Interim summary report EURL-*Salmonella*

Proficiency Test food-feed 2019

Detection of *Salmonella* in flaxseed

R.E. Diddens & K.A. Mooijman
RIVM, the Netherlands

Introduction

In March 2019, an EURL-*Salmonella* Proficiency Test for 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 as well as an ingredient of animal feed. Therefore, NRLs-*Salmonella*, which analyse food (products), as well as NRLs-*Salmonella*, which analyse animal feed were invited to participate in this Proficiency Test (PT).
In total 42 NRLs-*Salmonella* participated in this study: 37 NRLs from 28 EU-Member States (MS) and 5 NRLs from third countries (EU candidate MS and potential EU candidate MS and members of the European Free Trade Association (EFTA)). Of the 42 participants, 28 were NRLs-*Salmonella* for food & animal feed, 9 NRLs-*Salmonella* for food and 6 NRLs-*Salmonella* for animal feed.
This interim summary report consists of two parts. One part contains the individual NRL results, which was sent separately to the NRLs-*Salmonella*. The other part contains the overall results of all NRLs-*Salmonella,* which is described here.

Materials & Methods

Samples

Each NRL-*Salmonella* analysed in total 20 samples:

- 6 negative samples of 25 g flaxseed (no *Salmonella* added)
- 6 samples of each 25 g flaxseed with a low level of *Salmonella* Typhimurium (STm)
- 6 samples of each 25 g flaxseed with a high level of *Salmonella* Typhimurium (STm)
- 2 control samples (BPW and own positive control)

A batch of 35.5 kg flaxseed was obtained from a mill in the Netherlands. The flaxseed arrived at EURL-*Salmonella* in November 2018 and was tested negative for *Salmonella*. Until the PT-samples were prepared, the flaxseed was stored in a sealed, plastic container in a dark and dry place.

Early March 2019, subsamples were made by adding 25g flaxseed to sample bags. Each subsample was individually, artificially contaminated with a low or a high level of STm or no *Salmonella* at all (negative samples). Next, the samples were stored at 5 °C.

On Monday 18 March 2019, the food-feed samples were shipped to the NRLs-*Salmonella*. Upon arrival, the NRLs were requested to store the samples at 5 °C until the start of the analysis on Monday 25 March 2019.

The level of natural background flora in the flaxseed was tested in November 2018 (after receipt at the EURL-*Salmonella*) and in March 2019 (after storage). Table 1 shows the number of *Enterobacteriaceae* and aerobic bacteria in the obtained flaxseed.

Table 1. Number of *Enterobacteriaceae* and aerobic bacteria per gram flaxseed (negative for *Salmonella*)

Date	*Enterobacteriaceae* (cfu/g)	Aerobic bacteria (cfu/g)
21 November 2018	$4.6*10^6$	$7.0*10^6$
25 March 2019[a]	$4.0*10^5$	$1.6*10^6$

a. After storage at room temperature for 4 months and at 5 °C for 2 weeks

Table 2 shows the inoculum levels of the diluted culture with *Salmonella* Typhimurium (STm) used to artificially contaminate the flaxseed samples. Also a five tube Most Probable Number (MPN) test was performed on the artificially contaminated flaxseed samples with low and high level STm.

Table 2. Number of *Salmonella* Typhimurium in the inoculum for the artificial contamination of the flaxseed samples and in the flaxseed samples after storage at 5 °C for 2 weeks

Date of testing	Low level STm in cfu per sample	High level STm in cfu per sample
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 2 weeks

The NRLs-*Salmonella* had to analyse the following samples:

6x '25g flaxseed (negative, no *Salmonella* added)'
6x '25g flaxseed + low level of STm'
6x '25g flaxseed + high level of STm'

Additionally, two control samples had to be analysed, being:

1x only BPW (negative procedure control)
1x own positive control sample with *Salmonella* (own positive control)

The flaxseed samples were individually packed and labelled. The decoding of these samples can be found in the tables of the individual NRL results.

