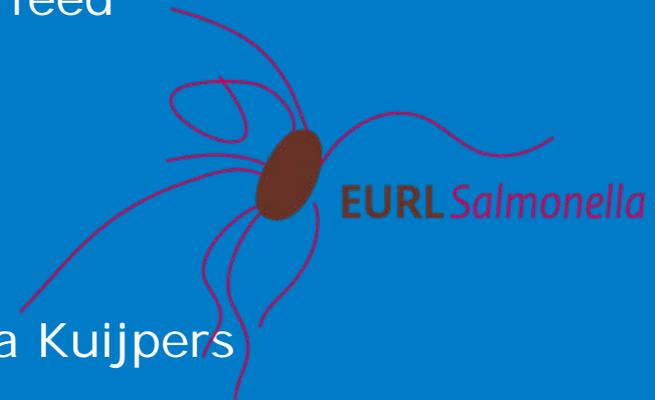




National Institute for Public Health
and the Environment
Ministry of Health, Welfare and Sport

EURL *Salmonella* Interlaboratory comparison study FEED IV 2018

Detection of *Salmonella* in
chicken feed



Angelina Kuijpers



Time table

Week (2018)	Dates	Subject
7	12 – 16 February	Mailing of the protocol and instructions for the web based test report and link to the NRLs by email.
8	19 February	Mailing of parcels to the NRLs
9	26 February	Performance of the study
12	Before 22 March	Deadline for completing the electronic submission of results.



EURL *Salmonella* detection study



Comparable to earlier studies

The number of samples to be tested and the type of samples are comparable to the studies organised since 2013:

- Artificial contamination of samples at laboratory EURL-*Salmonella* before sending to NRLs
- Use of webbased testreport
- Less control samples: one blank control & one own positive control
- No Standard Operating Procedure (SOP), Follow EN ISO 6579-1 2017 (and the underlying EN ISO documents) according to normal routine procedure for detection of *Salmonella* in 'official' samples.
- Reporting results positive or negative per sample



Pre-test: stability of samples (I)

chicken feed with *Salmonella*

PRE-TEST

- Artificially contamination of chicken feed with a diluted *Salmonella* culture
- *Tested:* *Salmonella* Mbandaka (SMb/dog food)
Salmonella Typhimurium (STM/chicken)
- Level of contamination/25 g feed: 10- 15 CFU and 100 CFU
- Stability of samples during: storage & transport (5 °C & 10 °C):
-*Salmonella* detection and influence of backgroundflora
- BRO feed 2014 low level 20 CFU/25g: 98% positive
aim 2018 low level ~10 CFU/25g: 60-70% positive





Pre-test: stability of samples (II) chicken feed

Different types of chicken feed tested:

1. Laying meal (COMPLETE FEED FOR LAYING HENS)
2. Laying meal with 4 grains (COMPLETE FEED FOR LAYING HENS)
3. Vitamix chicken (COMPLETE FEED FOR POULTRY)



Backgroundflora of 3 feed was comparable:

Enterobacteriaceae ~10⁴ CFU/g
 Aerobic ~10⁵ CFU/g

Fungi present in all samples but > 1. laying meal

Further experiments with Laying meal with 4 grains





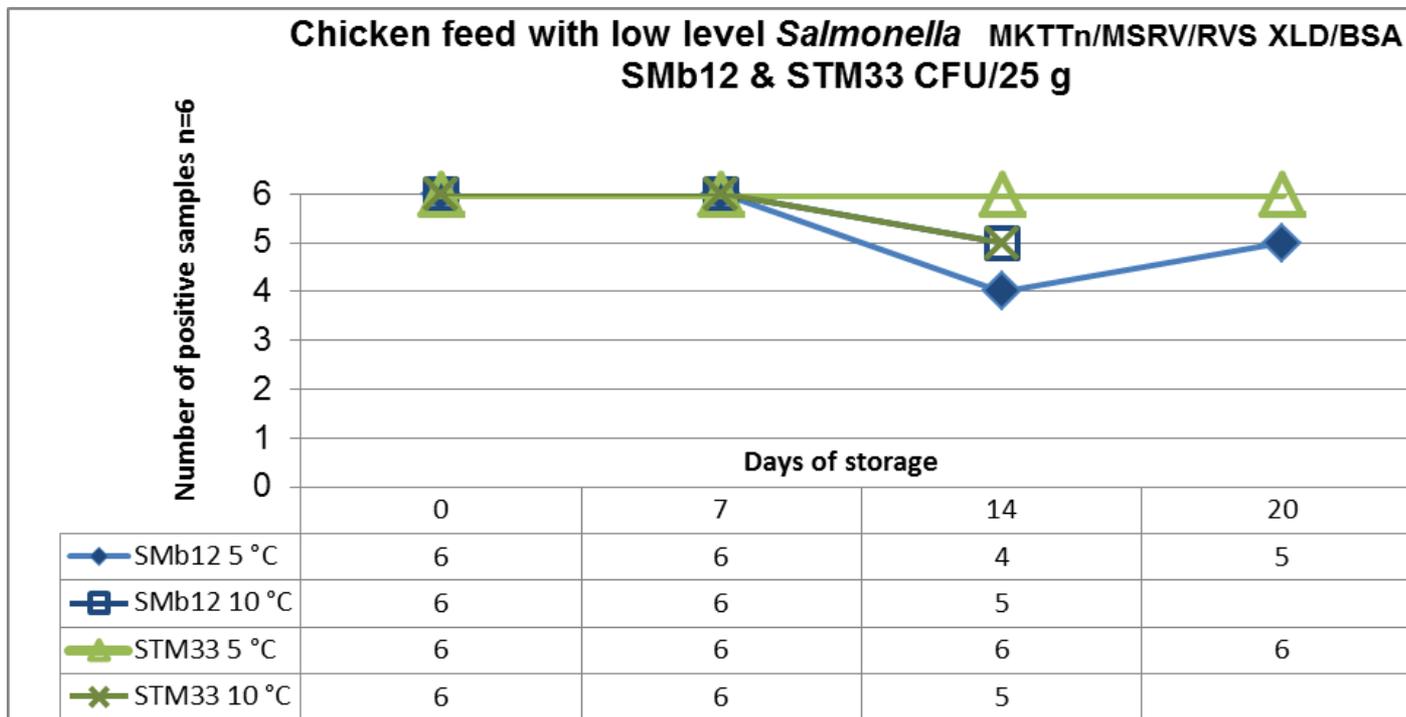
Pre-test: stability of samples (III)

