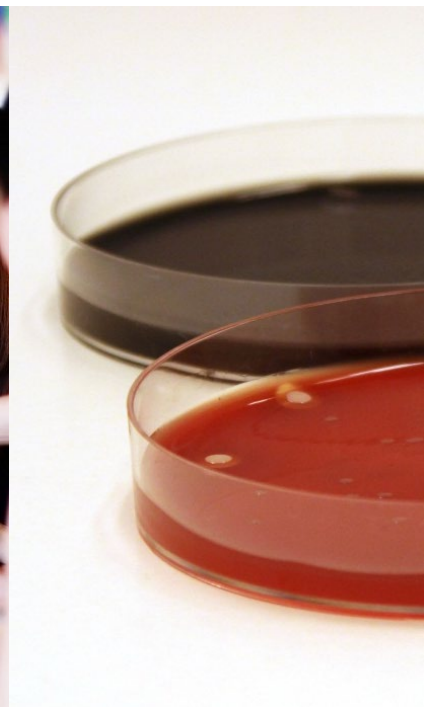


## Food Microbiology

October 2019

Jonas Ilbäck



*Edition*

Version 1 (2019-12-06)

*Editor in chief*

Maria Sitell, head of Biology department, Swedish Food Agency

*Responsible for the scheme*

Jonas Ilbäck, microbiologist, Biology department, Swedish Food Agency

PT October 2019 is registered as no. 2019/01956 at the Swedish Food Agency

*Proficiency Testing*  
**Microbiology – Food**  
October 2019

**Quantitative analyses**

- Aerobic microorganisms, 30 °C
- Aerobic microorganisms, 20 °C
- Contaminating microorganisms in dairy products
- Enterobacteriaceae
- Coliform bacteria, 30 °C
- Coliform bacteria, 37 °C
- Thermotolerant coliform bacteria
- *Escherichia coli*
- Presumptive *Bacillus cereus*
- Coagulase-positive staphylococci
- Enterococci

**Qualitative analyses**

- Gram-negative bacteria in pasteurized milk and cream

## Abbreviations

### Media

BA	Blood agar
BEA	Bile esculin agar
BcsA	<i>Bacillus cereus</i> selective agar
BGLB	Brilliant green lactose bile broth
BHI	Brain heart infusion broth
BP	Baird-Parker agar
CBC	Oxoid Brilliance™ <i>Bacillus cereus</i> agar
Compact Dry ETC	Compact Dry™ <i>Enterococcus</i>
COMPASS	COMPASS® <i>Enterococcus</i> agar
EC	<i>E. coli</i> broth
ENT	Slanetz & Bartley <i>Enterococcus</i> agar
IA	Iron agar
KEAA	Kanamycin esculin azide agar
LSB	Lauryl sulphate broth
LTLNB	Lactose tryptone lauryl sulphate broth
MPCA	Milk plate count agar
MYP	Mannitol egg yolk polymyxin agar
PCA	Plate count agar
PEMBA	Polymyxin pyruvate egg yolk mannitol bromothymol blue agar
Petrifilm AC	3M™ Petrifilm™ Aerobic Count
Petrifilm EB	3M™ Petrifilm™ Enterobacteriaceae
Petrifilm EC/CC	3M™ Petrifilm™ <i>E. coli</i> /Coliform Count
Petrifilm SEC	3M™ Petrifilm™ Select <i>E. coli</i>
Petrifilm Staph	3M™ Petrifilm™ Staph Express
Petrifilm Disk	3M™ Petrifilm™ Staph Express Disk
RPFA	Rabbit plasma fibrinogen agar
SFA	Sugar-free agar
TBX	Tryptone bile X-glucuronide agar
TEMPO AC	TEMPO® Aerobic Count
TEMPO BC	TEMPO® <i>Bacillus cereus</i>
TEMPO CC	TEMPO® Coliforms Count
TEMPO EB	TEMPO® Enterobacteriaceae
TEMPO EC	TEMPO® <i>E. coli</i>
TEMPO STA	TEMPO® Coagulase-positive staphylococci
TGE	Tryptone glucose extract agar
TSA	Tryptone soya agar
VRB	Violet red bile agar
VRBG	Violet red bile glucose agar

### Organisations

AFNOR	French National Standardization Association
AOAC	AOAC INTERNATIONAL
ISO	International Organization for Standardization
NMKL	Nordic Committee for Food Analyses
SLV/NFA	Livsmedelsverket/Swedish Food Agency, Sweden

## Contents

---

General information on results evaluation.....	4
Results of the PT round October 2019 .....	5
- General outcome .....	5
- Aerobic microorganisms, 30 °C and 20 °C .....	6
- Contaminating microorganisms in dairy products .....	9
- Enterobacteriaceae .....	11
- Coliform bacteria, 30 °C and 37 °C.....	13
- Thermotolerant coliform bacteria .....	16
- <i>Escherichia coli</i> .....	16
- Presumptive <i>Bacillus cereus</i> .....	19
- Coagulase-positive staphylococci .....	20
- Enterococci .....	23
- Gram-negative bacteria in pasteurized milk and cream .....	25
Outcome of the results of individual laboratory – assessment .....	26
- Box plot .....	27
Test material and quality control .....	33
- Test material .....	33
- Quality control of the mixtures .....	34
References .....	35

Annex 1: Results obtained by the participants

Annex 2: z-scores of all participants

## General information on results evaluation

### Statistical evaluation of the results

Highly deviating values that did not belong to a strictly normal distribution after  $\log_{10}$  transformation were identified as statistical outliers (Grubbs' test modified by Kelly (1)). In some cases, subjective adjustments were made to set limits based on knowledge of the mixture's contents. Outliers and false results were not included in the calculations of means and standard deviations. Results reported as "> value" were excluded from the evaluation. Results reported as "< value" were interpreted as being zero (negative result). All reported results are presented in Annex 1.

According to EN ISO/IEC 17043, for which the proficiency testing programme is accredited, it is mandatory for the participating laboratories to report method information for all their analyses. Method information is sometimes difficult to interpret, since many laboratories report a medium that is not included in the standard method they refer to. Results from laboratories that report contradictory data on methods/media have either been excluded from the method analysis, or been added to the group of "Others", together with results from methods and media that are only used by 1-2 laboratories.



Mean values and standard deviations are normally provided for the different analyses. When the total number of reported results for an analysis is fewer than 20, the median is provided instead of the mean value. For method groups with fewer than 5 results, only the number of false results and outliers are provided.

### Uncertainty of measurement for the assigned values

The uncertainty of measurement for an assigned value is calculated as the standard deviation divided by the square root of the number of correct results ("standard error"). The assigned value of evaluated parameters is the mean value of the participants results.




### Table and figure legends

#### Tables

N	number of laboratories that performed the analysis
n	number of laboratories with satisfactory result
m	mean value in $\log_{10}$ cfu ml <sup>-1</sup> (false results and outliers excluded)
s	standard deviation (false results and outliers excluded)
F	number of false positive or false negative results
<	number of low outliers
>	number of high outliers
	global results for the analysis
	values discussed in the text

#### Figures

Histograms of the analytical results for each mixture and parameter are presented. The mean value of the analysis results is indicated in each histogram.

	values within the interval of acceptance (Annex 1)
	outliers
	false negative results
*	values outside of the x-axis scale

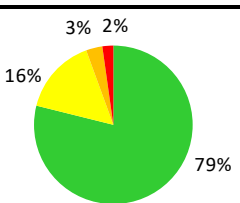
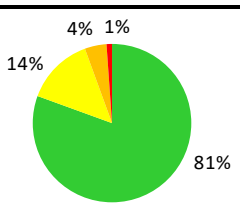
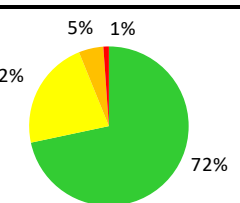
# Results of the PT round October 2019

## General outcome

Samples were sent to 185 laboratories, 46 in Sweden, 122 in other European countries, and 17 outside of Europe. Of the 180 laboratories that reported results, 85 (48 %) provided at least one result that received an annotation. In the previous round with similar analyses (October 2018) the proportion was 40 %.

Individual results for each analysis in the PT round are listed in Annex 1 and are also available on the website after logging in: <https://www2.slv.se/absint>.

**Table 1.** Composition of the test material and proportion of deviating results (N: number of reported results, F%: false positive or false negative, X%: outliers)

		Sample A				Sample B				Sample C			
<b>% participants with</b>													
<b>Microorganisms</b>		<i>Escherichia coli</i> <i>Serratia marcescens</i> <i>Staphylococcus hyicus</i>				<i>Enterococcus durans</i> <i>Escherichia coli</i> <i>Serratia marcescens</i> <i>Staphylococcus aureus</i>				<i>Bacillus cereus</i> <i>Pediococcus acidilactici</i> <i>Staphylococcus xylosum</i>			
<b>Analys</b>		<b>Target organism</b>	<b>N</b>	<b>F%</b>	<b>X%</b>	<b>Target organism</b>	<b>N</b>	<b>F%</b>	<b>X%</b>	<b>Target organism</b>	<b>N</b>	<b>F%</b>	<b>X%</b>
Aerobic micro-organisms	30°C	All	163	0	5	All	162	0	4	All	162	0	6
	20°C	All	35	3	0	All	35	3	3	All	35	3	6
Contaminating microorganisms		All	19	5	0	All	20	5	5	<i>S. xylosum</i> <i>B. cereus</i>	20	5	20
Enterobacteriaceae		<i>E. coli</i> <i>S. marcescens</i>	139	0	2	<i>E. coli</i> <i>S. marcescens</i>	139	1	2	-	139	1	0
Coliform bacteria	30°C	<i>E. coli</i> ( <i>S. marcescens</i> )	50	0	6	<i>E. coli</i> ( <i>S. marcescens</i> )	49	2	6	-	50	2	0
	37°C	<i>E. coli</i> ( <i>S. marcescens</i> )	101	2	2	<i>E. coli</i> ( <i>S. marcescens</i> )	101	1	1	-	101	1	0
Thermotolerant coliform bacteria		<i>E. coli</i>	50	2	6	<i>E. coli</i>	50	2	6	-	50	0	0
<i>Escherichia coli</i>		<i>E. coli</i>	118	1	4	<i>E. coli</i>	117	1	4	-	119	0	0
Presumptive <i>B. cereus</i>		( <i>S. marcescens</i> ) ( <i>S. hyicus</i> )	115	2	0	( <i>S. marcescens</i> ) ( <i>S. aureus</i> )	114	2	0	<i>B. cereus</i>	114	0	6
Coagulase-positive staphylococci		( <i>S. hyicus</i> )	104	17	0	<i>S. aureus</i>	101	1	6	( <i>S. xylosum</i> )	103	10	0
Enterococci		-	72	1	0	<i>E. durans</i>	72	0	6	( <i>P. acidilactici</i> )	72	36	0
Gram-negative bacteria in milk prod.		<i>E. coli</i> <i>S. marcescens</i>	13	0	-	<i>E. coli</i> <i>S. marcescens</i>	13	0	-	-	12	0	-

- no target organism or no value (microorganisms) = false positive before confirmation

■ Positive results are also considered correct for this analysis.

## Aerobic microorganisms 30 °C and 20 °C

### Sample A

All strains in the sample were target organisms. *S. hyicus* and *E. coli* were present in somewhat higher concentrations than *S. marcescens*.

### Sample B

All strains in the sample were target organisms. *S. marcescens* and *S. aureus* were present in somewhat higher concentrations than *E. coli* and *E. durans*.

### Sample C

All strains in the sample were target organisms. *S. xylosus* was present in somewhat higher concentration than *B. cereus* and *P. acidilactici*.

### General remarks

The choice of method and medium was very similar at the two temperatures. At 30 °C most laboratories used NMKL 86:2013 (26 %), 3M Petrifilm (23 %) and ISO 4833-1:2013 (21 %). The older NMKL 86:2006 and ISO 4833:2003 were still used by 9 % and 4 % of the laboratories, respectively. These methods are however similar, and are all based on incubation on PCA or MCPA at 30 °C for 72 h. With Petrifilm AC it is possible to use different time/temperature depending on which method validation that is followed. For example AOAC® 990.12 prescribes incubation at 35 °C for 48 h while AFNOR 3M 01/1-09/89 prescribes 30 °C for 72 h.

Incubation on MPCA was mostly used by laboratories within the dairy industry, and was then mainly performed according to ISO 4833-1:2013. Incubation on TSA was mainly attributed to the use of a company-specific method. At 20 °C, incubation on IA was done by laboratories that followed NMKL 184. This method is adapted for aerobic microorganisms and specific spoilage organisms in fish and fish products. The results for MPCA, TSA and IA were similar to those for PCA and Petrifilm AC.

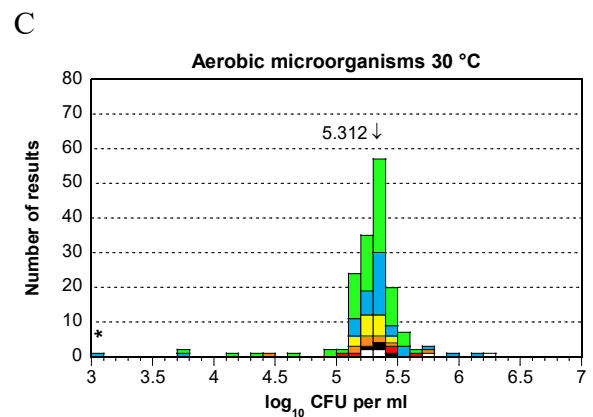
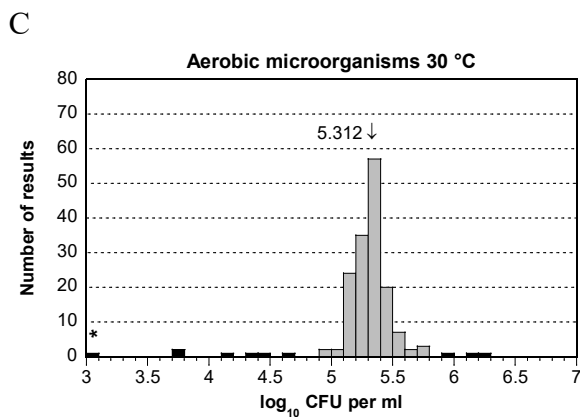
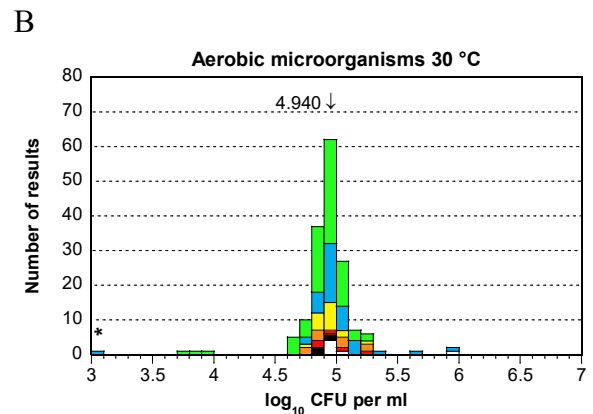
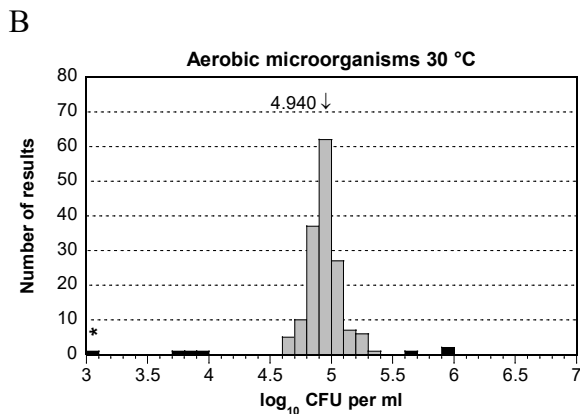
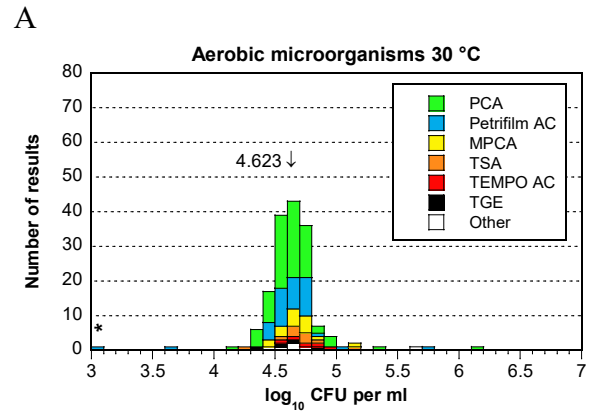
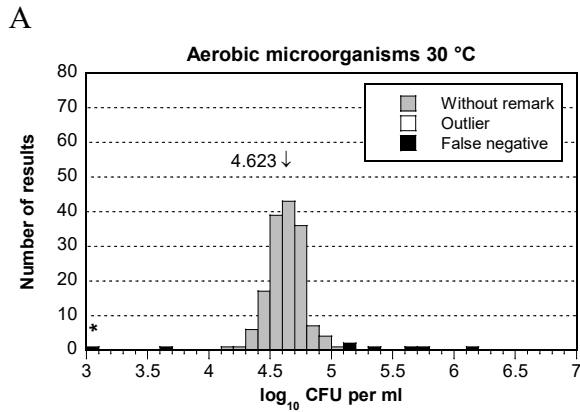
A smaller number of laboratories used TEMPO® AC (bioMérieux® SA, Marcy l'Etoile, France), which is based on MPN (Most Probable Number). With this method, the sample is incubated in a card that contains different-sized wells. A medium in the wells fluoresces when hydrolysed by the microorganisms. The number of microorganisms is then determined by the number and size of the fluorescing wells.

