	1
BGA	8
BGA (Modified)	6
BPLS	6
BSA	1
CHROMagar *Salmonella*	1
ChromoID *Salmonella*	1
Compass *Salmonella*	2
Rambach	9
Rapid *Salmonella* Agar	7
Salmonella Differential Agar (RajHans Medium)	1
SM(ID)2	3
XLT	1

Explanations of the abbreviations used are given in the 'List of abbreviations'.

The last step in the procedure for detection of *Salmonella* is the confirmation step. All participating laboratories performed one or several confirmation tests for *Salmonella*. An overview can be found in Table 7. Thirty-two laboratories performed a biochemical test and the majority performed one or more additional confirmation test(s).
Thirteen laboratories (also) used another confirmation test, such as MALDI-TOF, Chromogenic Agar, Wellcolex kit, MINI VIDAS and API 20E.

Table 7. Number of laboratories using different confirmation methods

Number of labs	Bio-chemical	Sero-logical	Sero-typing	PCR	Other
1	x				
11	x	x			
4	x	x	x		
1	x	x		x	x
3	x	x			x
6	x		x		
1	x		x	x	
2	x		x		x
1	x			x	
2	x				x
1		x	x		
2			x		
1			x	x	
3			x		x
1				x	
2					x

4.3 Control samples

4.3.1 General

Two empty safety bags were sent to each participating NRL-*Salmonella*, which were used for the control samples, being:

- a procedure control consisting only of BPW;
- a positive control with the laboratories' own *Salmonella* control strain.

Procedure control (BPW only)
All laboratories analysed the procedure control sample (no matrix, only BPW) correctly to be negative for *Salmonella*. Only laboratory 13 reported the procedure control as '*Salmonella* detected' and the laboratory was contacted by the EURL-*Salmonella* for a possible explanation. Laboratory 13 made a mistake when entering the result for the procedure control on the result form. *Salmonella* was not detected in the procedure control (only BPW) and this was confirmed by their raw data.

Positive control with Salmonella
The laboratories were asked to use their own positive control, normally used in their routine analysis for the detection of *Salmonella*.
All laboratories detected *Salmonella* in their positive control sample. Only laboratory 13 reported the positive control as '*Salmonella* not detected' and the laboratory was contacted by the EURL-*Salmonella* for a possible explanation. Laboratory 13 made a mistake when entering the result for the positive control. They detected *Salmonella* in the positive control, which was confirmed by their raw data.

The *Salmonella* serovars used by the participants for the positive control sample were: *S.* Enteritidis (15), *S.* Typhimurium (10), *S.* Nottingham (7), and ten participants used other *Salmonella* serovars. See Table 8.

Table 8. Salmonella *serovars used by participants for the positive control samples*

***Salmonella* serovar**	**Number of users**
S. Enteritidis	15
S. Typhimurium	10
S. Nottingham	7
S. Abaetetuba	2
S. Blegdam	2
S. Alachua, *S.* Harleystreet, *S.* Poona, *S.* Infantis, *S.* bongori serovar 66 : z41 : -, *S.* Tranoroa *(Salmonella enterica* subsp. *Salamae)*	1 (per serovar)

The concentration of *Salmonella* in the positive control samples used by the different participants varied between 2 and 10^8 cfu/sample (see Table 9). Thirteen laboratories used a concentration of 2 – 10 cfu/sample and six laboratories used a concentration of 10^3-10^8 cfu/sample. All other laboratories were in between those concentrations or did not know the concentrations of *Salmonella* added to their positive control sample.

Table 9. Concentration of Salmonella *in the positive control*

Concentration *Salmonella* (cfu/sample)	**Number of laboratories**
2-10	13
11-120	16
121-520	5*
10^3-10^8	6
High concentration	1
Not defined	1

* Including a reported *Salmonella* concentration of 100-300 cfu/sample

A positive control sample of a detection method should demonstrate that media are capable of supporting the growth of the target organisms in low numbers. To obtain information on the sensitivity of a method, the concentration of a positive control sample should preferably be just above the detection limit of the method. In the current study, the majority of the participants used a much higher concentration. Additionally, for a positive control, it may be advisable to use a rarely isolated serovar from the routine samples analysed in the laboratory. In this way, possible cross-contamination can be more easily detected.

Additionally, it also advisable to add a *Salmonella*–free matrix to the positive control sample. It is a more realistic control of the procedure. Preferably, a matrix which is similar to the samples tested.
Five laboratories (lab codes 12, 20, 28, 30 and 39) also used a matrix with their positive control. The matrices used were: meat product, fishmeal, minced meat, chia seeds and food.