chicken feed

6 samples with SMb12 and 6 with STM33 tested

3 weeks at 5 °C & 10 °C

Test 6 samples after 1, 2 and 3 weeks of storage





Pre-test: stability of samples (III)

chicken feed

Backgroundflora: *samples tested*

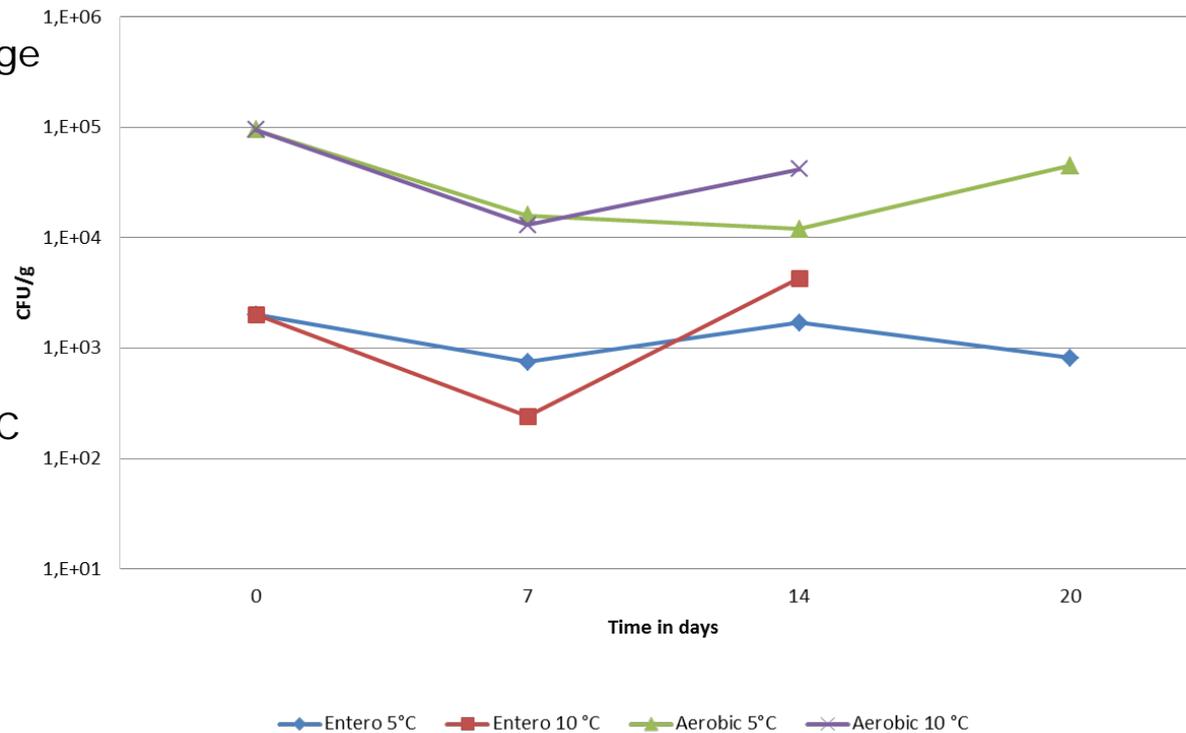
3 weeks at 5 °C & 10 °C

Test after 1, 2 and 3 weeks of storage

Enterobacteriaceae 10^3 - 10^4 CFU/g

Aerobic 10^4 - 10^5 CFU/g

Stable for 2/3 weeks at 5 °C & 10 °C





Pre-test: stability of samples (IV)

chicken feed with *Salmonella* Mbandaka

CONCLUSION

- Matrix: chicken feed, Laying meal with 4 grains
- Artificial contamination of chicken feed with a diluted culture :
 - SMb low level (8 - 10 CFU/25g feed)
 - SMb high level (50 -100 CFU/25g feed)
 - Blank
- After contamination of the feed with SMb
Storage at the EURL and the NRLs at 5 °C





Study design

Artificially contaminated	Samples (n=18): 25 g chicken feed
S. Mbandaka low	6
S. Mbandaka high	6
Blank	6

