Interim summary report

EURL-*Salmonella* Proficiency Test Food-Feed 2023

Detection of *Salmonella* spp. in flaxseed

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1. Introduction

In March 2023, the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*) organised a Proficiency Test (PT) for the detection of *Salmonella* in food and feed for the National Reference Laboratories for *Salmonella* (NRLs-*Salmonella*). The matrix under analysis was flaxseed, which is used as a food product as well as an ingredient of animal feed. NRLs-*Salmonella* which analyse food samples and NRLs-*Salmonella* which analyse animal feed products were invited to participate in this PT.
There were in total 51 participants in this PT: NRLs-*Salmonella* from 27 EU Member States and 6 NRLs-*Salmonella* from third countries (EU candidate Member State, members of the European Free Trade Association (EFTA), and United Kingdom).
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.

2. Materials & Methods

2.1 Samples

The samples of this PT consisted of flaxseed with different concentrations of *Salmonella* Typhimurium.

Each NRL-*Salmonella* had to analyse in total 16 samples:
- 4 negative samples of each 25 g flaxseed (no *Salmonella* added);
- 6 samples of each 25 g flaxseed with a low level of *Salmonella* Typhimurium (STm);
- 4 samples of each 25 g flaxseed with a high level of *Salmonella* Typhimurium (STm);
- 1 procedure control (BPW only)
- 1 positive control (laboratories' own *Salmonella* control strain)

A batch of 25 kg flaxseed was obtained on 09-01-2023 from a mill in the Netherlands. The batch was used for performing pre-tests and for preparation of the PT samples. The batch flaxseed was stored at room temperature until sample preparation. The absence of *Salmonella* was checked by analysing in total ten randomly taken samples of 25 g from the flaxseed batch, following EN ISO 6579-1:2017/A1:2020. *Salmonella* was not detected in any of the tested samples.

By the end of February 2023, the samples for the PT were prepared. For this, 784 subsamples of each 25 g flaxseed were weighed into (plastic) sample bags. In total, 224 subsamples were individually, artificially contaminated with a high level, and 336 subsamples with a low level of the diluted overnight culture of *S*. Typhimurium; 224 subsamples were not contaminated with *Salmonella* (negative samples). The decoding of these samples can be found in the table of the individual NRLs-*Salmonella* results. Next, the samples were stored at 5 °C until shipment.

On Monday 20 March 2023, the PT samples were shipped to the NRLs-*Salmonella*. During transport the samples were kept cool by using frozen cooling elements and the temperature during transport was registered by an electronic temperature device ('temperature probe'). Upon arrival, the NRLs-*Salmonella*

were requested to store the samples, together with the temperature probe, at 5 °C until the start of the analysis on Monday 27 March 2023.

The level of natural background flora in the flaxseed was tested at the EURL-*Salmonella* on 10 January 2023 (shortly after receipt of the flaxseed) and on 27 March 2023, at the start of the PT, following EN ISO 4833-1:2013 and EN ISO 21528-2:2017. Table 2.1 shows the number of aerobic bacteria and *Enterobacteriaceae* in the flaxseed.

Table 2.1 Number of aerobic bacteria and Enterobacteriaceae *per gram flaxseed*

Date	Aerobic bacteria (cfu/g)	*Enterobacteriaceae* (cfu/g)
10 January 2023	$1{,}1 \times 10^7$	$5{,}9 \times 10^6$
27 March 2023[a]	$4{,}0 \times 10^6$	$2{,}1 \times 10^6$

[a] After storage at room temperature for 9 weeks and at 5 °C for 13 days

Table 2.2 shows the (low and high) inoculation levels of the diluted culture of *Salmonella* Typhimurium used to artificially contaminate the flaxseed samples. Also a five tube Most Probable Number (MPN) test was performed on the artificially contaminated PT samples with low and high level STm. The MPN test was performed at the start of the PT at the EURL-*Salmonella*.