Analysis of samples according to EN ISO 6579-1

The prescribed method was EN ISO 6579-1:2017 (Microbiology of the food chain - Horizontal method for the detection, enumeration and serotyping of *Salmonella* - Part 1: Detection of *Salmonella* spp.) and the underlying EN ISO documents, e.g. the EN ISO 6887 series for preparation of test samples.
EN ISO 6579-1:2017 describes technical steps for the detection of *Salmonella* in food, animal feed and samples from the primary production stage. An important change in this document compared to the earlier version of EN ISO 6579 (2002), is the possibility to choose between RVS and MSRV, additional to MKTTn, for the selective enrichment of *Salmonella* from food and animal feed samples. It was also allowed to use all three selective enrichment media.

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-dispense volume of BPW)
- Resuscitate the sample for 20 to 30 minutes at 18 °C to 27 °C (room temperature)
- Mix for 60 s ± 5 s with a homogenizer

NRLs-*Salmonella* had to report the final confirmed results of the samples by indicating if *Salmonella* was ‘detected’ or ‘not detected’ per 25 g of flaxseed sample.

Additionally, the NRLs-*Salmonella* were given an opportunity to analyse the samples with a second detection method, if this is (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.

From the results of all laboratories the specificity, sensitivity and accuracy rates were calculated, as follows:

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

Good performance

Criteria for good performance used in the current EURL-*Salmonella* PT for detection in flaxseed of *Salmonella* are shown in Table 3.

Table 3. Criteria for good performance

Contaminated samples	Percentage positive	# pos samples/ total # samples
Negative samples*	20% max	1/6 max
Low level contamination	50%	3/6
High level contamination	80%	5/6
Control samples	**Percentage positive**	**# pos samples/ total # samples**
BPW	0%	0/1
Own positive control	100%	1/1

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

Results

General

On Monday 18 March 2019 the flaxseed samples were sent to 42 laboratories. The majority of the parcels were delivered at the NRLs within 1- 2 days.

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 40 laboratories varied between 0 and 7 °C. At two laboratories, a temperature was measured at a maximum of 9 °C.

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

Forty-one laboratories used the prescribed method EN ISO 6579-1:2017. One laboratory used EN ISO 6579:2002 (lab code 34).
Thirty-nine laboratories used MKTTn and RVS and/or MSRV as selective enrichment media. Two laboratories used only MSRV as selective medium (lab codes 1 and 24) and one laboratory used RVS and MSRV as selective media (lab code 9). These three laboratories did not used MKTTn as selective enrichment medium, which is prescribed in addition to MSRV and/or RVS in EN ISO 6579-1:2017 for analysis of food and feed samples.

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

Artificially contaminated flaxseed samples

Negative samples
Forty-one laboratories scored all six negative samples correct: *Salmonella* was not detected in the samples.
One laboratory (lab code 42) detected *Salmonella* in one of the negative samples. All negative samples should be tested negative. However, as no 100% guaranty about the *Salmonella* negativity of flaxseed can be given, one positive out of six negative samples (80% neg.) will still be considered as acceptable. However, a false positive result for a negative sample may also been caused by cross-contamination, exchange of samples or by misinterpretation of the results.

Samples with a low level of *Salmonella* Typhimurium
Forty-one laboratories detected *Salmonella* in all six low contaminated flaxseed samples. One laboratory (lab code 21) detected *Salmonella* in five out of six low level contaminated samples. See Figure 1.

Samples with a high level of *Salmonella* Typhimurium
All laboratories detected *Salmonella* in all six high contaminated flaxseed samples. See Figure 2.

In Table 4 the specificity, sensitivity and accuracy rates are given for the flaxseed samples.

Controls

BPW (negative procedure control)
Forty-one laboratories analysed the procedure control sample (no matrix, only BPW) correctly negative for *Salmonella*. Only laboratory 13 reported the procedure control blank as positive.