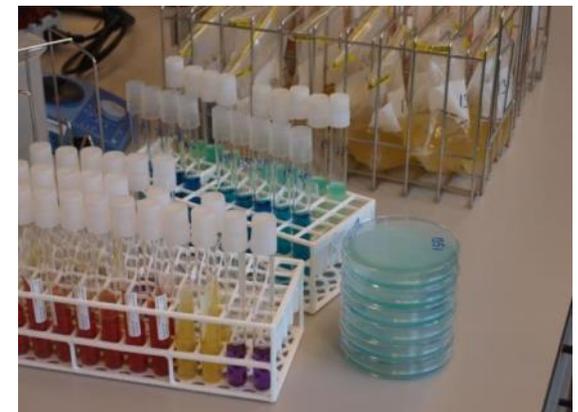
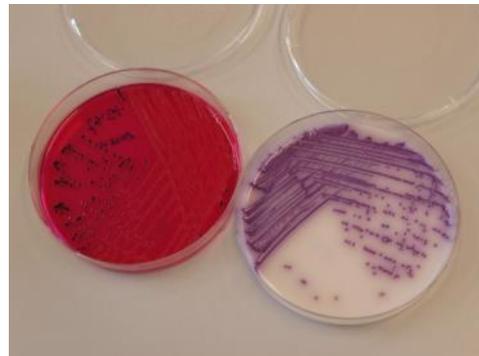
Number and level of samples according to ISO/TS 22117 (2010)

Control samples	n=2
Procedure control blank: BPW	1
Positive control: Own control with <i>Salmonella</i>	1



Analytical methods in study

- Follow ISO 6579-1 (choose between RVS and MSRV)
 - Selective enrichment in : MKTTn and RVS or MKTTn and MSRV or MKTTn, RVS and MSRV
- Isolation medium
 - XLD and 2nd agar for choice
- Confirmation





Participants

35 Laboratories

- 30 NRLs from 28 EU-Member States (MS)
- 5 NRLs from third countries
 - EU candidate MSs or potential EU candidate MSs
 - members of EFTA countries (European Free Trade Association)
 - non-EU



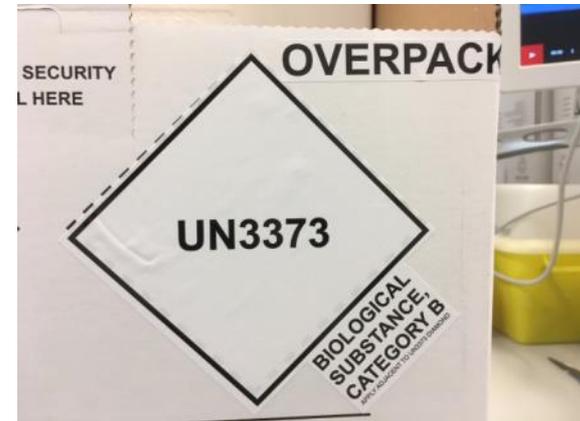


Transport of samples

Cooling devices & Temperature logger

Packages all transported as Biological Substances Cat. B UN3373 by DHL door-to-door

- **Transport time:**
 - 28 parcels **1 day**
 - 5 parcels **2 days**
 - 1 parcel (non-EU MS) **3 days** delay at the customs
 - 1 parcel (non-EU MS) **7 days** delay at the customs





Transport of samples

Cooling devices & Temperature I
Packages all transported as Biological Sub

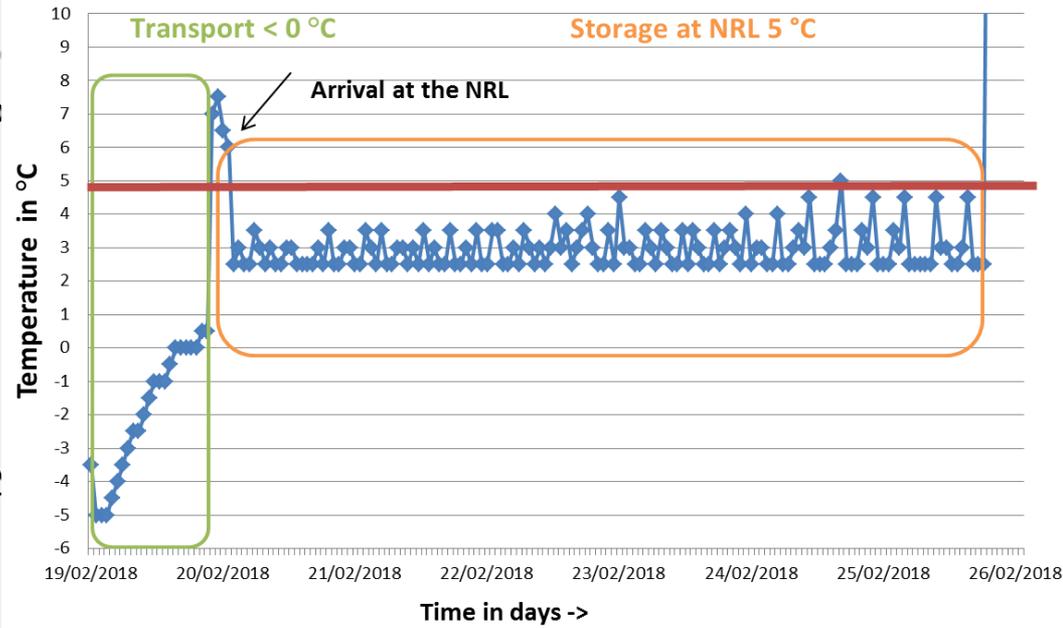
Temperature during

-transport:

- 32 NRLs between -5 °C and +1 °C
- 1 NRL between +1 °C and +5 °C
- 2 NRLs 1 day -5 °C and +1 °C second day
- 1 NRL 2 days -5 °C and +1 °C 4 days +1