### Results from analysis of aerobic microorganisms, 30 °C

Medium	Sample A						Sample B						Sample C					
	N	n	m	s	F	< >	N	n	m	s	F	< >	N	n	m	s	F	< >
All results	163	155	4.623	0.140	0	2 6	162	155	4.940	0.125	0	4 3	162	152	5.312	0.133	0	7 3*
PCA	80	78	4.607	0.141	0	0 2	80	77	4.920	0.123	0	3 0	79	75	5.301	0.123	0	4 0
Petrifilm AC	41	38	4.635	0.133	0	2 1	40	37	4.976	0.116	0	1 2	41	37	5.329	0.128	0	2 2*
MPCA	17	16	4.645	0.105	0	0 1	17	17	4.930	0.114	0	0 0	17	17	5.274	0.093	0	0 0
TSA	10	9	4.628	0.178	0	0 1	10	10	4.965	0.197	0	0 0	10	9	5.317	0.186	0	1 0
TEMPO AC	5	5	4.742	0.168	0	0 0	5	5	4.988	0.158	0	0 0	5	5	5.370	0.242	0	0 0
TGE	4	4	-	-	0	0 0	4	4	-	-	0	0 0	4	4	-	-	0	0 0
Other	6	5	4.612	0.090	0	0 1	6	5	4.946	0.042	0	0 1	6	5	5.372	0.217	0	0 1

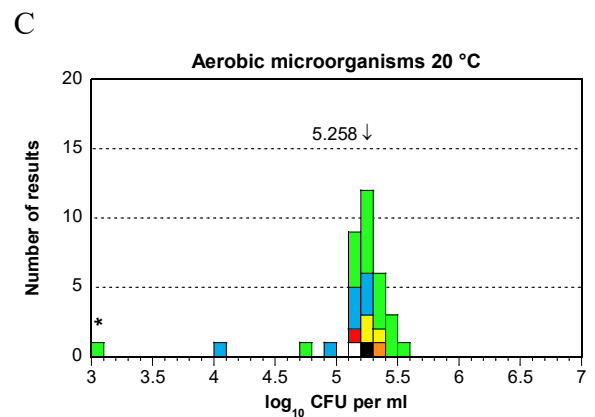
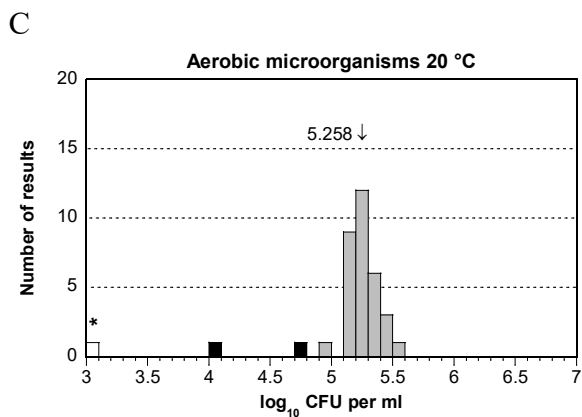
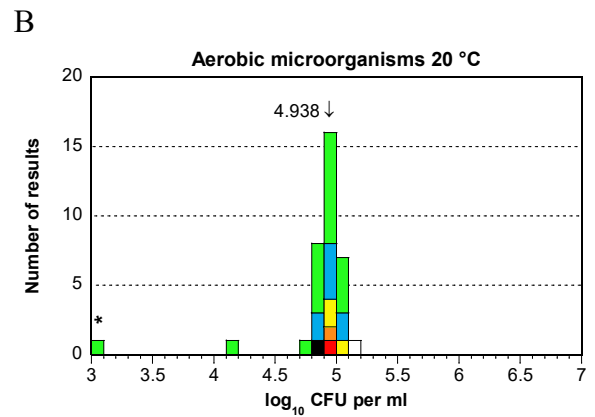
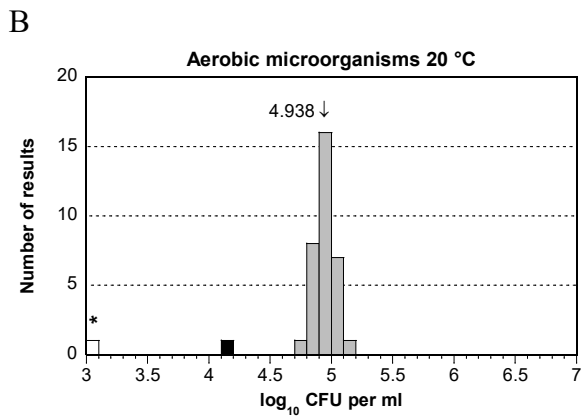
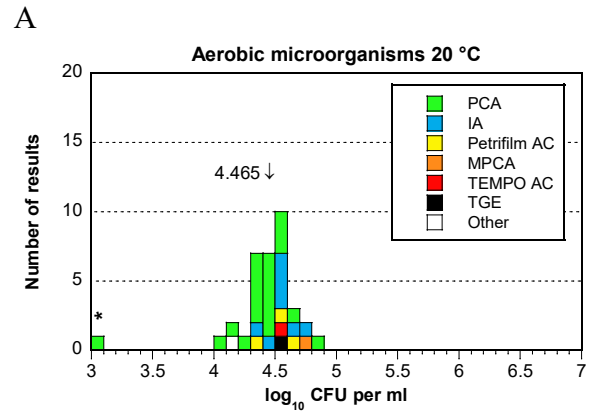
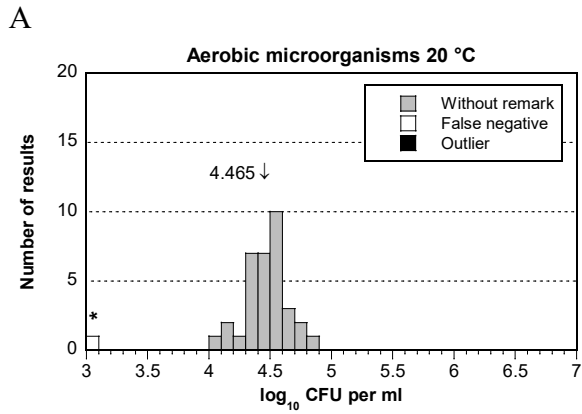
\*It has been communicated that one of the high outliers for sample C is caused by a calculation error, and that the correctly calculated result is within the limits of acceptance.





*Results from analysis of aerobic microorganisms, 20 °C*

Medium	Sample A						Sample B						Sample C					
	N	n	m	s	F	< >	N	n	m	s	F	< >	N	n	m	s	F	< >
All results	35	34	4.465	0.162	1	0 0	35	33	4.938	0.079	1 1	0 0	35	32	5.258	0.115	1 2	0 0
PCA	20	19	4.419	0.161	1	0 0	20	18	4.926	0.078	1 1	0 0	20	18	5.296	0.116	1 1	0 0
IA	8	8	4.533	0.111	0	0 0	8	8	4.939	0.077	0	0 0	8	7	5.174	0.106	0	1 0
Petrifilm AC	3	3	-	-	0	0 0	3	3	-	-	0	0 0	3	3	-	-	0	0 0
MPCA	1	1	-	-	0	0 0	1	1	-	-	0	0 0	1	1	-	-	0	0 0
TEMPO AC	1	1	-	-	0	0 0	1	1	-	-	0	0 0	1	1	-	-	0	0 0
TGE	1	1	-	-	0	0 0	1	1	-	-	0	0 0	1	1	-	-	0	0 0
Other	1	1	-	-	0	0 0	1	1	-	-	0	0 0	1	1	-	-	0	0 0



## Contaminating microorganisms in dairy products

### Sample A

All strains in the sample were target organisms. *S. hyicus* and *E. coli* were present in somewhat higher concentrations than *S. marcescens*.

### Sample B

All strains in the sample were target organisms. *S. marcescens* and *S. aureus* were present in somewhat higher concentrations than *E. coli* and *E. durans*.

### Sample C

All strains in the sample can form colonies on SFA. The strain of *P. acidilactici* has in earlier proficiency testing rounds formed very small (pin-point) colonies on SFA. Such colonies shall be excluded during the enumeration according to ISO 13559:2002 / IDF 153:2002. Since the strain of *S. xyloso* was present in a higher concentration than *B. cereus* and *P. acidilactici*, this should however not have had a significant impact on the result.

### General remarks

Only 20 laboratories performed the analysis and the results were therefore difficult to evaluate statistically. Outliers have therefore been determined manually. When determining outliers, consideration has been taken to the species and concentration of target organisms (Table 3), the mean value of all laboratories, and the distribution of results that is normally seen in this analysis.

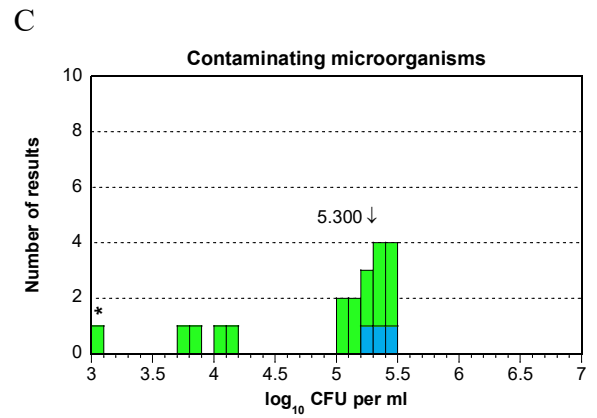
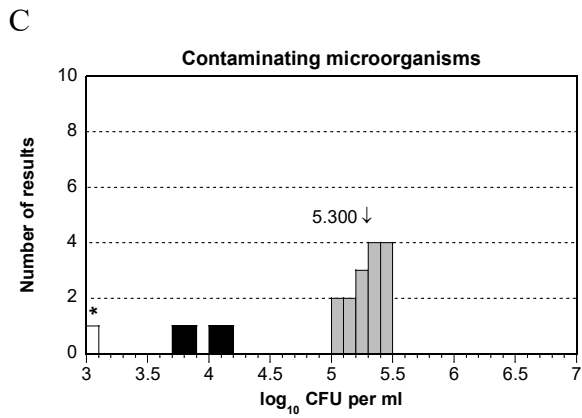
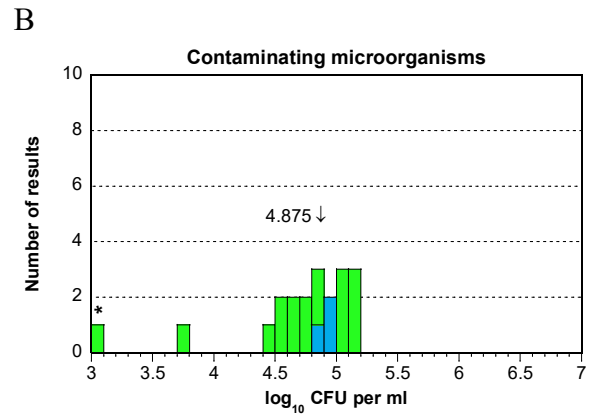
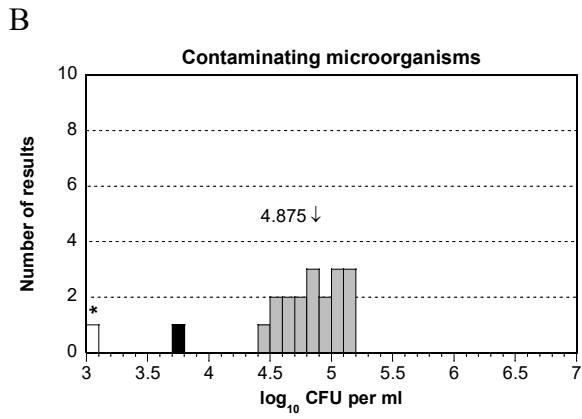
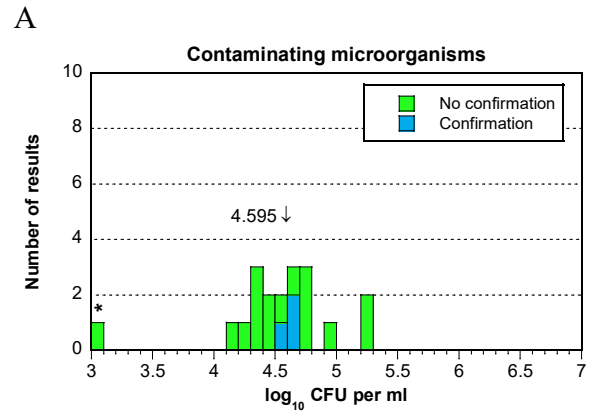
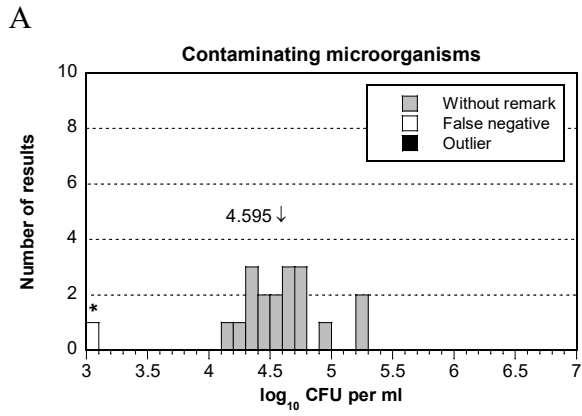
Nine of the 20 laboratories followed ISO 13559:2002 / IDF 153:2002. This was last reviewed by ISO in 2013 and remains current. One laboratory followed the older IDF 153:1999. Other laboratories either followed internal methods, or did not specify further which method they used. All laboratories except one used the medium SFA.

The goal of the analysis is to identify potential contaminating microorganisms in dairy products. According to ISO 13559:2002 / IDF 153:2002, lactic acid bacteria are in this sense not classified as contaminating microorganisms. Lactic acid bacteria are catalase negative and some laboratories therefore use confirmation with a catalase test. Such a test is however not included in ISO 13559:2002 / IDF 153:2002, and the method only specifies the enumeration of "characteristic contaminating microorganisms". Only three laboratories stated that they performed a confirmation test, but did not specify this further.