Own positive control with *Salmonella*
The laboratories were also asked to use their own positive control normally used in their routine analysis for the detection of *Salmonella*.
Forty-one laboratories scored good results with their own *Salmonella* positive control sample. Only laboratory 13 reported the positive control with *Salmonella* as negative.

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

Table 5 gives the correct scores for the control samples with an accuracy rate of 97.6%.

Table 4. Specificity, sensitivity and accuracy rates in the flaxseed samples

Control samples	Percentage positive	Total laboratories n = 42
Negative samples n = 6	No. of samples	252
	No. of negative samples	251
	Specificity in %	99.6%
Low level contamination n = 6	No. of samples	252
	No. of positive samples	251
	Sensitivity in %	99.6%
High level contamination n = 6	No. of samples	252
	No. of positive samples	252
	Sensitivity in %	100%
All flaxseed samples artificially contaminated with *Salmonella*	No. of samples	504
	No. of positive samples	503
	Sensitivity in %	99.8%
All flaxseed samples	No. of samples	756
	No. of positive samples	754
	Accuracy in %	99.7%

Table 5. Correct scores of control samples

Control samples	Percentage positive	Total laboratories n = 42
BPW	No. of samples	42
	No. of negative samples	41
	Correct score in %	97.6%
Own positive control	No. of samples	42
	No. of negative samples	41
	Correct score in %	97.6%
All control samples n=2	No. of samples	84
	No. of negative samples	82
	Accuracy in %	97.6%

Performance of the participants

Forty-one laboratories fulfilled the criteria of good performance.

One laboratory scored below the level of good performance. Laboratory 13 detected *Salmonella* in the BPW control sample and *Salmonella* was not detected in their own positive control sample.

The EURL-*Salmonella* will contact the laboratory for further explanations.