Table 2.2 Number of Salmonella *Typhimurium in the inoculum for artificial contamination of the flaxseed samples and in the PT samples after storage at 5 °C for 13 days*

Date	Low level STm in cfu per sample	High level STm in cfu per sample
14 March 2023 Inoculation of flaxseed	9	52
27 March 2023[a] MPN of flaxseed samples, inoculated with STm (95% confidence limit)	3,25 (1,1 – 10,3)	7 (2,3 – 21,5)

[a] After storage at 5 °C for 13 days

2.2 Analysis of samples following EN ISO 6579-1:2017/A1:2020

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.), including Amendment 1 (EN ISO 6579-1:2017/A1:2020), and the underlying EN ISO documents, e.g. the EN ISO 6887 series for preparation of test samples.

For this Proficiency Test the laboratories could follow their own procedure for preparation of the test samples for this type of matrix or follow the procedure below. This procedure is slightly adjusted from EN ISO 6887-4:2017 to avoid punctures in the plastic sample bags:

- Add the BPW to the 25 g 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 30 seconds (± 5 seconds) by hand;
- Continue with the non-selective pre-enrichment procedure as described in EN ISO 6579-1:2017(/A1:2020).

EN ISO 6579-1:2017(/A1:2020) describes the technical steps for the detection of *Salmonella* in food, animal feed samples, environmental samples from the food production area, and samples from the primary production stage. For food and feed samples, EN ISO 6579-1:2017(/A1:2020) prescribes the use of two selective enrichment media. In addition to Muller-Kauffmann tetrathionate-novobiocin broth (MKTTn) either Rappaport-Vassiliadis with Soya (RVS) broth or Modified semi-solid Rappaport-Vassiliadis agar (MSRV) agar shall be used or all three selective enrichment media.

In summary:
- pre-enrichment in:
  Buffered Peptone Water (BPW);
- selective enrichment in/on:
  Muller-Kauffmann tetrathionate-novobiocin (MKTTn) broth;
  Modified semi-solid Rappaport-Vassiliadis (MSRV) agar and/or;
  Rappaport-Vassiliadis 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(/A1:2020)) or reliable, commercially available identification kits.

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

Additionally, the NRLs-*Salmonella* were allowed 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(/A1:2020) were used to assess the performance of each NRL.

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

Specificity rate

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

Sensitivity rate

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

Accuracy rate

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

2.3 Performance analysis

Criteria for good performance used in the current EURL-*Salmonella* PT for detection of *Salmonella* in flaxseed are shown in Table 2.3.

In the past, the tools for subtyping isolates from 'false-positive' samples were limited, but nowadays it is well possible to precisely distinguish the inoculum strain from other *Salmonella* strains by using Whole Genome Sequencing (WGS). For that reason the new criterion for negative samples will become: 'No *Salmonella* detected in any of the negative samples. However, as 100% *Salmonella*-free matrix cannot be guaranteed, an incidental positive result with a *Salmonella* strain different from the inoculum strain may still be acceptable.' Therefore, the EURL-*Salmonella* asks the NRLs-*Salmonella* to conserve one *Salmonella* confirmed colony from each positive sample, so that in case of deviating results additional tests can be performed.

Table 2.3 Criteria for good performance

Contaminated samples	**Percentage positive**	**# pos samples/ total # samples**
Negative samples	0%*	0* / 4
Low level of *S.* Typhimurium	≥ 50%	≥ 3 / 6
High level of *S.* Typhimurium	≥ 75%	≥ 3 / 4
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, so that an incidental positive result with a *Salmonella* strain different from the inoculation strain is still considered as acceptable.

3. Results

3.1 General

NRLs-*Salmonella* which analyse both food and feed samples were able to request two different laboratory codes (separate as NRL-*Salmonella* food and as NRL-*Salmonella* feed) and these laboratories could also request two (similar) sets of samples. In total 51 laboratory codes were generated for this EURL-*Salmonella* PT.
On Monday 20 March 2023 the Proficiency Test samples were sent to all participants. Forty parcels were delivered at the NRLs-*Salmonella* within one or two days. NRLs-*Salmonella* which requested two sets of samples, received one parcel with both sets of samples. The parcel destined for laboratory 47 and 48 was held at customs and arrived after eight days at the laboratory. This laboratory started with the PT on 28 March 2023.