### Results from analysis of contaminating microorganisms in dairy products

Method	Sample A						Sample B						Sample C					
	N	n	Med*	s	F	< >	N	n	Med*	s	F	< >	N	n	Med*	s	F	< >
All results	19	18	4.595	0.302	1	0 0	20	18	4.875	0.212	1	1 0	20	15	5.300	0.143	1	4 0
No confirmation	16	15	4.570	0.332	1	0 0	17	15	4.850	0.228	1	1 0	17	12	5.310	0.156	1	4 0
Confirmation	3	3	-	-	0	0 0	3	3	-	-	0	0 0	3	3	-	-	0	0 0
Other	0	0	-	-	0	0 0	0	0	-	-	0	0 0	0	0	-	-	0	0 0

\* Med = median



## Enterobacteriaceae

---

### Sample A

The strains of *E. coli* and *S. marcescens* were target organisms. The strain of *E. coli* was present in a somewhat higher concentration than *S. marcescens*. On VRBG, both strains form red colonies, and are surrounded by typical precipitation zones. Both strains are oxidase-negative.

### Sample B

The same strains of *E. coli* and *S. marcescens* as in sample A were target organisms. In sample B however, the strain of *S. marcescens* was present in a higher concentration than *E. coli*.

### Sample C

No target organisms for the analysis was present in the sample.

### General remarks

As in previous proficiency testing rounds, most laboratories followed either NMKL 144:2005 (44 %) or a method with Petrifilm EB (25 %), while the ISO methods (different versions) were used by in total 21 % of the laboratories. The number of users of the new ISO 21528-2:2017 was higher than ISO 21528-2:2004 (11 % and 5 %, respectively). The new ISO 21528-1:2017 was however only used by two laboratories (1 %), while five laboratories (4 %) followed the older ISO 21528-1:2004.

ISO 21528-2:2017 is based on colony-count, while ISO 21528-1:2017 is based on MPN (Most Probable Number). The latter method is recommended when the expected concentration of Enterobacteriaceae is lower than 100 cfu g<sup>-1</sup>. A small number of laboratories used methods based on detection of fluorescence (TEMPO EB).

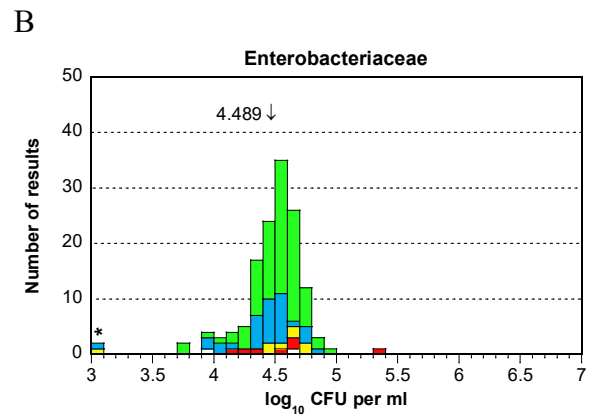
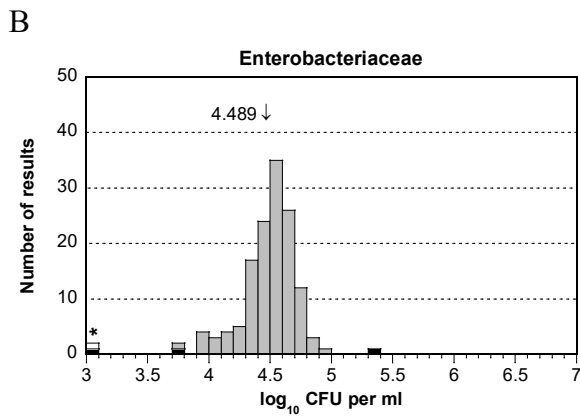
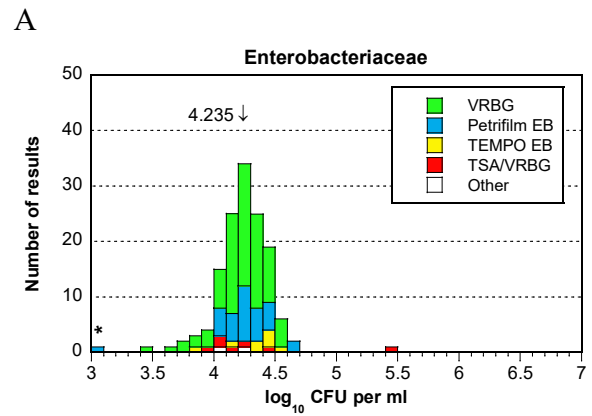
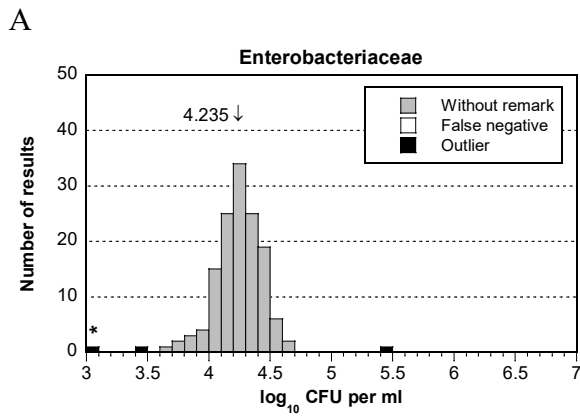
Enterobacteriaceae are Gram-negative and oxidase-negative bacteria, that ferment glucose with the formation of acid by-products. On VRBG, which is used in both NMKL 144 and ISO 21528-2, they therefore form pink/red colonies, with or without a bile salt precipitation zone. Enterobacteriaceae have a similar appearance on Petrifilm EB, which also contains a colour indicator that facilitates detection of acid by-products, and a plastic film for detection of gas production.

With NMKL 144:2005, presumptive colonies on VRBG are confirmed with an oxidase test. With ISO 21528-2:2017, presumptive colonies are confirmed both with an oxidase test and with a test for glucose fermentation. Oxidase-negative colonies that also ferment glucose in glucose oxidation/fermentation (OF) medium are confirmed as Enterobacteriaceae. In total, 62 % of the laboratories stated that they performed some kind of confirmation test; the majority of these specified that this consisted of an oxidase test.

No major differences could be seen between the different methods and media that were used. For sample B, there was however a tendency towards higher results for TEMPO EB, compared to other media. Such higher results for TEMPO EB have been seen in several previous proficiency testing rounds, and should be considered as normal.

Results from analysis of Enterobacteriaceae

Medium	Sample A					Sample B					Sample C				
	N	n	m	s	F < >	N	n	m	s	F < >	N	n	m	s	F < >
All results	139	136	4.235	0.177	0 2 1	139	135	4.489	0.207	1 2 1	138	136	-	-	2 - -
VRBG	88	87	4.224	0.180	0 1 0	88	87	4.511	0.195	0 1 0	89	88	-	-	1 - -
Petrifilm EB	34	33	4.265	0.154	0 1 0	34	33	4.426	0.224	0 1 0	33	33	-	-	0 - -
TEMPO EB	8	8	4.318	0.225	0 0 0	8	7	4.616	0.131	1 0 0	8	8	-	-	0 - -
TSA/VRBG	7	6	4.148	0.171	0 0 1	7	6	4.438	0.202	0 0 1	6	5	-	-	1 - -
Other	2	2	-	-	0 0 0	2	2	-	-	0 0 0	2	2	-	-	0 - -



## Coliform bacteria 30 °C and 37 °C

---

### Sample A

The strain of *E. coli* was target organism for the analysis. It forms red colonies with a precipitation zone on VRB. *S. marcescens* is a weak fermenter of lactose, and is capable of forming small colonies on VRB, with a less prominent precipitation zone. Both strains are oxidase-negative, but *S. marcescens* should be excluded after confirmation since, in contrast to *E. coli*, it does not produce gas in BGLB. *S. hyicus* is Gram-positive and is therefore inhibited by the presence of bile salts and crystal violet in VRB.

### Sample B

The same strain of *E. coli* as in sample A was target organism. As in sample A, *S. marcescens* may also have formed colonies on VRB. *E. durans* and *S. aureus* are Gram-positive and should not have grown on VRB.

The results at 37 °C were distributed around a fairly wide peak, with the majority of the results between  $\log_{10}$  4.0 and  $\log_{10}$  4.7 cfu ml<sup>-1</sup>. A wide distribution of the results was also seen at 30 °C. At this temperature, it was possible to discern two overlapping peaks, one at around  $\log_{10}$  4.2 and one at around  $\log_{10}$  4.5 cfu ml<sup>-1</sup>. The two peaks could not be separated statistically, but the low and high peak correspond well to the concentrations of *E. coli* and of *E. coli* + *S. marcescens*, respectively.

### Sample C

No target organism for the analysis was present in the sample.

### General remarks

Coliform bacteria are Gram-negative rods that ferment lactose with the production of gas and acid by-products. On VRB they form characteristic red colonies due to uptake of crystal violet and neutral red from the medium. The colonies are normally surrounded by a red/pink precipitation zone, which is formed due to the precipitation of bile salts when the pH decreases. Petrifilm CC and Petrifilm EC/CC are based on VRB, but also have a plastic film that facilitates detection of gas production.

At both temperatures, the most common methods were NMKL 44:2004, ISO 4832:2006 and 3M™ Petrifilm™. Both NMKL 44:2004 and ISO 4832:2006 prescribe incubation on VRB, but the confirmation steps differ somewhat. NMKL 44:2004 states that all presumptive colonies on VRB shall be confirmed with BGLB. In contrast, with ISO 4832:2006 only atypical colonies require further confirmation. Such differences between the methods may (at least partially) explain why *S. marcescens* was counted as a coliform bacterium by some laboratories. Further, if the sample is suspected to contain stressed coliform bacteria, NMKL 44:2004 recommends pre-incubation on TSA. Such a pre-incubation could also contribute to higher results.

LSB in combination with BGLB was used by laboratories that followed ISO 4831 and NMKL 96 (various editions). ISO 4831:2006 is based on MPN (Most Probable Number) and is adapted for use when the expected concentration of coliform bacteria is lower than or equal to 100 cfu g<sup>-1</sup>. NMKL 96 is also based on MPN, and is adapted for the analysis of coliform bacteria in fish and seafood. It is recommended when the expected concentration of microorganisms is lower than or equal to 300 cfu g<sup>-1</sup>. In some previous proficiency testing rounds, users of these methods have had problems with correctly determining higher concentrations – such as those in samples A and B.

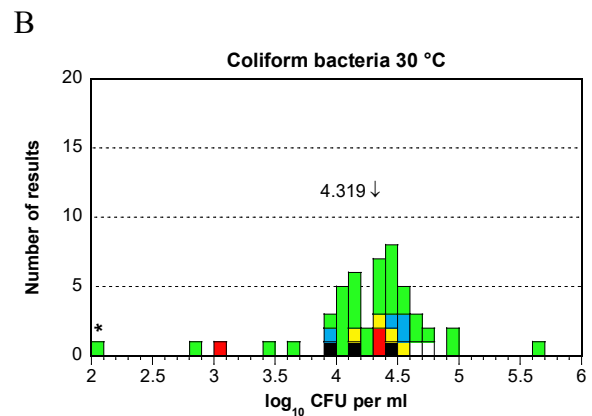
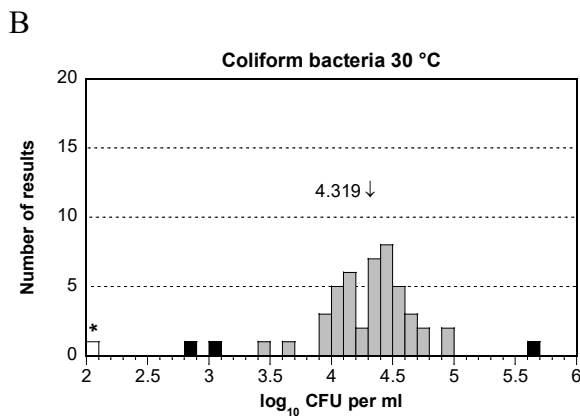
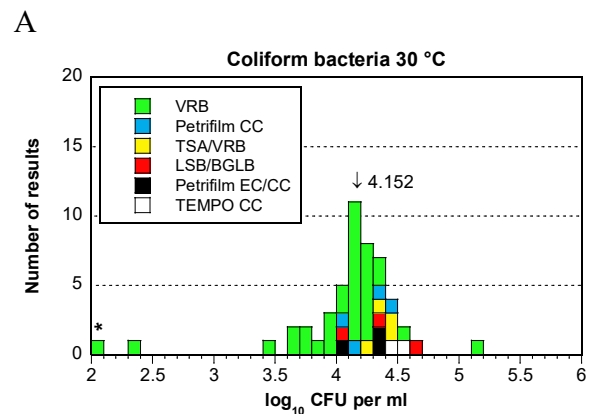
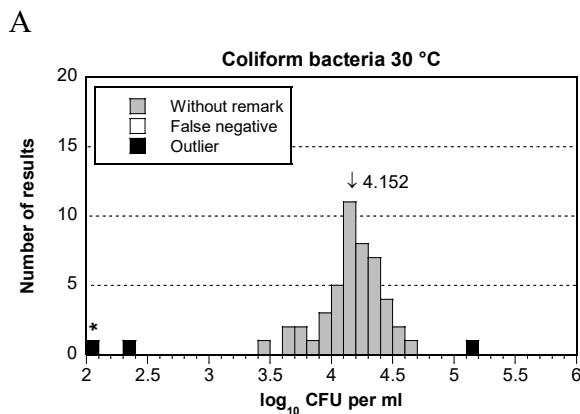
However in this proficiency testing round, only one deviating result was reported by laboratories that used either of these methods.

A wider range of media were used at 37 °C, compared to at 30 °C. At 37 °C, four laboratories used RAPID'E. coli 2 agar, which detects β-galactosidase and β-glucuronidase activity. On this medium, coliform bacteria (Gal+/Gluc-) form blue/green colonies, while *E. coli* (Gal+/Gluc+) form pink/purple colonies. Two laboratories used TEMPO CC. One laboratory used Compact Dry EC, on which coliform bacteria form red or red/violet colonies, while *E. coli* forms blue colonies.

Confirmation of some kind was performed by 75 % of the laboratories at 30 °C and by 50 % at 37 °C. Confirmation was less often reported by laboratories that used Petrifilm CC and Petrifilm EC/CC, which is reasonable since confirmation is not required with those methods.