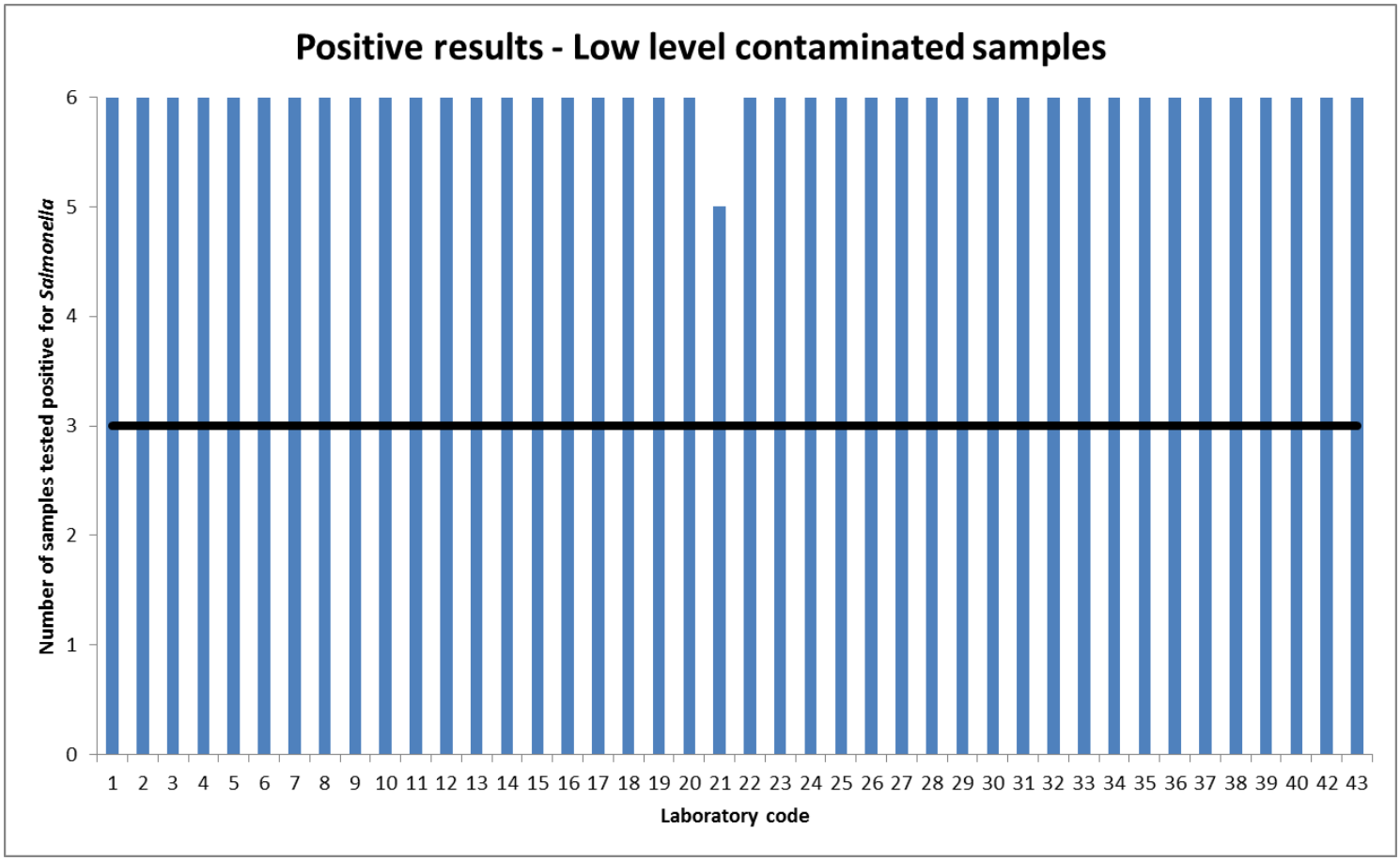

Figure 1. Number of flaxseed samples artificially contaminated with a low level of *Salmonella* Typhimurium (n=6), tested positive for *Salmonella* by each participant.