-storage at the NRL

- 27 NRLs between +1°C and +5°C
- 8 NRLs between +5°C and +10°C





Material & Methods

Follow as much as possible your routine procedure

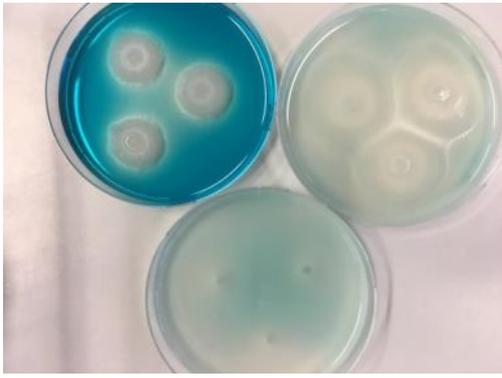
- Addition of 225 ml BPW to plastic filter bags (samples)
(BPW at least at room temperature)
- Homogenisation (according ISO 6887)
(before homogenisation 20-30 min room temperature)
- Incubation BPW sample at 37 °C for 18 h
- Selective enrichment media MKTTn and RVS and/or MSR/V
- Isolation on XLD and 2nd plate & Confirmation





Inoculation level of *S. Mbandaka*

Date of testing	Low level SMb CFU/sample 25 g	High level SMb CFU/sample 25 g
13 February 2018 Inoculum of animal feed	8	91



Performance of the study 26 February 2018



Backgroundflora chicken feed

Date of testing	Enterbacteriaceae CFU/g	Aerobic bacteria CFU/g
15 January 2018	$1 * 10^5$	$2 * 10^5$
26 February 2018 After storage for 6 weeks at +5 °C	$4 * 10^4$	$5 * 10^5$

Performance of the study 26 February 2018





Accreditation according to ISO/IEC 17025

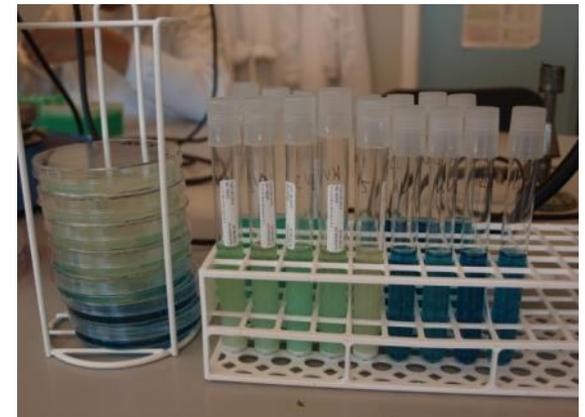
All 35 participants are accredited

Accredited method:

24 NRLs EN ISO 6579-1:2017

15 NRLs ISO 6579: 2002 (MKTTn and RVS)

10 NRLs Annex D of ISO 6579: 2007 (MSRV)





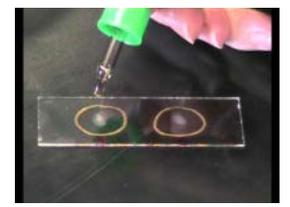
Control samples

The laboratories used their own routine positive control.

		#labs
Positive controls: (2 -10 ⁸ CFU/sample)	diluted culture	19
	lenticule disc	8
	freeze dried ampoule, culti-loop, kwik-stik	2 each
	Capsule, frozen cult	1 each



<i>Salmonella</i> serovar	Enteritidis	16
	Typhimurium	7
	Nottingham	4
	<i>Tennessee, bongori, Harleystreet, Alachua</i>	1 each
	<i>Blegdam, Infantis, Abaetetuba, Poona</i>	





Results control samples

Procedure control Blank (BPW)

- 34 NRLs analysed this sample correctly negative
- 1 NRL reported a positive result

Positive control (own) with *Salmonella*

- 32 NRLs analysed this sample correctly positive
- 3 NRLs reported a negative result

Correct scores (%)

Control samples

	MKTTn and RVS or/and MSRV XLD or 2nd plate
Procedure control (BPW)	97%
Positive control (own)	91%
All control samples	94%



Results chicken feed samples

Blank

- 31 NRLs scored all 6 blank chicken feed samples correctly negative.
- 3 laboratories found 1 sample positive for *Salmonella*.

Low SMb

- 9 NRLs detected *Salmonella* in 1 or 2 out of 6 samples.
- Most NRLs could not detect *Salmonella* in any of the 6 samples.

High SMb

- 33 NRLs detected *Salmonella* in at least 1 out of 6 samples.
- 2 NRLs found all samples negative for *Salmonella*.



We have a problem !!!!

All participants were informed about unexpected results on day 5 of the study, just before the weekend.....



Performance NRLs

Difficult to judge performance of NRLs

Therefore no judgment, but only description of results

Expected results control samples

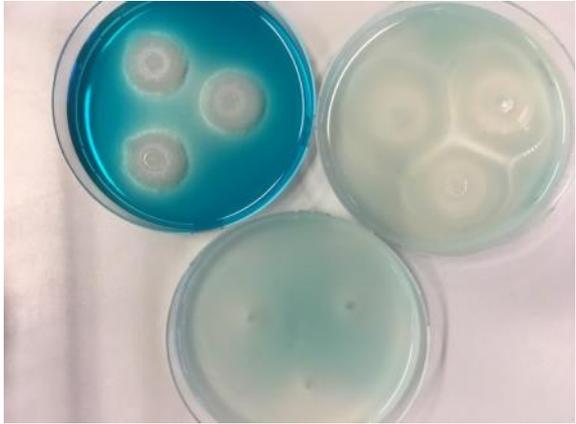
Control samples (n=2)	Percentage positive	No. of positive samples/ Total No. of samples
Procedure control blank: BPW	0 %	0/1
Positive control: Own control with <i>Salmonella</i>	100%	1/1