*Results from analysis of coliform bacteria, 30 °C*

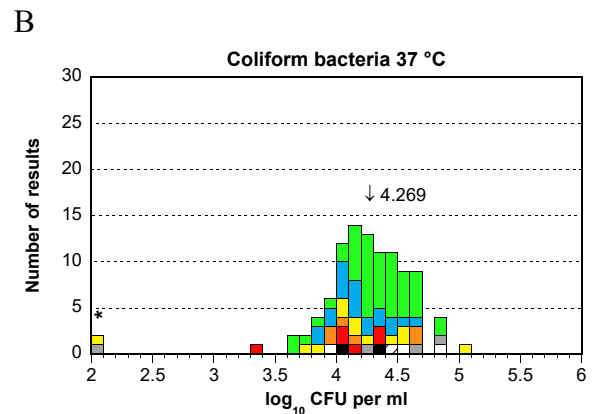
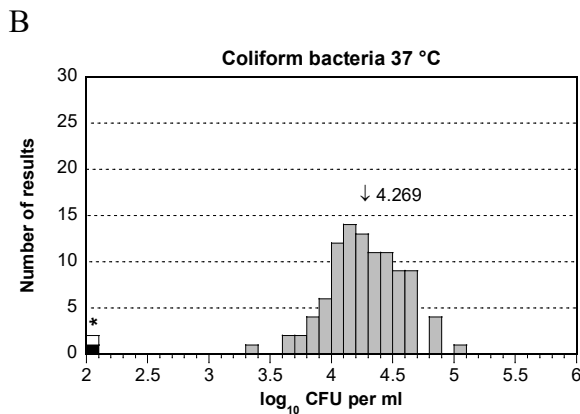
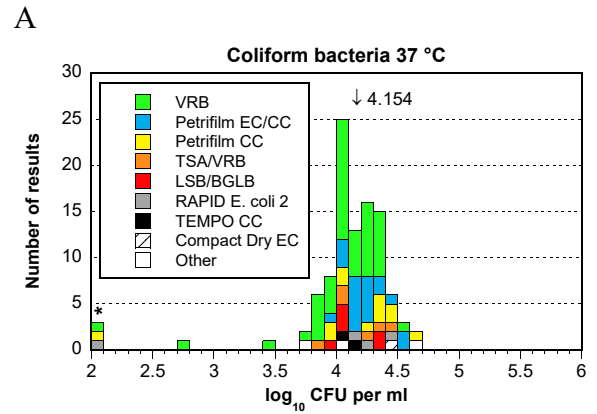
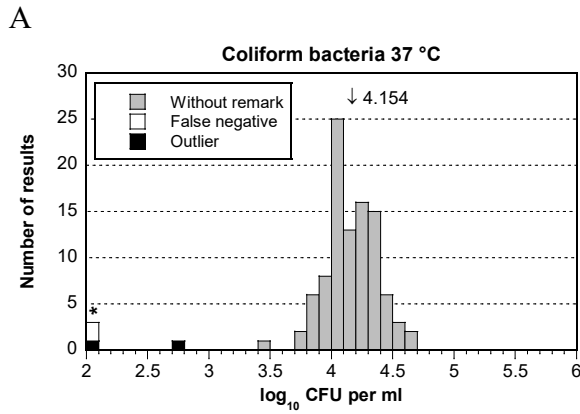
Medium	Sample A						Sample B						Sample C						
	N	n	m	s	F	< >	N	n	m	s	F	< >	N	n	m	s	F	< >	
All results	50	47	4.152	0.248	0	2 1	49	45	4.319	0.300	1	2 1	50	49	-	-	1	-	-
VRB	34	31	4.067	0.236	0	2 1	33	30	4.296	0.328	1	1 1	34	33	-	-	1	-	-
Petrifilm CC	4	4	-	-	0	0 0	4	4	-	-	0	0 0	4	4	-	-	0	-	-
TSA/VRB	4	4	-	-	0	0 0	4	4	-	-	0	0 0	4	4	-	-	0	-	-
LSB/BGLB	3	3	-	-	0	0 0	3	2	-	-	0	1 0	3	3	-	-	0	-	-
Petrifilm EC/CC	3	3	-	-	0	0 0	3	3	-	-	0	0 0	3	3	-	-	0	-	-
TEMPO CC	2	2	-	-	0	0 0	2	2	-	-	0	0 0	2	2	-	-	0	-	-





Results from analysis of coliform bacteria, 37 °C

Medium	Sample A							Sample B							Sample C						
	N	n	m	s	F	<	>	N	n	m	s	F	<	>	N	n	m	s	F	<	>
All results	101	97	4.154	0.210	2	2	0	101	99	4.269	0.304	1	1	0	101	100	-	-	1	-	-
VRB	47	45	4.093	0.204	1	1	0	47	47	4.316	0.281	0	0	0	47	46	-	-	1	-	-
Petrifilm EC/CC	20	20	4.217	0.153	0	0	0	20	20	4.178	0.227	0	0	0	20	20	-	-	0	-	-
Petrifilm CC	12	11	4.248	0.228	0	1	0	12	11	4.235	0.370	0	1	0	12	12	-	-	0	-	-
TSA/VRB	6	6	4.165	0.235	0	0	0	6	6	4.220	0.339	0	0	0	6	6	-	-	0	-	-
LSB/BGLB	6	6	4.129	0.168	0	0	0	6	6	4.047	0.383	0	0	0	6	6	-	-	0	-	-
RAPID'E.coli 2	4	3	-	-	1	0	0	4	3	-	-	1	0	0	4	4	-	-	0	-	-
TEMPO CC	2	2	-	-	0	0	0	2	2	-	-	0	0	0	2	2	-	-	0	-	-
Compact Dry EC	1	1	-	-	0	0	0	1	1	-	-	0	0	0	1	1	-	-	0	-	-
Other	3	3	-	-	0	0	0	3	3	-	-	0	0	0	3	3	-	-	0	-	-



## **Thermotolerant coliform bacteria and *Escherichia coli***

---

### **Sample A**

The strain of *E. coli* was target organism for both analyses. It produces both gas and indole in LTL SB. The strain is also positive for  $\beta$ -glucuronidase.

### **Sample B**

The same strain of *E. coli* as in sample A was target organism for both analyses.

### **Sample C**

No target organism was present in the sample.

### **General remarks**

NMKL 125:2005 was the most commonly used method for the analysis of thermotolerant coliform bacteria (58 % of the laboratories). It describes the analysis of both thermotolerant coliform bacteria and of *E. coli*. Thermotolerant coliform bacteria are in the method defined as those that form typical dark red colonies surrounded by a zone of precipitation on VRB after 24 h at 44 °C. The colonies are confirmed by inoculation either in EC or in LTL SB at 44 °C. In both of these media, thermotolerant coliform bacteria produce gas as a consequence of lactose fermentation. Thermotolerant coliform bacteria that also produce indole either in LTL SB or in tryptone broth are counted as *E. coli*.

For the analysis of *E. coli*, most laboratories used methods based on 3M™ Petrifilm™ (either Petrifilm EC/CC or Petrifilm SEC), followed by NMKL 125:2005 and ISO 16649-2:2001. Both Petrifilm EC/CC and Petrifilm SEC include substrates that facilitate detection of  $\beta$ -glucuronidase, and thus *E. coli* form blue-green colonies on these media. The plastic film in Petrifilm EC/CC and Petrifilm SEC also facilitates detection of gas production due to lactose fermentation. ISO 16649-2:2001 is also based on detection of  $\beta$ -glucuronidase activity. The method uses TBX, on which *E. coli* form typical blue colonies after 18-24 h at 44 °C. No further confirmation of  $\beta$ -glucuronidase positive colonies is required according to ISO 16649-2:2001.

In the analysis of *E. coli*, 89 % of the laboratories that followed NMKL 125:2005 stated that they performed some kind of confirmation. Laboratories that used Petrifilm or those that followed ISO 16649-2:2001 less often reported confirmation of *E. coli*, which is reasonable, since neither of these methods require confirmation. No obvious difference in the results could however be seen between laboratories that performed a confirmation and those that did not.

Among the less frequently used methods were ISO 7251 and NMKL 96 (different editions). ISO 7251 is an MPN-based method for the detection of *E. coli*. NMKL 96 is also based on MPN, and is adapted for the analysis of coliform bacteria, thermotolerant coliform bacteria and *E. coli* in fish and seafood.

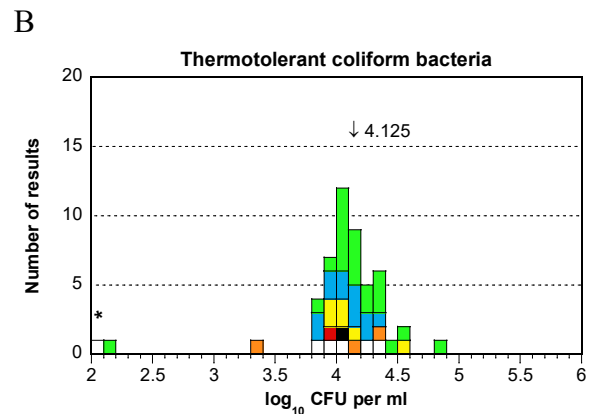
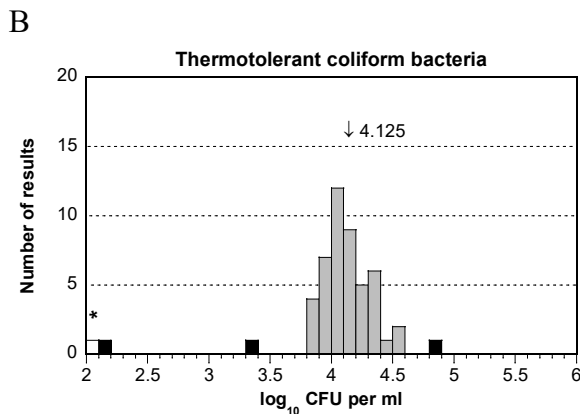
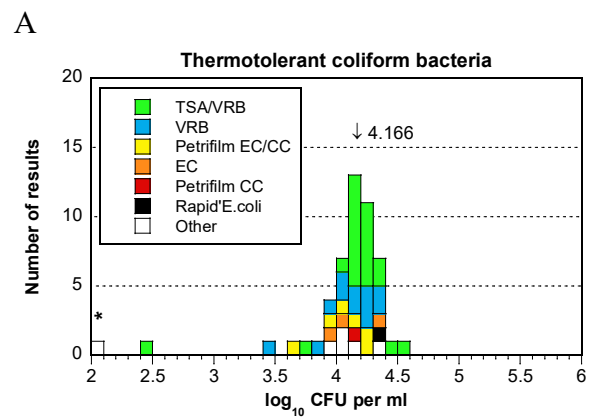
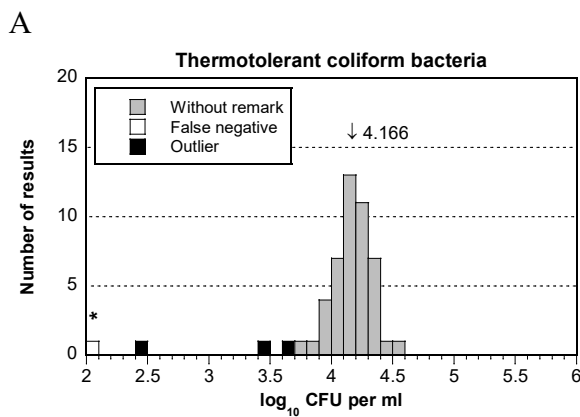
In the analysis of *E. coli*, a few laboratories used TEMPO EC. This was associated with somewhat higher results, compared to other media. That has not been seen in previous proficiency testing rounds, and may simply be due to chance and the low number of users of TEMPO EC. In previous proficiency testing rounds, the results for *E. coli* have however occasionally been somewhat lower for TBX, and somewhat higher for TSA/VRB, compared to other media. At those times, the differences have been assumed to be due to performing, or not performing, a pre-incubation at a lower temperature. Here, the mean values for TSA/VRB and TBX did not deviate significantly

from compared to other media, and the results were within one standard deviation from the mean value of all results.

When analysing *E. coli* the incubation is normally done at 42-44 °C or 35-37 °C, depending on which method that is followed. The mean values for these two temperature groups did not differ. It was also not possible to identify any obvious difference in the number of outliers and false results.

*Results from analysis of thermotolerant coliform bacteria*

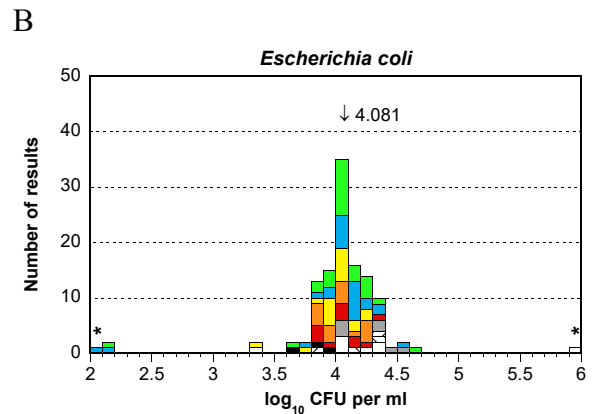
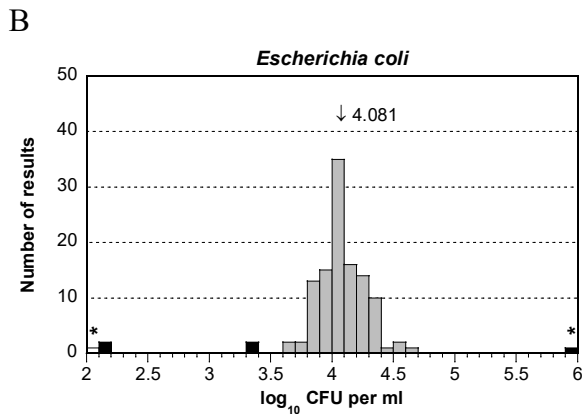
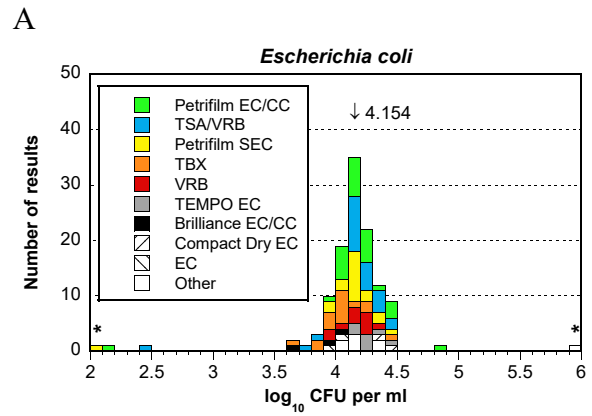
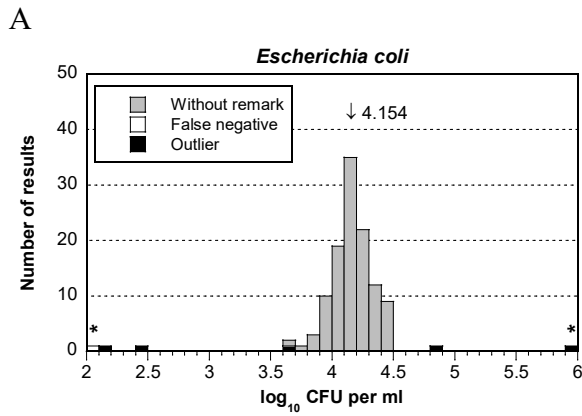
Medium	Sample A							Sample B							Sample C						
	N	n	m	s	F	<	>	N	n	m	s	F	<	>	N	n	m	s	F	<	>
All results	50	46	4.166	0.154	1	3	0	50	46	4.125	0.187	1	2	1	50	50	-	-	0	-	-
TSA/VRB	21	20	4.198	0.155	0	1	0	21	19	4.157	0.188	0	1	1	21	21	-	-	0	-	-
VRB	12	11	4.141	0.151	0	1	0	12	12	4.076	0.183	0	0	0	12	12	-	-	0	-	-
Petrifilm EC/CC	6	5	4.128	0.146	0	1	0	6	6	4.120	0.223	0	0	0	6	6	-	-	0	-	-
EC	3	3	-	-	0	0	0	3	2	-	-	0	1	0	3	3	-	-	0	-	-
Petrifilm CC	1	1	-	-	0	0	0	1	1	-	-	0	0	0	1	1	-	-	0	-	-
RAPID'E.coli	1	1	-	-	0	0	0	1	1	-	-	0	0	0	1	1	-	-	0	-	-
Other	6	5	4.115	0.162	1	0	0	6	5	4.103	0.211	1	0	0	6	6	-	-	0	-	-



Results from analysis of *Escherichia coli*

Method	Sample A						Sample B						Sample C						
	N	n	m	s	F	< >	N	n	m	s	F	< >	N	n	m	s	F	< >	
All results	118	112	4.154	0.157	1	3 2	117	111	4.081	0.182	1	4 1	119	119	-	-	0	-	-
Petrifilm EC/CC	26	24	4.175	0.140	0	1 1	26	25	4.076	0.182	0	1 0	24	24	-	-	0	-	-
TSA/VRB*	24	23	4.195	0.151	0	1 0	24	22	4.108	0.169	1	1 0	24	24	-	-	0	-	-
Petrifilm SEC	19	18	4.159	0.132	1	0 0	18	17	4.030	0.139	0	1 0	19	19	-	-	0	-	-
TBX	16	15	4.052	0.163	0	1 0	16	16	4.036	0.150	0	0 0	17	17	-	-	0	-	-
VRB	11	11	4.142	0.123	0	0 0	11	11	4.044	0.178	0	0 0	11	11	-	-	0	-	-
TEMPO EC	7	7	4.267	0.102	0	0 0	7	7	4.266	0.200	0	0 0	7	7	-	-	0	-	-
Brilliance EC/CC	3	3	-	-	0	0 0	3	3	-	-	0	0 0	3	3	-	-	0	-	-
Compact Dry EC	2	2	-	-	0	0 0	2	2	-	-	0	0 0	2	2	-	-	0	-	-
EC	2	2	-	-	0	0 0	2	2	-	-	0	0 0	2	2	-	-	0	-	-
Other	8	7	4.162	0.131	0	0 1	8	6	4.189	0.153	0	1 1	10	10	-	-	0	-	-

\* The group TSA/VRB includes three laboratories that used TSA/VRBG.