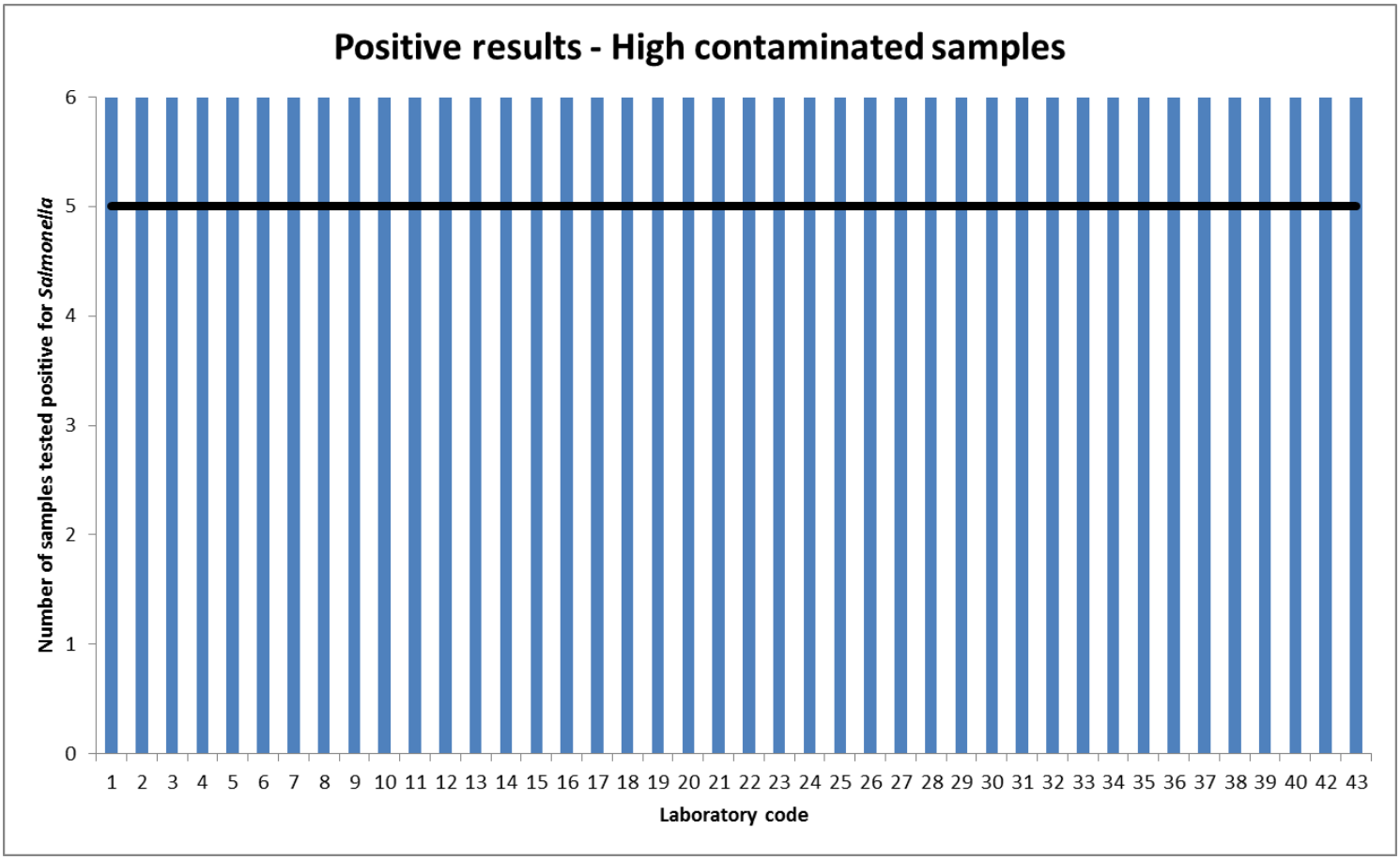

Figure 2. Number of flaxseed samples artificially contaminated with a high level of *Salmonella* Typhimurium (n=6), tested positive for *Salmonella* by each participant.

List of abbreviations

BPW	Buffered Peptone Water
cfu	colony forming units
EFTA	European Free Trade Associations
EU	European Union
EURL	European Union Reference Laboratory
ISO	International Organization for Standardization
MKTTn	Muller-Kauffmann TetraThionate-novobiocin broth
MPN	Most Probable Number
MS	Member State
MSRV	Modified Semi-solid Rappaport Vassilliadis agar
NRL	National Reference Laboratory
PT	Proficiency Test
RVS	Rappaport Vassilliadis with Soya
STm	*Salmonella* Typhimurium

References

EN ISO 6579-1: 2017. Microbiology of the food chain – Horizontal method for the detection, enumeration and serotyping of *Salmonella* spp. Part 1: Detection of *Salmonella* spp.

EN ISO 6579: 2002. Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp.

EN ISO 6887-1 & -4: 2017. Microbiology of the food chain - Preparation of test samples, initial suspension and decimal dilutions for microbiological examination - series for preparation of test samples. Part 1: General rules for the preparation of the initial suspension and decimal dilutions. Part 4: Specific rules for the preparation of miscellaneous products

EN ISO/TS 22117: 2010. Microbiology of food and animal feeding stuffs — Specific requirements and guidance for proficiency testing by interlaboratory comparison.

EN ISO 22117: 2019. Microbiology of the food chain — Specific requirements and guidance for proficiency testing by interlaboratory comparison.

Robin Diddens E-mail: robin.diddens@rivm.nl Tel.: + 31 30 274 7039
Kirsten Mooijman E-mail: kirsten.mooijman@rivm.nl Tel.: + 31 30 274 3537

EURL-*Salmonella*
National Institute for Public Health and the Environment (RIVM)
Centre for Zoonoses and Environmental Microbiology (Z&O – Pb 63)
Antonie van Leeuwenhoeklaan 9, P.O. Box 1
3720 BA Bilthoven, the Netherlands

Website: http://www.eurlsalmonella.eu/