Samples (n=6) Chicken feed	Percentage positive	No. of positive samples/ Total No. of samples
Blank	20 % at max	1/6 at max



Mean number of positive samples found by all participants

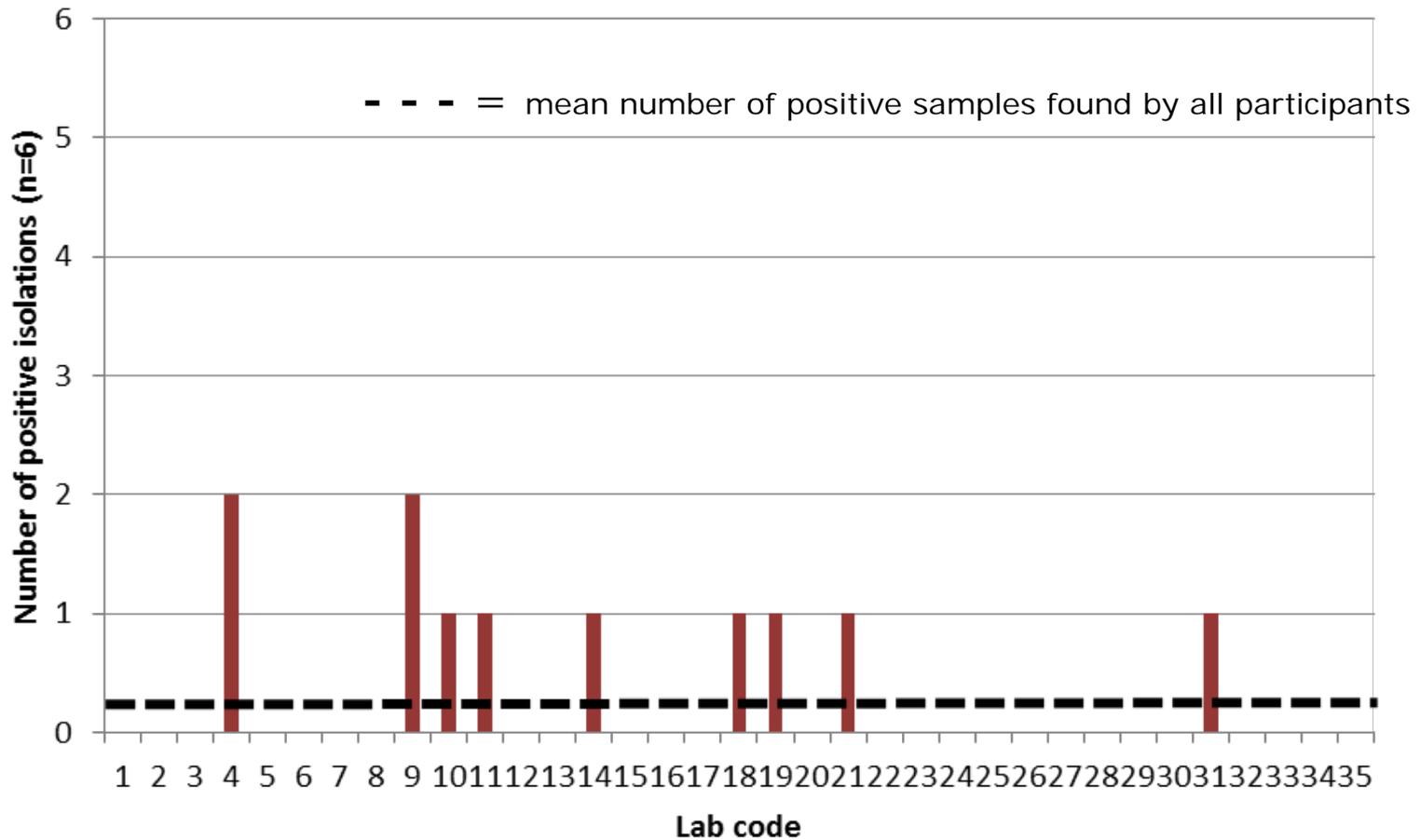
Samples (n=12) Chicken feed	Percentage positive	Mean No. of positive samples/ Total No. of samples
S. Mbandaka low	5 %	0.3/6
S. Mbandaka high	52 %	3.1/6