## **Presumptive *Bacillus cereus***

---

### **Sample A**

No target organism for the analysis was present in the sample. Laboratories may mistakenly have included either *S. marcescens* or *S. hyicus*, which may form atypical colonies on BcsA.

### **Sample B**

No target organism for the analysis was present in the sample. Laboratories may mistakenly have included either *S. marcescens* or *S. aureus*. During the initial quality control of the sample at the Swedish Food Agency, small atypical colonies were observed on blood agar. Upon confirmation, they formed atypical colonies without blue colouring on BcsA.

### **Sample C**

The strain of *B. cereus* was target organism. On BA it forms typical irregular, grey-white colonies surrounded by a zone of haemolysis. On BcsA it forms blue colonies with a lecithinase zone. At the Swedish Food Agency, in addition to *B. cereus* we observed two other types of colonies on BA. These are atypical shiny colonies without a zone of haemolysis. Upon confirmation on BcsA, only *B. cereus* forms typical blue colonies with a lecithinase zone.

### **General remarks**

Most laboratories followed either NMKL 67:2010 (54 %) or ISO 7932:2004 (23 %), which differ somewhat. NMKL 67:2010 is based on primary incubation on BA. On this medium, *B. cereus* forms large, irregular grey colonies, surrounded by a distinct zone of haemolysis. Colonies are confirmed either on BcsA or on Cereus-Ident agar. On BcsA presumptive *B. cereus* form bluish colonies that are surrounded by a blue zone of precipitation, due to lecithinase activity on egg yolk present in the medium. On Cereus-Ident agar, presumptive *B. cereus* are blue/turquoise and possibly surrounded by a blue ring. The colour is a result of *B. cereus* phosphatidylinositol phospholipase C (PI-PLC) cleavage of the chromogenic substrate X-myoinositol-1-phosphate present in Cereus-Ident agar. In contrast to the NMKL method, ISO 7932:2004 prescribes plating onto MYP, followed by confirmation on BA. On MYP, presumptive *B. cereus* form large pink colonies that are normally surrounded by a zone of precipitation, again as a consequence of lecithinase activity. The ISO method uses haemolysis on BA as the method for confirmation.

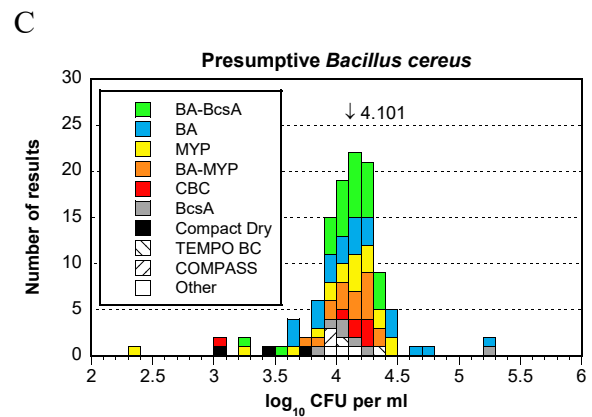
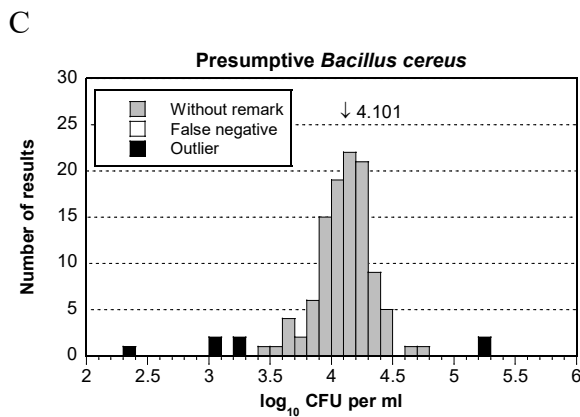
In addition to BA, BcsA and MYP, the chromogenic medium CBC was used by seven laboratories. Cleavage of the substrate X-Gluc present in CBC by *B. cereus*  $\beta$ -glucuronidase results in white colonies with a blue/green centre. Other media that were used to a lesser extent were Compact Dry X-BC, TEMPO BC and COMPASS® *Bacillus cereus* agar.

As in previous proficiency testing rounds the reporting of method data was in several cases unclear. For example, several laboratories reported combinations of method and media that were incompatible. As a general rule, this report shows the methods and media stated by the laboratories, regardless if these are compatible or not. Despite these uncertainties, the results and mean values for the different methods and media are very similar.

Results from analysis of presumptive *Bacillus cereus*

Medium	Sample A						Sample B						Sample C						
	N	n	m	s	F	< >	N	n	m	s	F	< >	N	n	m	s	F	< >	
All results	115	113	-	-	2	- -	114	112	-	-	2	- -	114	107	4.101	0.216	0	5	2
BA-BcsA*	29	29	-	-	0	- -	29	29	-	-	0	- -	29	28	4.122	0.163	0	1	0
BA	25	24	-	-	1	- -	24	23	-	-	1	- -	25	24	4.106	0.309	0	0	1
MYP	19	19	-	-	0	- -	19	19	-	-	0	- -	19	17	4.135	0.203	0	2	0
BA-MYP	18	18	-	-	0	- -	18	17	-	-	1	- -	17	17	4.095	0.173	0	0	0
CBC	7	7	-	-	0	- -	7	7	-	-	0	- -	7	6	4.162	0.070	0	1	0
BcsA*	7	6	-	-	1	- -	7	7	-	-	0	- -	7	6	4.052	0.140	0	0	1
Compact Dry X-BC	3	3	-	-	0	- -	3	3	-	-	0	- -	3	2	-	-	0	1	0
TEMPO BC	2	2	-	-	0	- -	2	2	-	-	0	- -	2	2	-	-	0	0	0
COMPASS B. cereus	2	2	-	-	0	- -	2	2	-	-	0	- -	2	2	-	-	0	0	0
Other	3	3	-	-	0	- -	3	3	-	-	0	- -	3	3	-	-	0	0	0

\* The use of PEMBA has been interpreted as the use of BcsA.



## Coagulase-positive staphylococci

### Sample A

The sample contained a strain of *S. hyicus*, which is normally included among coagulase-positive staphylococci. However in tests at the Swedish Food Agency, the strain in sample A displays no, or only weak, coagulase activity. On RPFA it forms grey/white colonies, without a zone of precipitation. With methods based on rabbit plasma, it should therefore normally not be considered as coagulase-positive.

At the Swedish Food Agency, the strain is only characterised with media and confirmation methods based on rabbit plasma. It is therefore possible that there is a variation in how it performs on other media and with other methods for confirmation. For example, we have been informed that it may form atypical black colonies on Petrifilm Staph. If these colonies are surrounded by a pink DNase zone upon confirmation with Petrifilm Disk, the strain should be counted as confirmed with that method. It is likely that the assessment of *S. hyicus* was problematic with Petrifilm Staph. This is since eight of the 19 laboratories that analysed with this method reported a negative result, while eleven instead reported a positive result.

In total, 18 laboratories reported a positive result. The median for these was  $\log_{10}$  4.23 cfu ml<sup>-1</sup>, which corresponds to the concentration of *S. hyicus* in the sample.

***Due to the characteristics of the current strain, both positive and negative results are considered as correct. The results are therefore not evaluated further, and no z-scores are calculated for the analysis.***

#### **Sample B**

The strain of *S. aureus* was target organism. On RPFA it forms typical convex grey colonies, surrounded by a zone of precipitation.

#### **Sample C**

No target organism was present in the sample. False positive results are likely due to detection of *S. xylosus*, which on RPFA may form atypical grey colonies without a zone of precipitation. The ten false positive results that were reported ranged between  $\log_{10}$  0.95 and  $\log_{10}$  5.17 cfu ml<sup>-1</sup>, with a median of  $\log_{10}$  4.53 cfu ml<sup>-1</sup>. At the Swedish Food Agency quality control, the concentration of *S. xylosus* was determined to be 5.25 cfu ml<sup>-1</sup>.

#### **General remarks**

Most laboratories (38 %) followed NMKL 66:2009. The use of 3M™ Petrifilm™ was higher (17 %) than previously, while the use of ISO 6888-1:1999 (15 %) and ISO 6888-2:1999 (12 %) was similar to previous proficiency testing rounds. Both ISO 6888-1:1999 (based on BP) and ISO 6888-2:1999 (based on RPFA) were last reviewed by ISO in 2015 and remain current. An alternative confirmation by stab-culture in RPFA has however been added for ISO 6888-1 (ISO 6888-1:1999/Amd 2:2018).

NMKL 66:2009 prescribes incubation on BP and/or RPFA. On BP, *S. aureus* forms characteristic convex, shiny colonies that have a grey/black colour due to reduction of tellurite in the medium. Proteolysis of egg yolk in the medium (due to lecithinase activity) normally causes a clear zone around the colonies. An opaque halo may also form near the colony, due to precipitation caused by lipase activity. The colonies are confirmed by a positive result in a coagulase test. When using RPFA, the coagulase activity is instead tested directly in the medium, and no further confirmation is required. In comparison, ISO 6888-1:1999 stipulates surface spreading on BP followed by confirmation with a coagulase test, whereas 6888-2:1999 stipulates the use of RPFA. Petrifilm Staph is based on a modified Baird-Parker agar. It also contains a chromogenic indicator that causes *S. aureus* to form red/purple colonies.

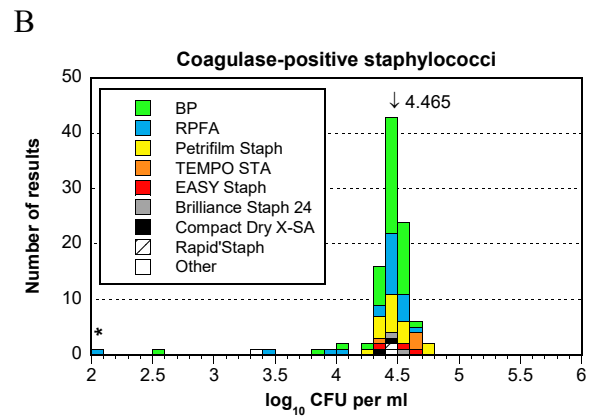
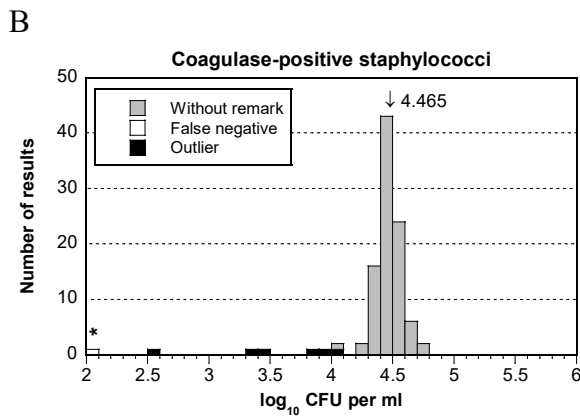
In summary, the results were very similar for the most common media BP, RPFA and Petrifilm Staph, in all three samples. The exception was the high number of positive results for Petrifilm Staph in sample A. Slightly lower mean values have in previous proficiency testing rounds been observed for Petrifilm Staph, but no such trend could be seen this time. Several media were used by only a small number of laboratories, which make them difficult to evaluate. However altogether, only one result with an annotation was reported by the laboratories that used either of the media TEMPO STA, EASY Staph®, Brilliance™ Staph 24, Compact Dry™ X-SA and Rapid Staph.

In total, 71 % of the laboratories reported that they performed some kind of confirmation. Traditionally, confirmation of coagulase-positive staphylococci is by detection of extracellular or bound coagulase (tube coagulase test and slide coagulase test respectively). Another common confirmation is a latex agglutination test. This is based on latex particles coated either with fibrinogen or with IgG that binds to protein A on the bacterial cell surface. Antibodies targeted against polysaccharides on the

bacterial cell surface are also used in variations of this test. The majority of the positive results in sample A were reported by laboratories that used Petrifilm Staph, of which most reported confirming the colonies with Petrifilm Disk. This confirmation method is based on detection of extracellular DNase, which is produced by the majority of coagulase-positive *S. aureus*, but also by the coagulase-positive staphylococci *S. intermedius* and *S. hyicus*. Toluidin blue O in the disks visualises DNase activity as a pink zone around the colonies.

*Results from analysis of coagulase-positive staphylococci*

Medium	Sample A						Sample B						Sample C						
	N	n	m	s	F	< >	N	n	m	s	F	< >	N	n	m	s	F	< >	
All results	104	86	-	-	18	- -	101	94	4.465	0.108	1	6	0	103	93	-	-	10	- -
BP	48	44	-	-	4	- -	46	44	4.449	0.101	0	2	0	48	43	-	-	5	- -
RPFA	23	20	-	-	3	- -	23	19	4.480	0.076	1	3	0	22	21	-	-	1	- -
Petrifilm Staph	19	8	-	-	11	- -	18	18	4.463	0.124	0	0	0	19	16	-	-	3	- -
TEMPO STA	4	4	-	-	0	- -	4	4	-	-	0	0	0	4	4	-	-	0	- -
EASY Staph	3	3	-	-	0	- -	3	3	-	-	0	0	0	3	3	-	-	0	- -
Brilliance Staph 24	2	2	-	-	0	- -	2	2	-	-	0	0	0	2	2	-	-	0	- -
Compact Dry X-SA	2	2	-	-	0	- -	2	2	-	-	0	0	0	2	1	-	-	1	- -
Rapid'Staph	1	1	-	-	0	- -	1	1	-	-	0	0	0	1	1	-	-	0	- -
Other	2	2	-	-	0	- -	2	1	-	-	0	1	0	2	2	-	-	0	- -





## Enterococci

---

### Sample A

No target organism for the analysis was present in the sample.

### Sample B

The strain of *E. durans* was target organism. On Slanetz & Bartley *Enterococcus* agar (ENT) it forms typical raised, dark red colonies. Upon confirmation on BEA a faint tan/black colour is normally seen in the medium after 2 hours, and a distinct black colour after 24 hours.

### Sample C

No *Enterococcus* was present. Laboratories may however have detected *P. acidilactici*, which may form atypical, faint pink colonies on ENT. Upon confirmation on BEA, no tan/black colour is usually seen after 2 hours, but a faint tan/black colour can be seen after 24 hours. This can likely explain why 26 laboratories reported a false positive result. The majority of these results were between  $\log_{10}$  4.0 and  $\log_{10}$  4.4 cfu ml<sup>-1</sup>, which corresponds well to the concentration of *P. acidilactici* in the sample.

***Due to the characteristics of the current strain, both positive and negative results are considered as correct. The results are therefore not evaluated further, and no z-scores are calculated for the analysis.***

**Comment:** In a previous proficiency testing round (October 2003), the same strain of *P. acidilactici* was distinguished since, in contrast to *Enterococcus*, it does not grow in BHI with 6.5 % salt or in BHI with pH 9.6. Confirmation with growth in BHI is included in the older NMKL 68:2004.