4.3.2 *Correct scores of the control samples*

Table 10 shows the number of correct scores found with the control samples. The calculations were performed for the results of all participants and for the EU-MS only.

*Table 10. Correct scores found with the control samples by all participants ('All') and by the laboratories of the EU Member States only ('EU-MS')**

Control samples	Percentage positive	All n = 42	EU-MS n = 37
Procedure control (only BPW)	No. of samples	42	37
	No. of negative samples	42	37
	Correct score in %	100%	100%
Positive control with *Salmonella*	No. of samples	42	37
	No. of positive samples	42	37
	Correct score in %	100%	100%
All control samples n=2	No. of samples	84	74
	No. of correct samples	84	74
	Accuracy in %	100%	100%

* Laboratory 13 switched the reported results of the procedure control and the positive control. The correct scores and accuracy in this table were calculated using the raw data.

4.4 Artificially contaminated flaxseed samples

4.4.1 General

Table 11 shows the results of the flaxseed samples artificially contaminated with *Salmonella* Typhimurium. It shows that the storage temperature of -12 °C of one of the parcels, as well as the technical deviations (see Chapter 4.2.4.), did not influence the final results. *Salmonella* was correctly detected in all artificially contaminated flaxseed samples.

Table 11. Number of positive results found with the artificially contaminated flaxseed samples at each laboratory

Lab code	Number of samples in which *Salmonella* is detected		
	negative n=6	Low level STm n=6	High level STm n=6
Criteria of good performance	**≤1**	**≥3**	**≥5**
21	0	5	6
All other NRLs-*Salmonella*	0	6	6

Negative flaxseed samples

All laboratories scored the six negative flaxseed samples correctly by not detecting *Salmonella* in these samples. However, one laboratory (laboratory code 42) originally reported one negative sample as positive. All negative samples should have tested negative. However, because no 100% guarantee on the *Salmonella*-negative status of the flaxseed could be given, one positive sample out of six negative samples (80% negative) was still considered acceptable. For this reason, this one positive sample had no influence on the performance of laboratory 42. Still, the EURL-*Salmonella* contacted the laboratory for additional information on this particular sample, to learn more about the possible natural contamination of the flaxseed. It turned out that the sample was not correctly transformed from the laboratorial software of lab 42 to the

result form. This was confirmed by their raw data, showing that this sample also correctly tested negative for *Salmonella*.

Flaxseed samples artificially contaminated with a low level of Salmonella
Forty-one laboratories detected *Salmonella* in all six flaxseed samples that were contaminated with a low level of *Salmonella*. One laboratory (lab code 21) detected *Salmonella* in five out of six flaxseed samples contaminated with a low level of *Salmonella*, which is well above the level of good performance. The level of good performance for the low-level samples for this PT was set at the detection of *Salmonella* in at least three out of six samples.

Figure 6 shows the number of samples in which *Salmonella* was detected per laboratory.

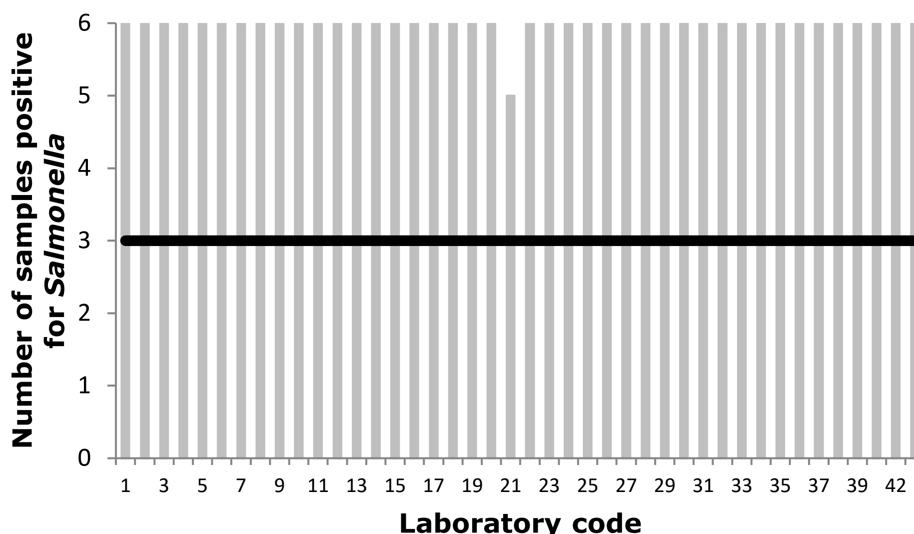

Figure 6. Number of flaxseed samples artificially contaminated with a low level of Salmonella *Typhimurium (n=6) that tested positive per laboratory*
▬▬ : level of good performance

Flaxseed samples artificially contaminated with a high level of Salmonella

All laboratories detected *Salmonella* in all six flaxseed samples contaminated with a high level of *Salmonella* (see Figure 7).