Results chicken feed samples

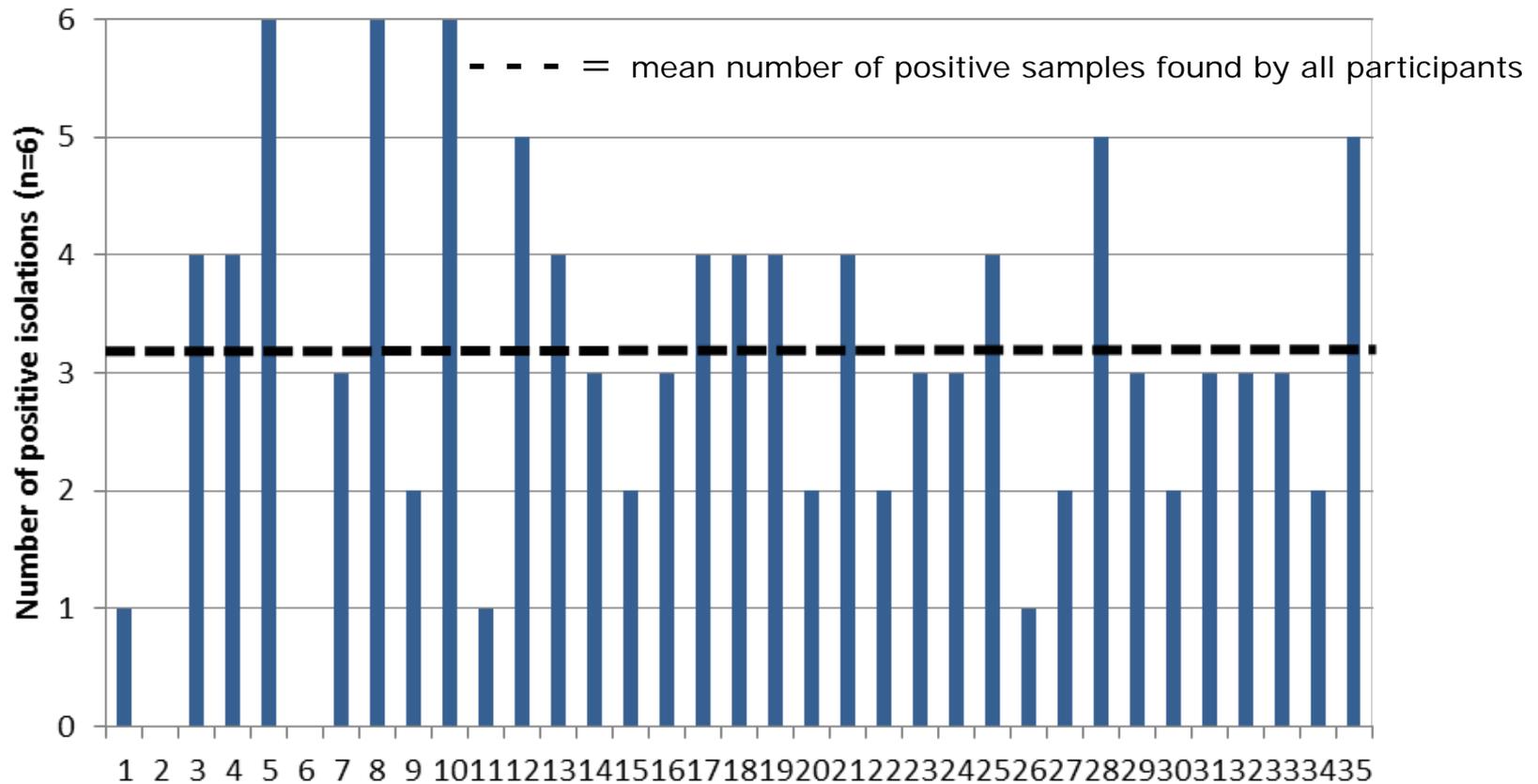
Low level





Results chicken feed samples

High level





Specificity, sensitivity & accuracy (%)

chicken feed samples

		MKTTn and RVS or/and MSRV XLD or 2nd plate
Specificity	Blank	99%
Sensitivity	Low SMb	5%
	High SMb	52%
	All SMb samples	29%
Accuracy	All samples	52%



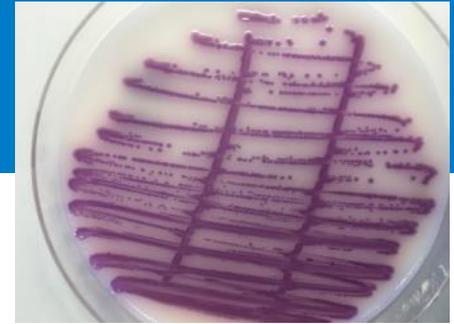


Level of contamination *S. Mbandaka*

Date of testing	Low level SMb CFU/sample 25 g	High level SMb CFU/sample 25 g
13 February 2018 Inoculum of animal feed	8	91
26 February 2018 Feed inoculated with SMb and stored at 5°C for 13 days	0 MPN (0-0.7)	1.1 MPN (0.4-3)

Performance of the study 26 February 2018
MPN: Most Probable Number (5 tube), (95 % confidence interval)





Results control samples procedure (BPW) and positive control

The results of the control samples compared with the expected results

The EURL contacted the 3 laboratories for their deviating results of their control sample (s). Explanations given:

- reporting error
- technical problems with the incubator during pre-enrichment
(no positive results at all)



Results chicken feed samples

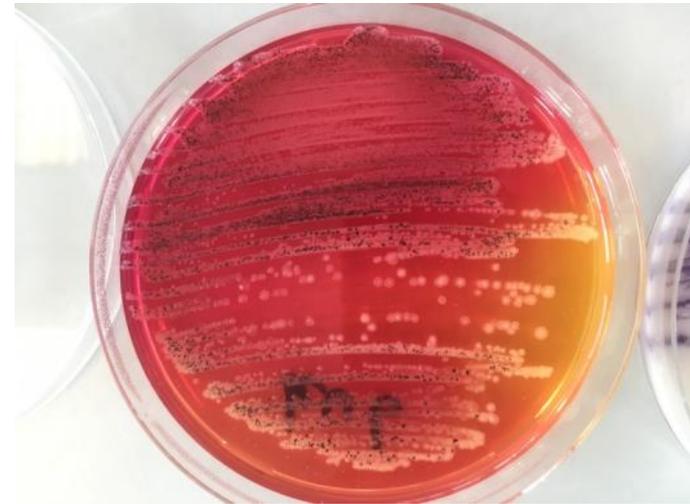
Blank

- 3 laboratories found 1 sample positive for *Salmonella* (2x SMb)

high amount of disturbing backgroundflora
(*Citrobacter*, *Enterobacter*, *Pseudomonas*, *Proteus*, *E. coli*)

Clarification:

- *exchange*
- *cross-contamination*
- *misinterpretation of the results ?*



Contaminated samples

- an unexpected high number of negative results of the artificially contaminated chicken feed samples → Decided not to set criteria



What happened with the *Salmonella* in the chicken feed ?