The temperature during transport and storage was registered using a temperature probe. The temperature of all parcels during transport was below 2,5 °C, except for the parcel of laboratories 47 and 48. The temperature of this parcel reached a maximum of 11 °C, when held at customs.

The measured storage temperature of the samples at the laboratories varied between 0 and 9 °C.

All laboratories used the prescribed method EN ISO 6579-1:2017(/A1:2020). Fifty laboratories used MKTTn and RVS and/or MSRV as selective enrichment media. One laboratory used only MSRV as selective medium (laboratory 25). This laboratory did not used MKTTn as selective enrichment medium, which is prescribed in addition to MSRV and/or RVS in EN ISO 6579-1:2017(/A1:2020) for analysis of food and feed samples.

Nineteen laboratories also used a second detection method for analysing the samples, but the results of this second method were not used to assess the performance of these laboratories. Fifteen laboratories used a real-time PCR as additional method, three laboratories used VIDAS and one laboratory a PCR. The results of the second detection methods were all similar to the reported results obtained with EN ISO 6579-1:2017(/A1:2020) by the laboratories.

3.2 Artificially contaminated flaxseed samples

Negative samples

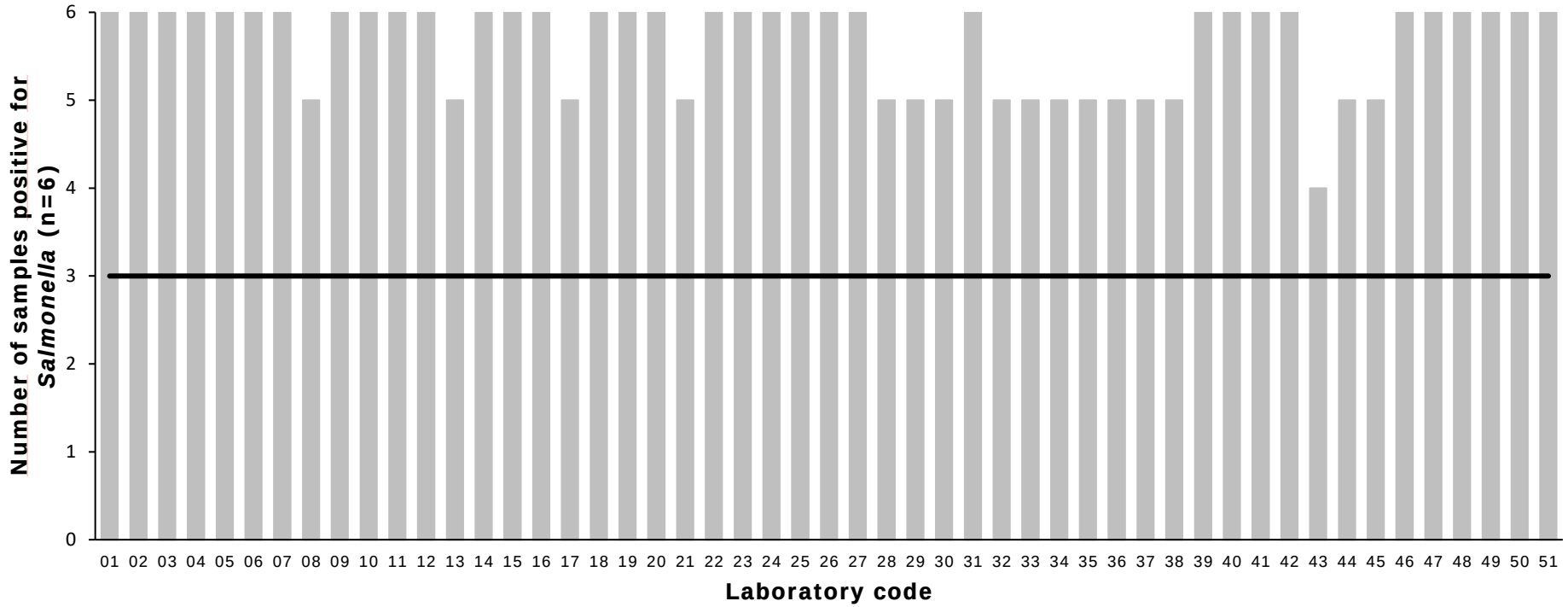
All fifty-one laboratories scored all four negative samples correctly: *Salmonella* was not detected. Only laboratory 14 reported one of the negative samples as positive for *Salmonella*. This laboratory made an administrative error and the result had to be 'Not detected'. The communication was done after the deadline of the PT, but before the intended results were shared with the laboratory. An explanation was given how this error came to light after the deadline of reporting and raw data confirmed the (negative) result of this sample.