### General remarks

A clear majority of the laboratories (64 %) followed NMKL 68:2011. Among the less frequently used methods were the drinking water method ISO 7899-2:2000 (7 %), IDF 149A:1997 (6 %) and the older NMKL 68:2004 (3 %). Most of the remaining laboratories used company-specific methods. It should be mentioned that according to ISO, IDF 149A:1997 has been replaced by ISO 27205:2010/IDF 149:2010.

With NMKL 68:2011 enterococci are defined as Gram-positive, catalase-negative and oval cocci that hydrolyse esculin at 44 °C. Incubation is done on ENT at 44 °C. On this medium, enterococci reduce the colourless substrate 2,3,5-trifenylnitroimidazolium chloride to red formazan and form slightly raised colonies with a pink/red/maroon colour. They can sometimes also have a colourless edge. When stressed enterococci are suspected (e.g. in frozen foods) a pre-incubation in TSA for 2 hours at 37 °C is recommended, followed by overlay with ENT. Dark red colonies with typical morphology are counted as enterococci without further confirmation. Non-typical colonies are confirmed by sub-culturing on BEA. On BEA the substrate esculin is hydrolysed by  $\beta$ -glucosidase present in enterococci, which results in the formation of esculetin and glucose. Esculetin together with iron ions present in the medium then form a black precipitate. Colonies that cause a tan/black colour in the medium after 2-24 hours are counted as enterococci. The drinking water method ISO 7899-2:2000 is based on membrane filtration followed by incubation on ENT at 37 °C. The confirmation is similar to the NMKL method, but is done by transferring the whole membrane filter from ENT to BEA (possibly with the addition of azide), and with incubation only for 2 hours. With the older NMKL 68:2004 confirmation is not done with BEA, but with a catalase test, as well as tests for the growth in BHI with 6.5 % salt and in BHI with pH

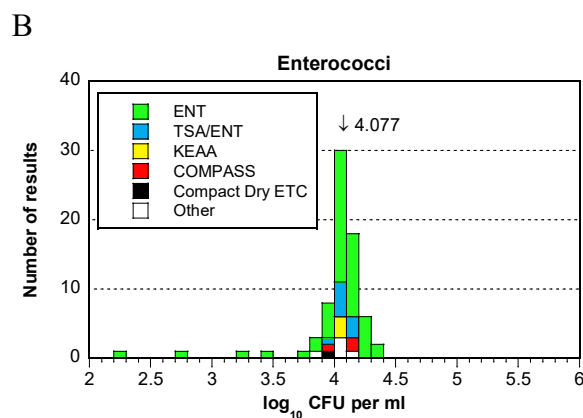
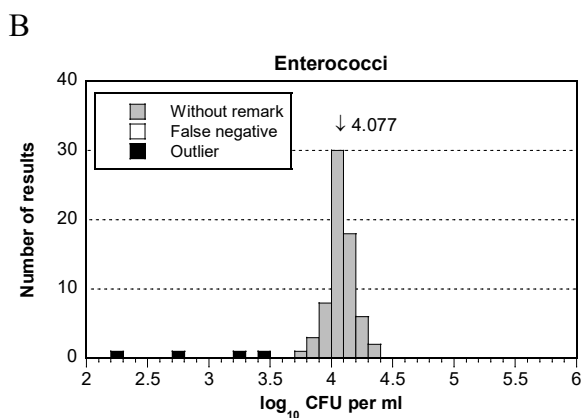
9.6. Despite this, both laboratories that analysed according to NMKL 68:2004 reported false positive results for sample C.

In total, 83 % of the laboratories incubated either on ENT or on TSA/ENT. A smaller number of laboratories used KEAA, COMPASS® *Enterococcus* agar or Compact Dry ETC. KEAA was used by laboratories that followed IDF 149A:1997. With KEAA, the esculin hydrolysis is tested directly in the medium. Similar to BEA, COMPASS detects β-glucosidase activity, but is instead based on the substrate X-Gluc. Enterococci therefore form blue colonies on this medium.

Confirmation of some kind was in total reported by 79 % of the laboratories. For sample C, performing or not performing a confirmation does not appear to have had an impact on the outcome (80 % confirmation among the negative results and 77 % confirmation among the positive results).

#### Results from analysis of enterococci

Medium	Sample A						Sample B						Sample C						
	N	n	m	s	F	< >	N	n	m	s	F	< >	N	n	m	s	F	< >	
All results	72	71	-	-	1	- -	72	68	4.077	0.109	0	4	0	72	46	-	-	26	- -
ENT	51	50	-	-	1	- -	51	47	4.089	0.116	0	4	0	51	32	-	-	19	- -
TSA/ENT	9	9	-	-	0	- -	9	9	4.069	0.071	0	0	0	9	5	-	-	4	- -
KEAA	3	3	-	-	0	- -	3	3	-	-	0	0	0	3	2	-	-	1	- -
COMPASS	3	3	-	-	0	- -	3	3	-	-	0	0	0	3	2	-	-	1	- -
Compact Dry ETC	1	1	-	-	0	- -	1	1	-	-	0	0	0	1	1	-	-	0	- -
Other	5	5	-	-	0	- -	5	5	4.030	0.095	0	0	0	5	4	-	-	1	- -



## Gram-negative bacteria in pasteurized milk and cream

---

### Sample A

The strains of *E. coli* and *S. marcescens* are Gram-negative.

### Sample B

The strains of *E. coli* and *S. marcescens* are Gram-negative.

### Sample C

No Gram-negative microorganism were present in the sample.

### General remarks

All reported results were correct. Ten laboratories followed NMKL 192:2011. One laboratory followed the ISO method for Enterobacteriaceae, ISO 21528-2:2017. The remaining two laboratories followed a company-specific method. Twelve of the 13 laboratories incubated on VRBG, while one used MacConkey agar.

NMKL 192:2011 is a qualitative method for detecting recontamination of Gram-negative bacteria in pasteurised milk and cream. Gram-negative bacteria do not survive high temperature/short time pasteurization (HTST), where the temperature is raised to 72 °C for at least 15 seconds. Presence of Gram-negative bacteria therefore indicates recontamination, something that may limit the shelf-life of the product. With the method the unopened package of milk/cream is pre-incubated at 25 °C for 24 h followed by plating and incubation of 10 µl on VRBG. Presence of five or more colonies on VRBG is considered a positive result, regardless of colony morphology and colour. When needed, confirmation can be done with potassium hydroxide (KOH). Colonies that form a viscous string after 5-10 seconds of stirring in KOH are considered as Gram-negative bacteria.

### Results from analysis of Gram-negative bacteria in pasteurized milk and cream

Method	Sample A			Sample B			Sample C		
	N	n	F	N	n	F	N	n	F
All results	13	13	0	13	13	0	12	12	0
NMKL 192:2011	10	10	0	10	10	0	10	10	0
ISO 21528-2:2017	1	1	0	1	1	0	0	0	0
Other	2	2	0	2	2	0	2	2	0

## **Outcome of the results of individual laboratory - assessment**

### **Reporting and evaluation of results**

The reported results of all participating laboratories are listed in Annex 1, together with the minimum and maximum accepted values for each analysis. Results that received a remark (false results and outliers) are highlighted in yellow, with bold font.

It is the responsibility of the participating laboratories to correctly report results according to the instructions. When laboratories incorrectly report their results, for example by stating “pos” or “neg” for quantitative analyses, the results cannot be correctly processed. Such incorrectly reported results are normally excluded. Inclusion and further processing of such results may still be done, after manual assessment in each individual case.

Z-scores (see below) for individual analyses are shown in Annex 2 and can be used as a tool by laboratories when following up on the results.

The laboratories are not grouped or ranked based on their results. The performance of a laboratory as a whole can be evaluated from the number of false results and outliers that are listed in Annex 1 and below the box plots.

Information on the results processing and recommendations for follow-up work are given in the Scheme Protocol (2). Samples for follow-up can be ordered, free of charge via our website: [www.livsmedelsverket.se/en/PT-extra](http://www.livsmedelsverket.se/en/PT-extra)

### **Z-scores, box plots and deviating results**

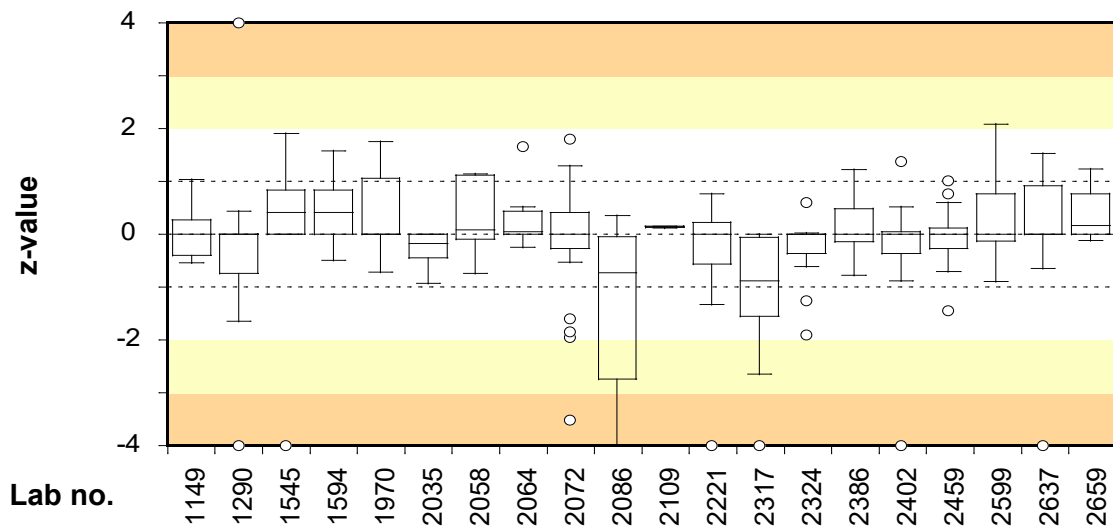
In order to allow comparison of the results from different analyses and mixtures, all results are transformed into standard values (z-scores). For quantitative analyses, a z-score is either positive or negative, depending on whether the individual result is higher or lower than the mean value calculated from all laboratory results for each analysis.

The box plots are based on the z-scores listed in Annex 2, and give a comprehensive view of the achievement of each laboratory. A small box, centred around zero, indicates that the results of the individual laboratory, with false results excluded, are close to the general mean values calculated for all laboratory results. The range of z-scores is indicated by the size of the box and, for most laboratories, by lines and/or circles above and beneath the box. For each laboratory, the number of false results and outliers are also listed in the tables below the box plots.

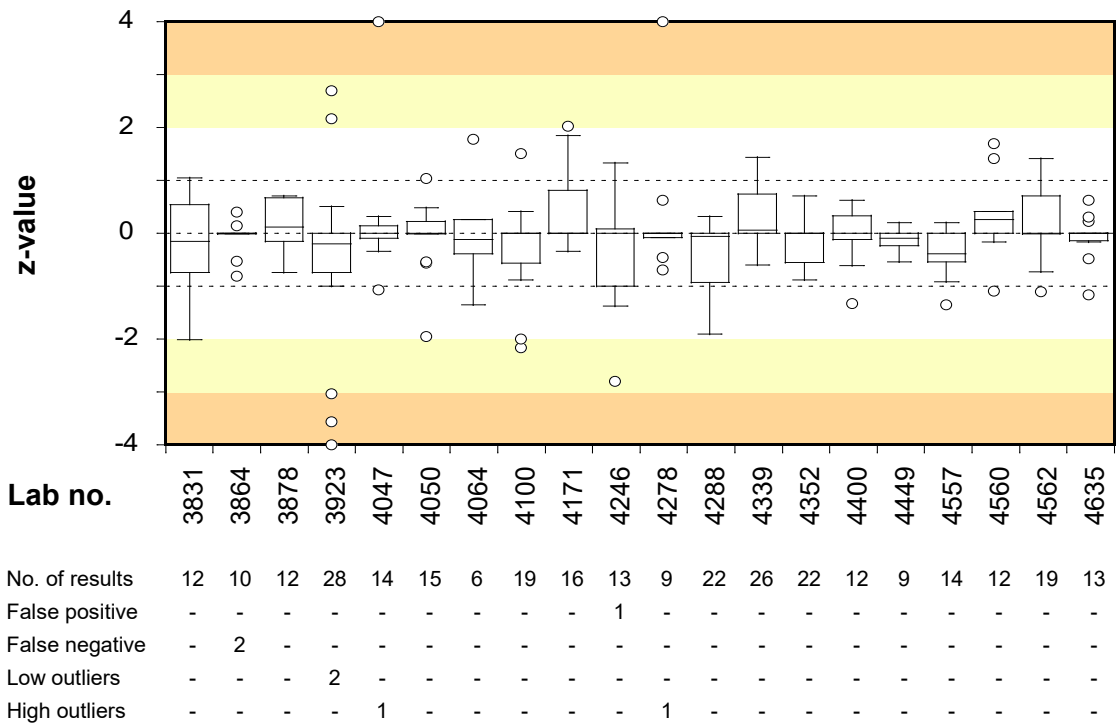
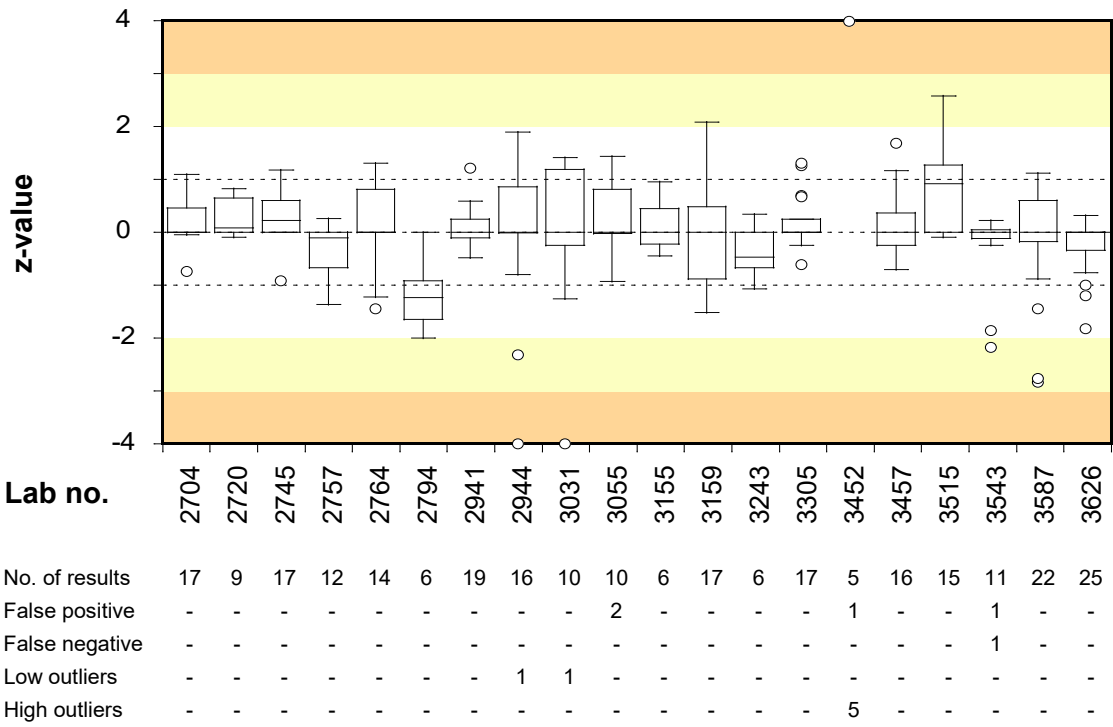
### Box plots and numbers of deviating results for each laboratory