- The study showed an unexpected high number of negative results.

This was not expected from the pre-tests with the same:

- chicken feed
- strain om *S. Mbandaka*



- High number (1 log) of backgroundflora: especially *Enterobacteriaceae* present in the batch of chicken feed used in the study (also mentioned by the participants).

This may influence the detection of *Salmonella* negatively but is not likely to be the only clarification

Checking if problems were caused by:

- Mistake with inoculation level ?
- Inhomogeneous contamination of the samples ?



The inoculation of *Salmonella*?

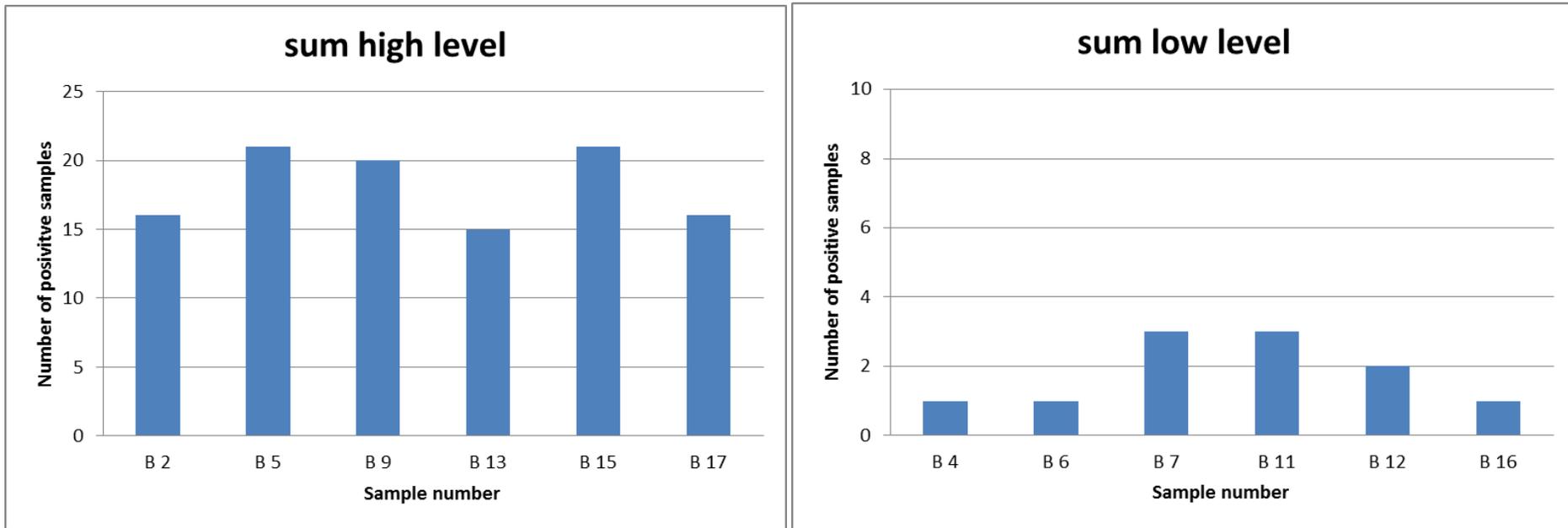
The EURL repeated the artificial contamination of animal feed samples using the same batch animal feed and *Salmonella* strain (SMb) with 10, 100 and 1000 CFU/25 g (n=5) and next store the samples at 5 °C for 13 days.

Inoculum CFU/25 g	No of positive samples/ total samples
10	0/5
100	1/5
1000	5/5
~ 3.3 CFU/sample (MPN 1-10)	

'Confirmation' of almost 2 log CFU reduction of SMb after addition to the chicken feed samples.