Samples with a low level of *Salmonella* Typhimurium

Thirty-four laboratories detected *Salmonella* in all six low contaminated flaxseed samples. Sixteen laboratories detected *Salmonella* in five out of six low level contaminated samples and one laboratory detected *Salmonella* in four out of six low level contaminated samples. Both cases still fulfils the criteria 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 of the six samples. See Figure 3.1.

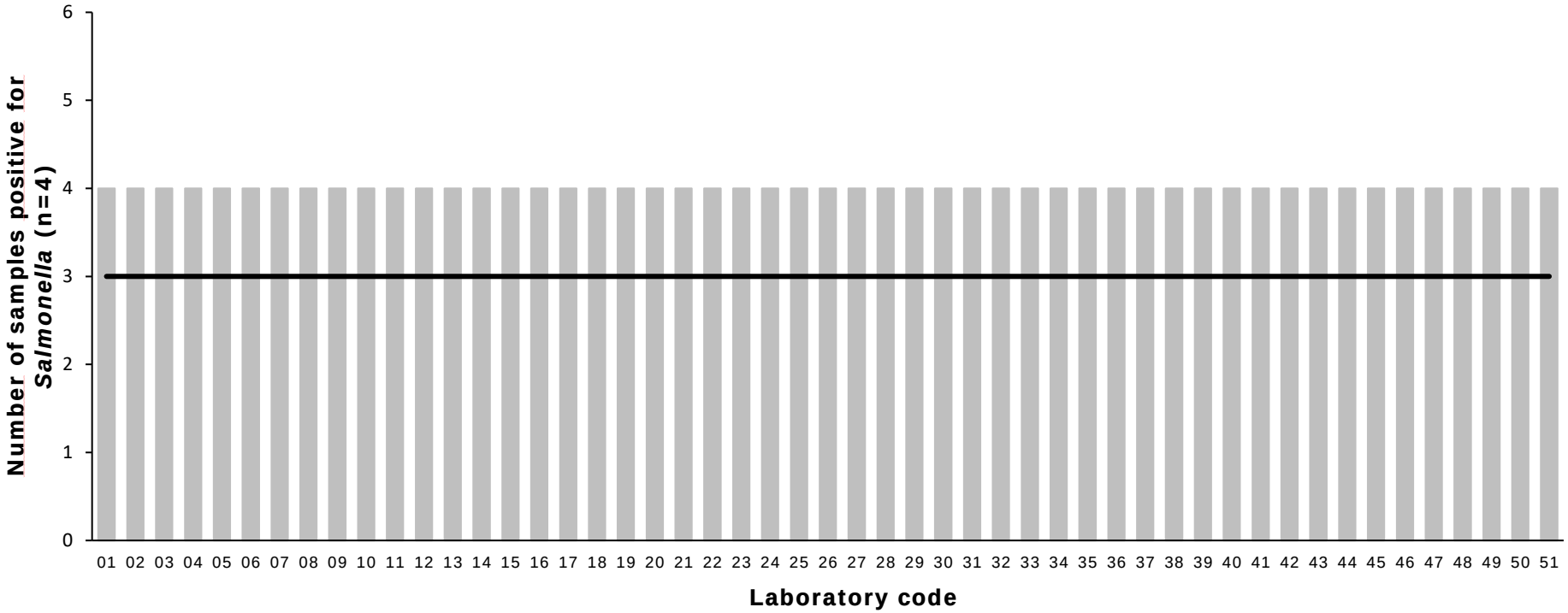
Samples with a high level of *Salmonella* Typhimurium

All laboratories detected *Salmonella* in all four flaxseed samples contaminated with a high level of *Salmonella* Typhimurium. The results are shown in Figure 3.2.