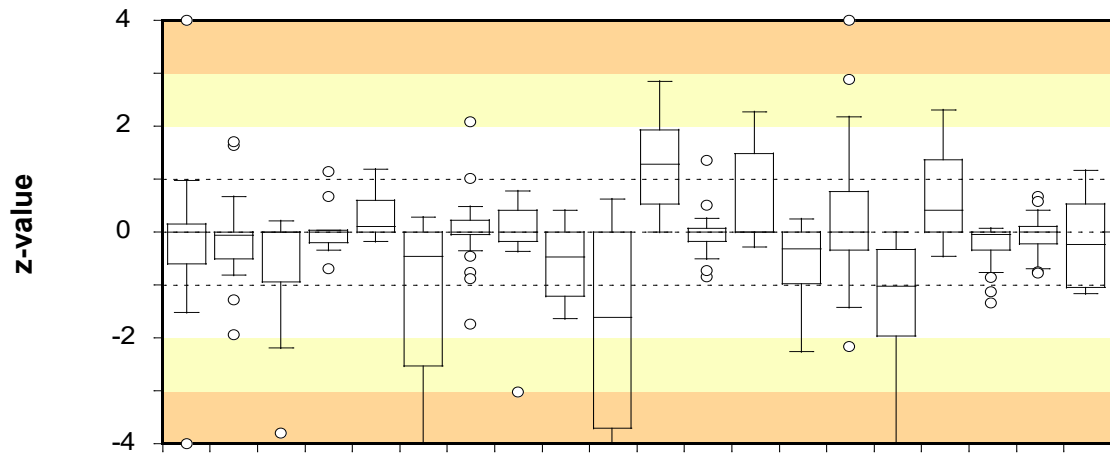
- Z-scores are calculated according to the formula:  $z = (x-m)/s$ , where  $x$  is the result of the individual laboratory,  $m$  is the mean of the results of all participating laboratories, and  $s$  is the standard deviation of the participating laboratories, after removing outliers and false results.
- Outliers are included in the figures after being calculated to z-scores in the same way as for other results.
- False results do not generate any z-scores, and are not included in “No. of results”.
- Correct results for qualitative analyses and correct negative results for quantitative analyses without target organism generate a z-score of 0.
- The laboratory median value is illustrated by a horizontal line in the box.
- The box includes 50 % of a laboratory’s results (25 % of the results above the median and 25 % of the results below the median). The remaining 50 % are illustrated by lines and circles outside the box.
- A circle is for technical reasons shown in the plot when a value deviates to certain degree\* from the other values. This does not by itself indicate that the value is an outlier.
- z-scores  $>+4$  and  $<-4$  are positioned at  $+4$  and  $-4$ , respectively, in the plot.
- The background is divided by lines and shaded fields to simplify identifying the range in which the results are located.

\*  $< [lowest\ value\ in\ the\ box - 1,5 \times (highest\ value\ in\ the\ box - lowest\ value\ in\ the\ box)]$   
or  
 $> [highest\ value\ in\ the\ box + 1,5 \times (highest\ value\ in\ the\ box - lowest\ value\ in\ the\ box)]$ .

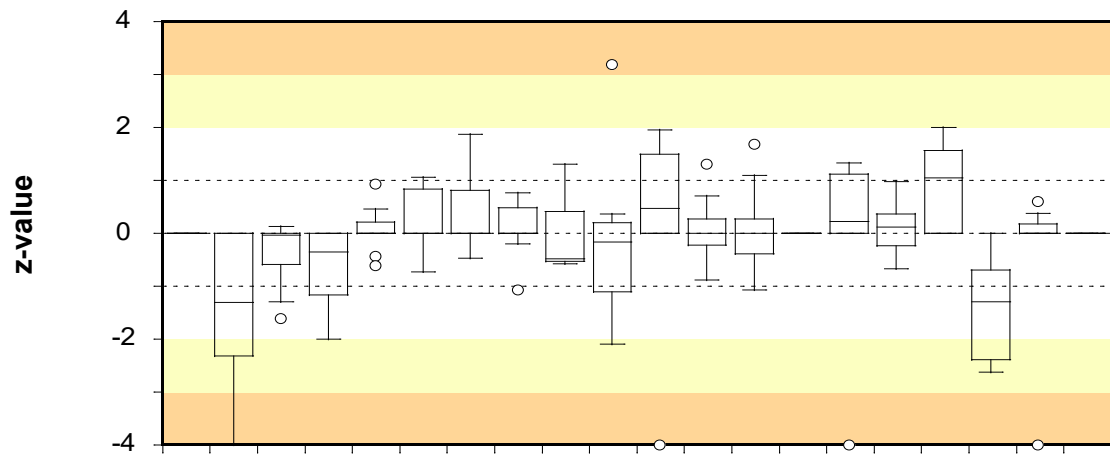


Lab no.	1149	1290	1545	1594	1970	2035	2058	2064	2072	2086	2109	2221	2317	2324	2386	2402	2459	2599	2637	2659
No. of results	14	26	19	28	28	6	6	9	28	11	3	25	15	16	14	12	16	23	17	17
False positive	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	1	-	-	-
False negative	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Low outliers	-	1	1	-	-	-	-	-	1	3	-	2	1	-	-	1	-	-	1	-
High outliers	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

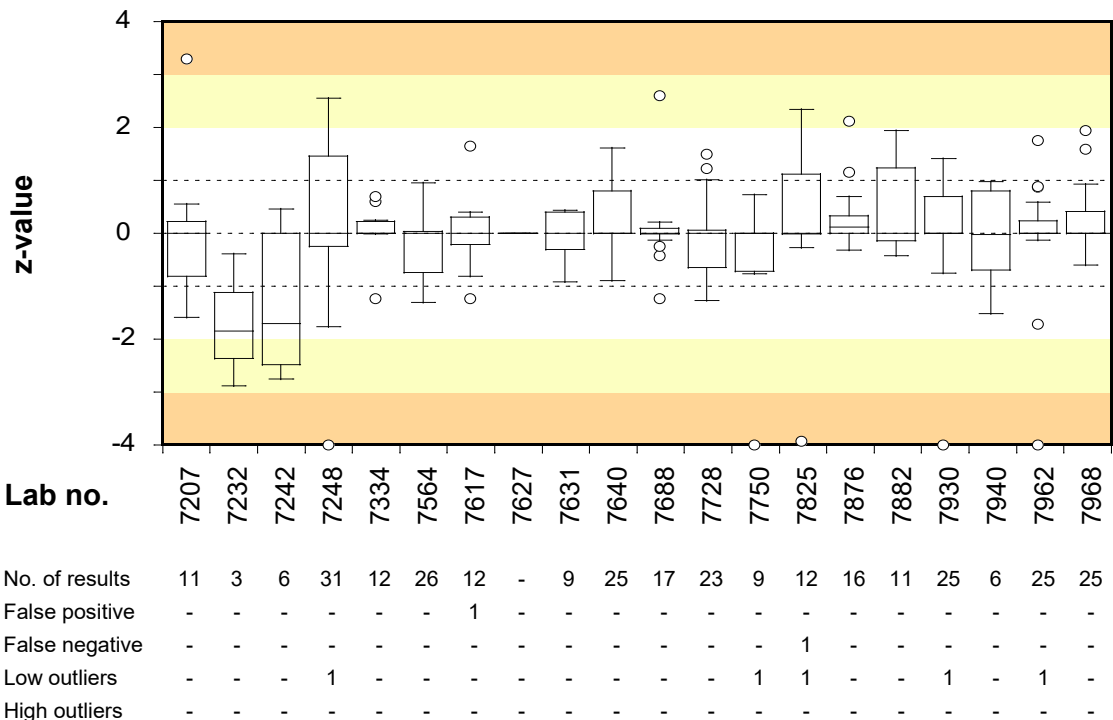
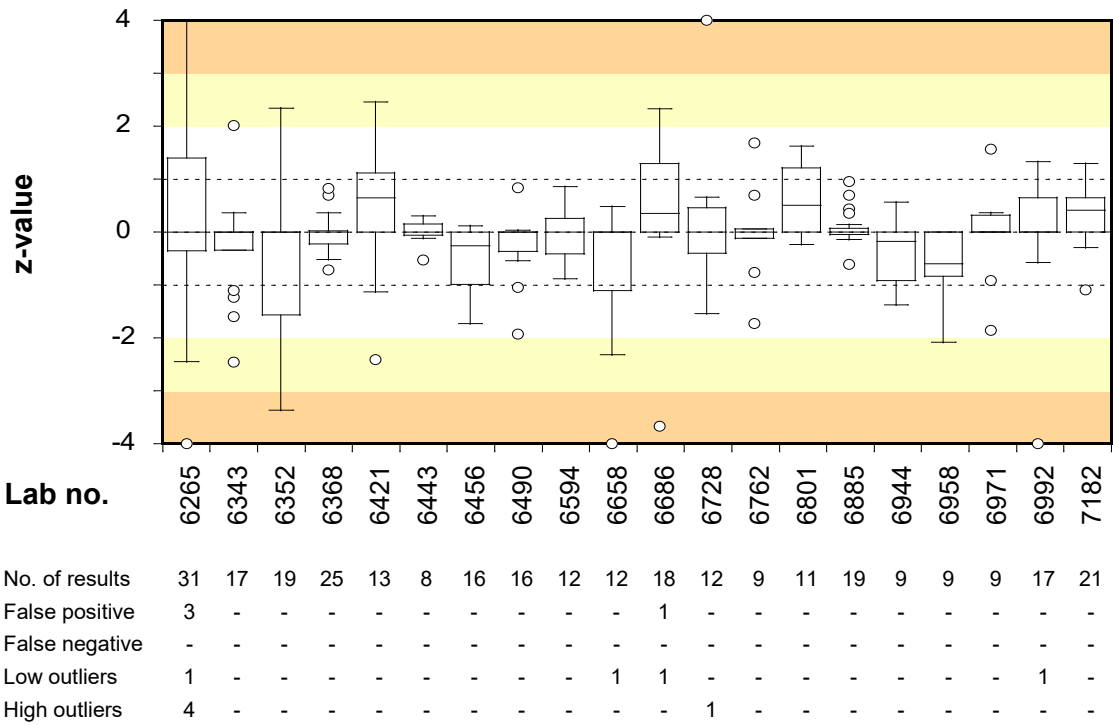




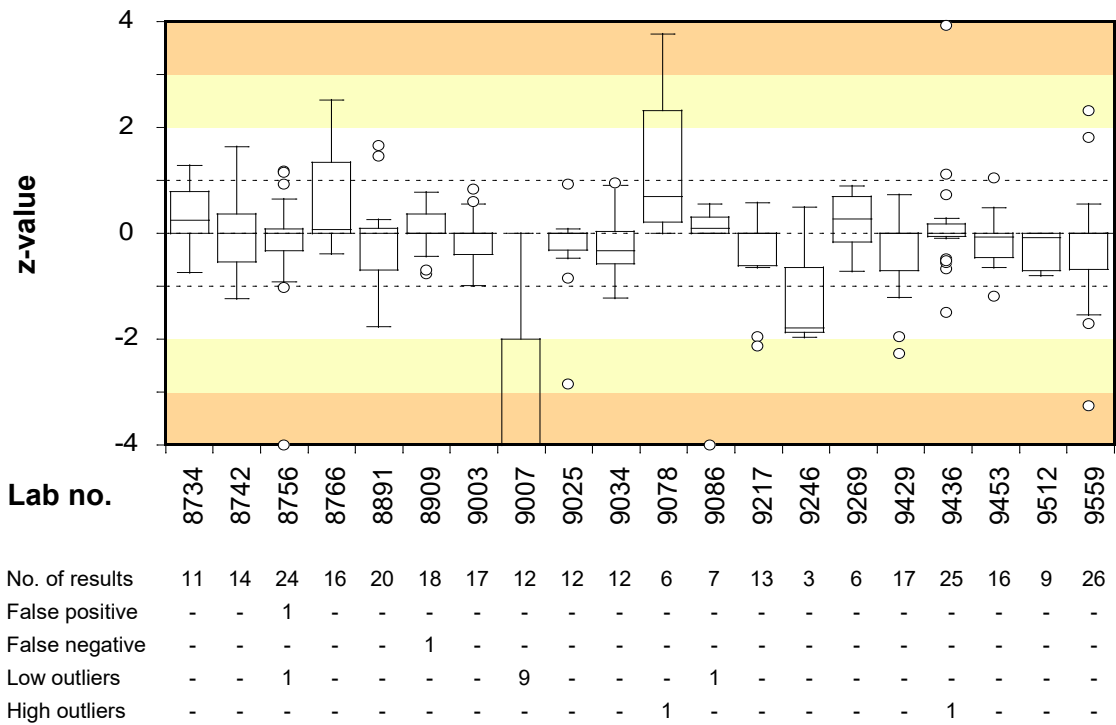
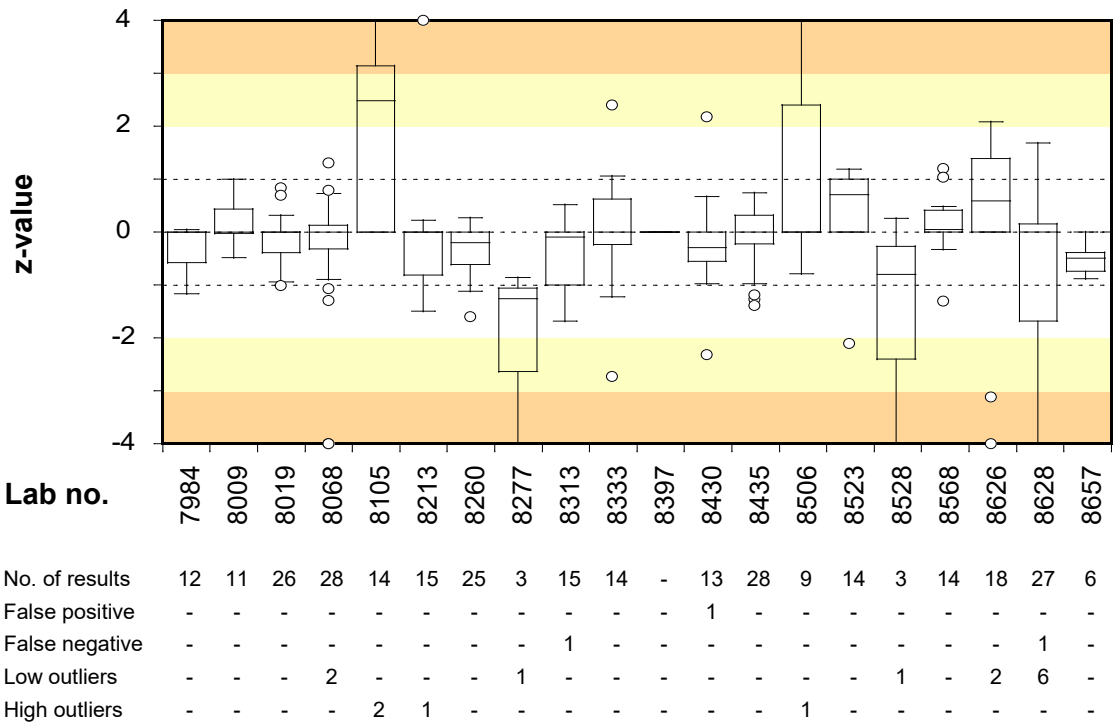
Lab no.	4664	4683	4710	4840	4889	4951	4980	4983	5018	5100	5119	5128	5182	5201	5204	5220	5290	5329	5333	5338
No. of results	22	23	21	10	25	10	23	9	25	8	12	14	12	9	25	11	10	19	25	6
False positive	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-
False negative	-	-	-	-	-	2	-	-	-	1	-	-	-	-	-	-	-	-	-	-
Low outliers	1	-	1	-	-	2	-	-	-	2	-	-	-	-	-	1	-	-	-	-
High outliers	2	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-