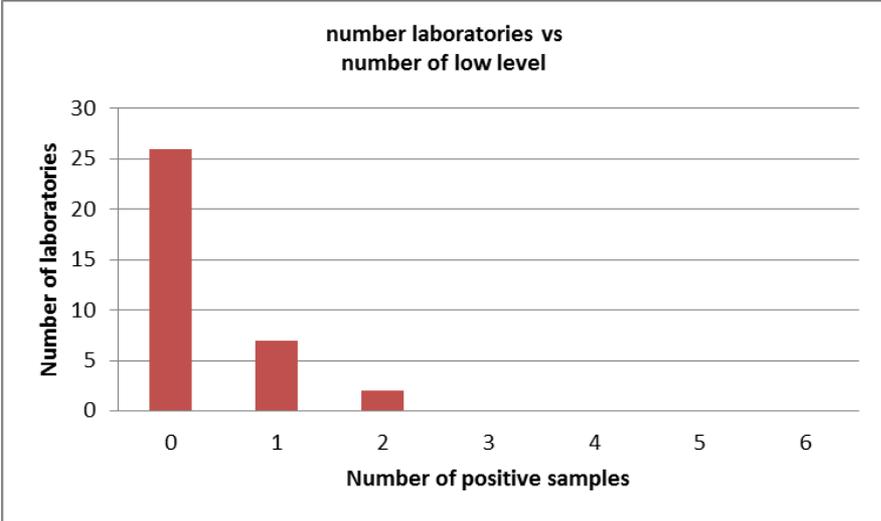
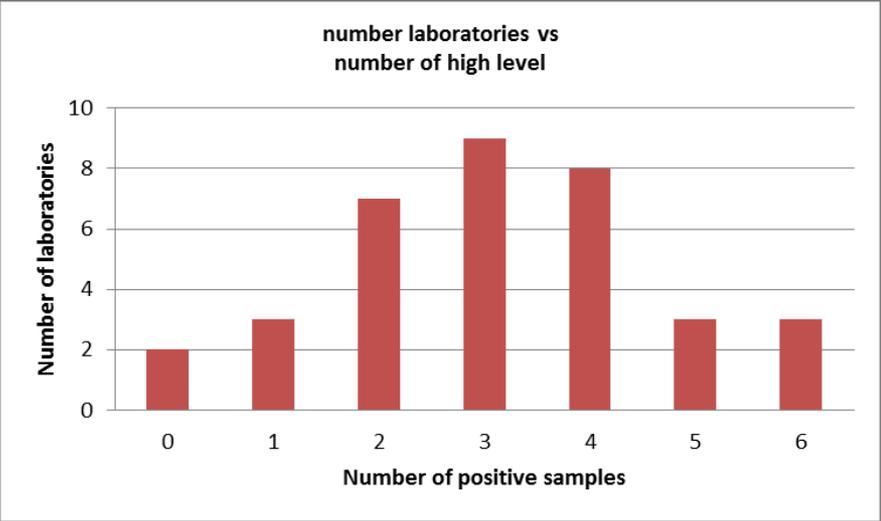
Distribution of all positive results over the samples



The number of positive samples found by all participants was evenly distributed over the different samples of both high and low level contaminated samples. (confirmed with Chi-square test)



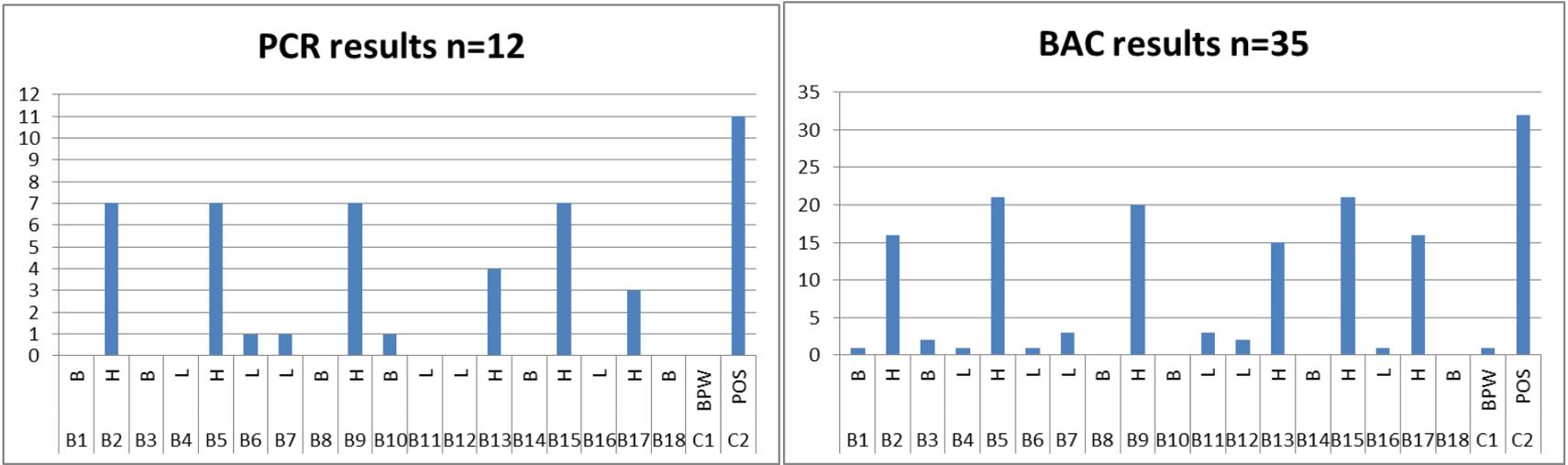
Distribution of positive results of over the laboratories



The number of participants with the same number of positive (high level) samples was binomially distributed.



Positive results by PCR vs culture method



The PCR and culture method gave the similar results and equal distribution of positive results over the samples



Conclusions (I)

- Unexpected high number of negative samples
- Low contaminated samples
 - 26 NRLs with 0 positive samples
 - 9 NRLs with 1-2 positive samples
 - Sensitivity rate: 5%
- High contaminated samples
 - 2 NRLs with 0 positive samples
 - 19 NRLs with 1-3 positive samples
 - 14 NRLs with 4-6 positive samples
 - Sensitivity rate: 52%
- Blank samples
 - still 3 false positive blank samples explanations:
exchange of samples ?





Conclusions (II)

- Final concentration high inoculum *S. Mbandaka* was approx. 1 CFU/25 g
-decrease of almost 2 log CFU
- Confirmed by additional experiments
- Contaminated samples equally distributed (homogeneous)

Explanations ?

- High background flora (only partly explanation)
- Differences between batches chicken feed (no problems in pre-test)
-caused by what ?
Presence of inhibitory substances ?

Suggestions ?





Conclusions (III)

Due to problems with samples it is not possible to judge the performance of the NRLs in this study.



Finally

THANKS !!!

The participants for all the extra work and the stress during this study.

Apologies for the inconvenience





Questions ??



Angelina Kuijpers