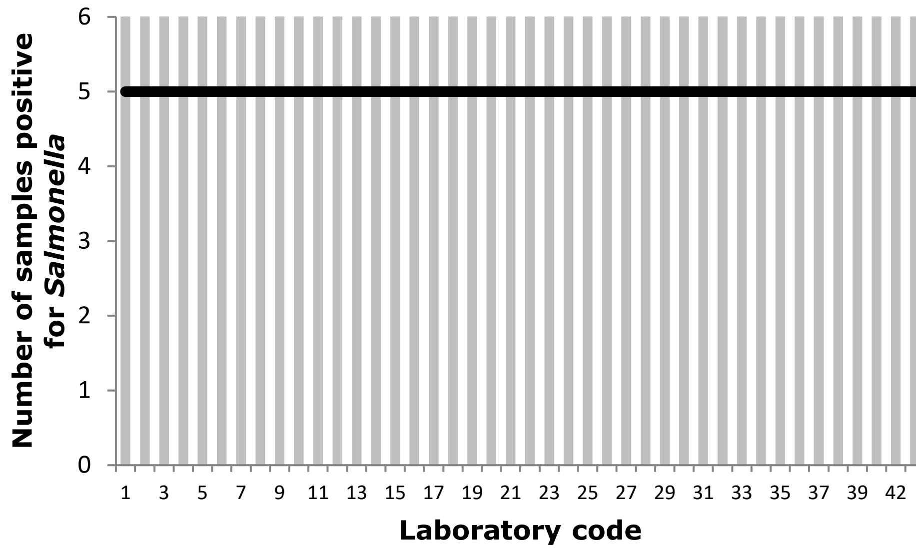

Figure 7. Number of flaxseed samples artificially contaminated with a high level of Salmonella *Typhimurium (n=6) that tested positive per laboratory*
▬▬▬ : level of good performance

4.4.2 *Specificity, sensitivity and accuracy rates of the flaxseed samples*

Table 12 shows the specificity, sensitivity and accuracy rates of the flaxseed samples tested in this Proficiency Test. The calculations were performed on the results of all participants and on the results of the EU-MS participants only. Only minor differences were seen between the two groups and only at the contaminated flaxseed samples with a low level of *Salmonella* Typhimurium.

*Table 12. Specificity, sensitivity and accuracy rates found by all participants ('All') and by the laboratories of the EU Member States only ('EU-MS') with the artificially contaminated flaxseed samples**

Flaxseed samples	Percentage positive	All n = 42	EU-MS n = 37
Negative samples n = 6	No. of samples	252	222
	No. of negative samples	252	222
	Specificity in %	100%	100%
Low level contamination n = 6	No. of samples	252	222
	No. of positive samples	251	221
	Sensitivity in %	99,6%	99,5%
High level contamination n = 6	No. of samples	252	222
	No. of positive samples	252	222
	Sensitivity in %	100%	100%
All flaxseed samples artificially contaminated with *Salmonella*	No. of samples	504	444
	No. of positive samples	503	443
	Sensitivity in %	99,8%	99,8%
All flaxseed samples	No. of samples	756	666
	No. of correct samples	755	665
	Accuracy in %	99,9%	99,8%

* Laboratory 42 made a reporting error for one negative sample. The specificity and accuracy in this table were calculated using the raw data.

4.5 Second detection method

Thirteen laboratories also used a second method for the detection of *Salmonella* in the flaxseed samples. An overview of the methods used per laboratory can be found in Table 13. Only validated methods were used. Seven laboratories use this second detection method routinely for sample analysis.

The results of the second detection methods were all equal to the reported results obtained with EN ISO 6579-1:2017.