:level of good performance

Figure 3.1 Number of positive Salmonella *isolations per laboratory found in the flaxseed samples artificially contaminated with a low level of* Salmonella *Typhimurium (n=6)*



: *level of good performance*

Figure 3.2 Number of positive Salmonella *isolations per laboratory found in the flaxseed samples artificially contaminated with a high level of* Salmonella *Typhimurium (n=4)*

In Table 3.1 the specificity, sensitivity and accuracy rates are given for the flaxseed samples.

*Table 3.1 Specificity, sensitivity and accuracy rates of the flaxseed samples**

Samples		**All participants n = 51**
Negative samples n = 4	No. of samples	204
	No. of negative samples	204
	Specificity in %	**100%**
Low level of *Salmonella* Typhimurium n = 6	No. of samples	306
	No. of positive samples	288
	Sensitivity in %	**94%**
High level of *Salmonella* Typhimurium n = 4	No. of samples	204
	No. of positive samples	204
	Sensitivity in %	**100%**
All flaxseed samples artificially contaminated with *Salmonella*	No. of samples	510
	No. of positive samples	492
	Sensitivity in %	**96%**
All flaxseed samples	No. of samples	714
	No. of correct samples	696
	Accuracy in %	**97%**

*Laboratory 14 made an administrative error when reporting one of the negative samples. The specificity and accuracy in this table were calculated using the correct raw data.

3.3 Control samples

Procedure control (BPW only)
All laboratories analysed the procedure control sample (BPW only) correctly: *Salmonella* was not detected.

Own 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 own *Salmonella* positive control sample.

The *Salmonella* serovars used by the majority of the participants for the positive control sample were: *S.* Typhimurium (15), *S.* Enteritidis (13), *S.* Nottingham (7), and sixteen participants used other *Salmonella* serovars.

Table 3.2 gives the correct scores for the control samples with an accuracy rate of 100%.

Table 3.2 Correct scores of the control samples

Control samples		All participants n = 51
Procedure control (BPW only) n=1	No. of samples	51
	No. of negative samples	51
	Correct score in %	**100%**
Positive control (Own *Salmonella* control) n=1	No. of samples	51
	No. of positive samples	51
	Correct score in %	**100%**
All control samples n=2	No. of samples	102
	No. of correct samples	102
	Accuracy in %	**100%**

4. Performance of the participants

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

One laboratory (laboratory 14) initially scored a moderate performance, because the laboratory made an administrative error when reporting the results of a negative sample. One of the (negative) samples was mistakenly reported as positive for *Salmonella*. The error was detected by lab 14 and communicated with the EURL-*Salmonella* after the reporting-deadline of the PT, but before the intended results were shared with the laboratory. An explanation was given how this error came to light and raw data confirmed the (negative) result of this sample. It was therefore decided to give laboratory 14 a 'Good performance*', with the above additional explanation.

5. 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-Vassiliadis
NRL	National Reference Laboratory
PCR	Polymerase Chain Reaction
PT	Proficiency Test
RVS	Rappaport-Vassiliadis medium with Soya
STm	*Salmonella* Typhimurium
XLD	Xylose Lysine Deoxycholate agar
WGS	Whole Genome Sequencing

6. 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 6579-1:2017/A1:2020. Microbiology of the food chain - Horizontal method for the detection, enumeration and serotyping of *Salmonella* - Part 1: Detection of *Salmonella* spp. - Amendment 1 Broader range of incubation temperatures, amendment to the status of Annex D, and correction of the composition of MSRV and SC (ISO 6579-1:2017/Amd 1:2020).

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.

7. Contact

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