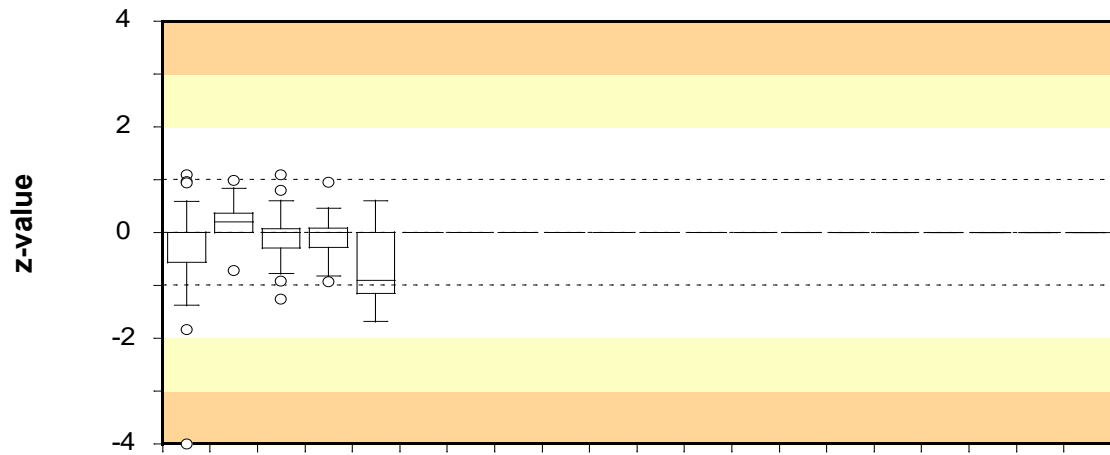


Lab no.	5342	5352	5419	5446	5545	5553	5615	5654	5701	5801	5808	5883	5950	5993	6109	6175	6224	6232	6253	6258
No. of results	-	21	19	17	10	17	17	9	3	10	12	14	34	-	9	6	9	6	11	-
False positive	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-
False negative	-	-	-	-	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Low outliers	-	1	-	-	-	-	-	-	-	-	1	-	-	-	1	-	-	-	1	-
High outliers	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-









Lab no.	9662	9747	9890	9903	9950
No. of results	25	9	20	16	12
False positive	-	-	-	-	-
False negative	-	-	-	-	-
Low outliers	1	-	-	-	-
High outliers	-	-	-	-	-

## Test material and quality control

### Test material

Each laboratory received three sample mixtures with freeze-dried microorganisms, designated A-C. The test material was freeze-dried in portions of 0.5 ml in vials, as described by Peterz and Steneryd (3). Before analysing the samples, the contents of each vial should be dissolved in 254 ml of sterile diluent. The organisms present in the mixtures are listed in Table 2.

**Table 2.** *Microorganisms in the samples*

Sample <sup>1</sup>	Microorganism	Strain	
		SLV no. <sup>2</sup>	Reference <sup>3</sup>
A	<i>Escherichia coli</i>	SLV-477	CCUG 43601
	<i>Serratia marcescens</i>	SLV-040	ATCC 13880
	<i>Staphylococcus hyicus</i>	SLV-546	Chicken
B	<i>Enterococcus durans</i>	SLV-078	CCUG 44816
	<i>Escherichia coli</i>	SLV-477	CCUG 43601
	<i>Serratia marcescens</i>	SLV-040	ATCC 13 880
	<i>Staphylococcus aureus</i>	SLV-280	Egg, 1989
C	<i>Bacillus cereus</i>	SLV-518	CCUG 44741
	<i>Pediococcus acidilactici</i>	SLV-213	CCUG 45146
	<i>Staphylococcus xylosus</i>	SLV-283	Cheese, 1989

<sup>1</sup> The links between the mixtures and the randomised sample numbers are shown in Annex 1.

<sup>2</sup> Internal strain identification no. at the Swedish Food Agency

<sup>3</sup> Origin or culture collection (CCUG: Culture Collection University of Gothenburg, Sweden ; ATCC: American Type Culture Collection)

### Quality control of the samples mixtures

In order to allow comparison of all freeze-dried samples, it is essential to have aliquots of homogeneous sample mixtures and equal volume in all vials. Quality control is performed on 10 randomly chosen vials in conjunction with manufacturing of the samples or on 5 vials if an “old” sample mixture was used and the last quality control was performed more than 6 months ago. Homogeneity of a sample mixture is approved if, for each analysis, the values obtained for the test of reproducibility (T) and the test “Index of dispersion” between vials ( $I_2$ ) do not simultaneously exceed 2.6 and 2.0, respectively. (For definitions of T and  $I_2$ , see references 4 and 5 respectively.)

**Table 3.** Concentration mean ( $m$ ), T and  $I_2$  values from the quality control of the sample mixtures;  $m$  is expressed in  $\log_{10}$  cfu (colony forming units) per ml of sample.

Analysis and method	A <sup>1</sup>			B <sup>1</sup>			C <sup>1</sup>		
	m	T	$I_2$	m	T	$I_2$	m	T	$I_2$
Aerobic microorganisms, 30 °C NMKL method no. 86:2013	4.66	1.28	0.69	5.01	1.36	2.27	5.32	1.17	0.65
Aerobic microorganisms, 20 °C NMKL method no. 86:2013	4.67	1.39	1.22	4.98	1.19	0.79	5.29	1.21	0.92
Contaminating microorganisms ISO method no. 13559:2002 IDF method no. 153:2002	4.69	1.27	0.68	5.03	1.13	0.41	5.32	1.19	0.84
Enterobacteriaceae NMKL method no. 144:2005	4.36	1.20	0.97	4.67	1.25	0.59	-	-	-
Coliform bacteria 30 °C NMKL method no. 44:2004	4.17	1.63	4.75	4.16	1.17	0.09	-	-	-
Coliform bacteria 37 °C NMKL method no. 44:2004	4.19	1.62	4.81	4.13	1.15	0.06	-	-	-
Thermotolerant coliform bacteria NMKL method no.125:2005	4.30	2.04	1.19	4.13	1.30	1.16	-	-	-
<i>Escherichia coli</i> NMKL method no. 125:2005	4.30	2.04	1.19	4.13	1.30	1.16	-	-	-
Presumptive <i>Bacillus cereus</i> NMKL method no. 67:2010	-	-	-	-	-	-	4.19	1.57	0.81
Coagulase-positive staphylococci NMKL method no. 66:2009	4.38*	1.59*	1.17*	4.55	1.53	1.54	5.25*	1.13*	0.34*
Enterococci NMKL method no. 68:2011	-	-	-	4.07	1.34	1.21	4.20*	1.29*	1.34*
Gram-negative bacteria in pasteurized milk and cream. Detection of recontamination. NMKL method no. 192:2011	Pos.	-	-	Pos.	-	-	Neg.	-	-

- No target organism and therefore no value

<sup>1</sup> n = 5 vials analysed in duplicate

\* Not a target organism for the analysis

## References

1. Kelly, K. 1990. Outlier detection in collaborative studies. *J. Assoc. Off. Anal. Chem.* 73:58–64.
2. Anonym, 2018. Verksamhetsprotokoll. Mikrobiologi. Dricksvatten & Livsmedel, Livsmedelsverket.
3. Peterz, M., Steneryd, A.C. 1993. Freeze-dried mixed cultures as reference samples in quantitative and qualitative microbiological examinations of food. *Journal of Applied Bacteriology*. 74:143–148.
4. Mooijman, K.M., During, M. & Nagelkerke, N.J.D. 2003. MICROCRM: Preparation and control of batches of microbiological materials consisting of capsules. RIVM report 250935001/2003. RIVM, Bilthoven, Holland.
5. Heisterkamp, S.H., Hoekstra, J.A., van Strijp-Lockefeer, N.G.W.M., Havelaar, A.H., Mooijman, K.A., in't Veld, P.H., Notermans, S.H.W., Maier, E.A. ; Griepink, B. 1993. Statistical analysis of certification trials for microbiological reference materials. Luxembourg: Commission of the European Communities, Report EUR 15008 EN.











Lab no.	Vial	Aerobic microorg. 30 °C			Aerobic microorg. 20 °C			Contaminating microorganisms			Enterobacteriaceae			Coliform bacteria 30 °C			Coliform bacteria 37 °C			Thermotolerant coliform bacteria			Escherichia coli			Presumptive Bacillus cereus			Coagulase-positive Staphylococci			Enterococci			Gram-neg bacteria in dairy prod.			Lab no.
		A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	
9950	1 2 3	4.5	4.73	5.16	-	-	-	4.34	4.62	5.06	-	-	-	-	-	-	4.28	4.18	0	-	-	-	-	-	-	0	0	3.85	-	-	-	-	-	-	-	-	-	9950
<b>N</b>		163	162	162	35	35	35	19	20	20	139	139	138	50	49	50	101	101	101	50	50	50	118	117	119	115	114	114	104	101	103	72	72	72	13	13	12	<b>N</b>
<b>Min</b>		1.69	2.00	2.34	0	0	0	0	0	0	1.08	0	0	1.43	0	0	0	0	0	0	0	0	0	0	0	0	0	2.30	0	0	0	0	2.22	0	-	-	-	<b>Min</b>
<b>Max</b>		6.13	5.92	6.24	4.81	5.12	5.57	5.29	5.16	5.49	5.43	5.30	5.27	5.14	5.62	1.60	4.69	5.00	0.95	4.56	4.86	0	6.20	6.20	0	4.00	3.69	5.28	4.65	4.78	5.17	0.95	4.36	4.37	-	-	-	<b>Max</b>
<b>Med</b>		4.63	4.94	5.31	4.49	4.93	5.25	4.60	4.88	5.30	4.23	4.53	0	4.18	4.36	0	4.14	4.25	0	4.17	4.09	0	4.15	4.05	0	0	0	4.11	0	4.46	0	0	4.08	0	-	-	-	<b>Med</b>
<b>m</b>		4.623	4.940	5.312	4.465	4.938	5.258	4.620	4.858	5.281	4.235	4.489	0	4.152	4.319	0	4.154	4.269	0	4.166	4.125	0	4.154	4.081	0	0	0	4.101	0	4.465	0	0	4.077	0	pos	pos	neg	<b>m</b>
<b>s</b>		0.140	0.125	0.133	0.162	0.079	0.115	0.302	0.212	0.143	0.177	0.207	0	0.248	0.300	0	0.210	0.304	0	0.154	0.187	0	0.157	0.182	0	0	0	0.216	0	0.108	0	0	0.109	0	-	-	-	<b>s</b>
<b>F+</b>		0	0	0	0	0	0	0	0	0	0	0	2	0	0	1	0	0	1	0	0	0	0	0	0	2	2	0	18	0	10	1	0	26	0	0	0	<b>F+</b>
<b>F-</b>		0	0	0	1	1	1	1	1	1	0	1	0	0	1	0	2	1	0	1	1	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	<b>F-</b>
<b>&lt;</b>		2	4	7	0	1	2	0	1	4	2	2	0	2	2	0	2	1	0	3	2	0	3	4	0	0	0	5	0	6	0	0	4	0	-	-	-	<b>&lt;</b>
<b>&gt;</b>		6	3	3	0	0	0	0	0	0	1	1	0	1	1	0	0	0	0	0	1	0	2	1	0	0	0	2	0	0	0	0	0	0	-	-	-	<b>&gt;</b>
<b>&lt; OK</b>		4.13	4.61	4.94	4.07	4.78	4.98	4.17	4.48	5.00	3.66	3.75	0	3.45	3.49	0	3.45	3.32	0	3.79	3.80	0	3.62	3.62	0	0	0	3.44	0	4.09	0	0	3.78	0	-	-	-	<b>&lt; OK</b>
<b>&gt; OK</b>		5.07	5.30	5.75	4.81	5.12	5.57	5.29	5.16	5.49	4.67	4.91	0	4.66	4.94	0	4.69	5.00	0	4.56	4.57	0	4.47	4.60	0	0	0	4.79	0	4.78	0	0	4.36	0	-	-	-	<b>&gt; OK</b>

N = number of analyses performed  
Min = lowest reported result

Max = highest reported result  
Med = median value

m = mean value  
s = standard deviation

F+ = false positive  
F- = false negative

< = low outlier  
> = high outlier

< OK = lowest accepted value  
> OK = highest accepted value


	The results are not evaluated
	Outlier, false positive or false negative
	Results "larger than" are not evaluated







Lab nr.	Provnr.	Aerobic microorganisms 30 °C			Aerobic microorganisms 20 °C			Contaminating microorganisms			Enterobacteriaceae			Coliform bacteria 30 °C			Coliform bacteria 37 °C			Thermotolerant coliform bacteria			Escherichia coli			Presumptive Bacillus cereus			Coagulase-positive Staphylococci			Enterococci			Gram-neg bacteria in dairy prod.			Lab nr.
		A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C				
8657	1 3 2	-0.738	-0.562	-0.389																																8657		
8734	3 2 1	-0.738	0.799	-0.088				1.280	0.834																											8734		
8742	2 1 3	-1.239	-0.402	0.063																																	8742	
8756	3 1 2	-0.095	-0.642	-0.916	0.651	-0.106	-1.026																													8756		
8766	3 1 2	1.264	2.080	1.418																																8766		
8891	2 1 3	0.263	-0.482	-0.088				-0.164	-1.265	1.463																											8891	
8909	2 1 3	0.406	0.079	-0.766																																	8909	
9003	1 2 3	-0.524	-0.402	-0.991																																	9003	
9007	3 2 1	-4.000	-4.000	-4.000																																	9007	
9025	2 1 3	-0.166	0.079	-0.841																																	9025	
9034	2 1 3	0.907	0.079	-1.217	0.961	-0.360	-0.593																														9034	
9078	1 2 3	3.767	2.321	0.213																																	9078	
9086	2 3 1	0.113	-4.000	0.552																																	9086	
9217	3 1 2	-1.954	-0.642	-0.615																																	9217	
9246	2 1 3				0.499	-1.784	-1.961																														9246	
9269	3 1 2	0.549	-0.722	-0.163																																	9269	
9429	3 2 1																																				9429	
9436	1 3 2	-0.667	1.120	1.117																																	9436	
9453	1 3 2	-0.238	-0.642	-0.314				-1.192	-0.605	-0.144																											9453	
9512	1 2 3	-0.381	-0.802	-0.766																																	9512	
9559	3 2 1	-1.024	-0.642	-0.464	-1.700	2.314	-0.680	0.432	-0.982	0.554																											9559	
9662	3 2 1	-0.023	-0.562	-0.916	0.589	-0.106	-1.373																														9662	
9747	3 1 2	0.835	-0.722	0.289																																	9747	
9890	1 3 2	-0.238	-0.162	-0.314	-0.772	-1.252	0.100																														9890	
9903	2 1 3				0.466	-0.742	-0.333																														9903	
9950	1 2 3	-0.881	-1.683	-1.142				-0.927	-1.124	-1.542																												9950

 The results are not evaluated



## **Internal and external control for microbiological analyses of food and drinking water**

All analytical activities require work of a high standard that is accurately documented. For this purpose, most laboratories carry out some form of internal quality assurance, but their analytical work also has to be evaluated by an independent party. Such external quality control of laboratory competence is commonly required by accreditation bodies and can be done by taking part in proficiency testing (PT).

In a proficiency test, identical test material is analysed by a number of laboratories using their routine methods. The organiser evaluates the results and compiles them in a report.

### **The Swedish Food Agency's PT program offers**

- External and independent evaluation of laboratories analytical competence.
- Improved knowledge of analytical methods with respect to various types of organisms.
- Expert support.
- Tool for inspections regarding accreditation.
- Free extra material for follow-up analyses.

For more information, visit our website: <https://www2.slv.se/absint>

### **The Swedish Food Agency's reference material**

As a complement to the proficiency testing, but without specific accreditation, the Swedish Food Agency also manufactures a number of reference materials (RM) for internal quality control of food and drinking water microbiological analyses, including pathogens.

For more information, visit our website: [www.livsmedelsverket.se/en/RM-micro](http://www.livsmedelsverket.se/en/RM-micro)