Table 13. Details on the second detection methods used by thirteen laboratories during the Proficiency Test for the detection of Salmonella *in flaxseed*

Lab code	Second detection method	Validated	Validated by	Routinely used number of tests/year	Reference
4	MINI VIDAS SLM TEST (LOT:1607019150)	Yes	AFNOR; AOAC	NA	BIO-12/10-09/02; BIO-12/16-09/05;996.08; 020901
5	SureTect real-time PCR (Thermo Scientific)	Yes	Thermo Fischer Scientific	5000	AOAC 051303, AFFNOR UNI 03/07-11/13
11	PCR	Yes	AFNOR and others	NA	QUA 18/03 - 11/02
12	PCR	Yes	In-house validation	10000	R180001 and R18053
17	PCR	Yes	AFNOR BRD 07/06-07/04	2300	ISO 16140
22	qPCR	Yes	Intra laboratory validation	57	Malorny *et al*., (2004)
23	Real Time PCR	Yes	AFNOR	2500	AFNOR BRD 07/06 – 07/04
24	qPCR	Yes	AOAC Research Institute	NA	Certificate Nr.071204.
26	Real time PCR	Yes	In-house validation according to ISO 16140	NA	-
30	qPCR (iQ-Check *Salmonella* II kit, BIORAD)	Yes	AFNOR	400	AFNOR BRD 07/06-07/04
32	qPCR	Yes	AFNOR	1356	AFNOR BRD 07/06-07/04
35	PCR	Yes	In-house validation (method) and AFNOR (PCR kit)	NA	AFNOR AB1 29/02-09/10
36	PCR	Yes	In-house validation	NA	Malorny *et al*., (2004)

NA: Not Applicable

4.6 Performance of the NRLs

Forty-one laboratories fulfilled the criteria of good performance.

One laboratory scored a moderate performance. Laboratory 13 detected *Salmonella* in the procedure control, while *Salmonella* was not detected in their own positive control sample. Laboratory 13 made a mistake when entering the results for the two control samples and switched these results on the result form. This was confirmed by their raw data and no further actions were considered necessary for this laboratory.

5 Conclusions

Forty-one laboratories fulfilled the criteria of good performance for the EURL-*Salmonella* Proficiency Test for the detection of *Salmonella* in flaxseed samples.

One laboratory, laboratory 13, scored a moderate performance for this EURL-*Salmonella* Proficiency Test.

The accuracy rate for the control samples was 100%.

The specificity rate for the negative flaxseed samples was 100%.

The sensitivity rates for the contaminated flaxseed samples with low and high levels of *Salmonella* were respectively 99,6% and 100%.

The accuracy rate of all artificially contaminated flaxseed samples for all participating laboratories was 99,9%.

Thirteen laboratories also performed a second method for the detection of *Salmonella* in the flaxseed samples. The methods used were PCR, qPCR and mini VIDAS. The results of the second detection method were all equal to the reported results obtained with EN ISO 6579-1:2017.

List of abbreviations

AFNOR	Association Française de Normalisation (French Standardization Association)
API	Analytical Profile Index
AOAC	Association of Analytical Communities
ASAP	AES *Salmonella* Agar Plate
BGA	Brilliant Green Agar
BGA(mod)	Brilliant Green Agar (Modified)
BPLS	Brilliant green Phenol-red Lactose Sucrose
BPW	Buffered Peptone Water
BSA	Brilliance *Salmonella* Agar
cfu	colony forming units
DG-SANTE	Directorate-General for Health and Consumer Protection
EC	European Commission
EFTA	European Free Trade Association
EU	European Union
EURL	European Union Reference Laboratory
ISO	International Organization for Standardization
MALDI-TOF	Matrix-Assisted Laser Desorption Ionization – Time Of Flight (Mass Spectrometry)
MKTTn	Muller-Kauffmann TetraThionate-novobiocin broth
MPN	Most Probable Number
MS	Member State
MSRV	Modified Semi-solid Rappaport Vassilliadis agar
NRL	National Reference Laboratory
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
PT	Proficiency Test
qPCR	quantitative Polymerase Chain Reaction
RIVM	Rijksinstituut voor Volksgezondheid en het Milieu (National Institute for Public Health and the Environment)
RVS	Rappaport Vassilliadis with Soya
SM (ID)2	*Salmonella* Detection and Identification-2
STm	*Salmonella* Typhimurium
VRBG	Violet Red Bile Glucose agar
XLD	Xylose Lysine Deoxycholate agar
XLT	Xylose Lysine Tergitol-4

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RIVM

De zorg voor morgen begint vandaag