

Food Microbiology

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Proficiency Testing **Microbiology – Food**

October 2018



Accred. no. 1457
Proficiency testing
ISO/IEC 17043

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 dairy products

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
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
LTLSB	Lactose tryptone lauryl sulphate broth
MPCA	Milk plate count agar
MYP	Mannitol egg yolk polymyxin agar
MSA	Mannitol salt agar
PCA	Plate count agar
PEMBA	Polymyxin pyruvate egg yolk mannitol bromothymol blue agar
RPFA	Rabbit plasma fibrinogen agar
SFA	Sugar-free agar
TBX	Tryptone bile X-glucuronide agar
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/National Food Agency, Sweden

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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 samples 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 normally 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 measurement uncertainty 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 an evaluated parameter is the mean value of the participants' results.




Table and figure legends

Tables

N	number of laboratories that reported results for the analysis
n	number of laboratories with satisfactory result
m	mean value in \log_{10} cfu/ml (false results and outliers excluded)
s	standard deviation
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 results for each sample 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

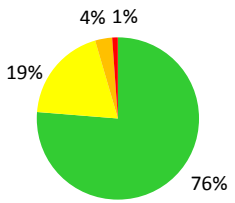
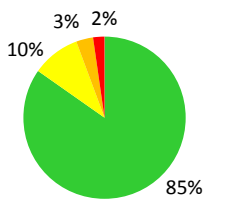
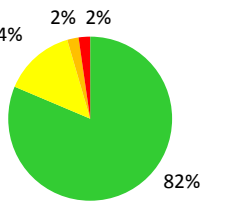
Results of the PT round October 2018

General outcome

Samples were sent to 183 laboratories, 47 in Sweden, 115 in other European countries, and 21 outside Europe. Of the 177 laboratories that reported results, 71 (40 %) provided at least one result that received an annotation. In the previous round with similar analyses (October 2017), the proportion was 39 %.

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 percentages of deviating results (N: number of reported results, F%: false positive or false negative, X%: outlier).

		Sample A				Sample B				Sample C			
% participants with													
Microorganisms		<i>Bacillus cereus</i> <i>Pediococcus acidilactici</i> <i>Staphylococcus xylosum</i>				<i>Enterococcus hirae</i> <i>Kocuria rhizophila</i> <i>Klebsiella pneumoniae</i>				<i>Enterococcus durans</i> <i>Escherichia coli</i> <i>Serratia marcescens</i> <i>Staphylococcus aureus</i>			
Analysis		Target organism	N	F%	X%	Target organism	N	F%	X%	Target organism	N	F%	X%
Aerobic micro-organisms	30°C	All	166	0	5	All	166	0	8	All	165	0	4
	20°C	All	29	0	0	All	29	0	0	All	29	0	0
Contaminating microorganisms		<i>S. xylosum</i> <i>B. cereus</i>	18	0	6	All	18	0	6	All	18	0	11
Enterobacteriaceae		-	137	0	0	<i>K. pneumoniae</i>	140	1	4	<i>E. coli</i> <i>S. marcescens</i>	140	0	5
Coliform bacteria	30°C	-	57	2	0	<i>K. pneumoniae</i>	57	0	7	<i>E. coli</i>	57	0	4
	37°C	-	94	1	0	<i>K. pneumoniae</i>	93	3	2	<i>E. coli</i>	92	2	1
Thermotolerant coliform bacteria		-	48	0	0	<i>K. pneumoniae</i>	46	0	2	<i>E. coli</i>	48	2	2
<i>E. coli</i>		-	119	0	0	-	119	3	0	<i>E. coli</i>	115	2	3
Presumptive <i>B. cereus</i>		<i>B. cereus</i>	109	1	5	-	109	1	0	(<i>S. aureus</i>) (<i>S. marcescens</i>)	109	2	0
Coagulase-positive staphylococci		(<i>S. xylosum</i>)	105	12	0	-	105	3	0	<i>S. aureus</i>	105	4	5
Enterococci		(<i>P. acidilactici</i>)*	66	32	0	<i>E. hirae</i>	66	0	5	<i>E. durans</i>	67	1	7
Gram-negative bacteria in dairy prod.		-	11	0	0	<i>K. pneumoniae</i>	11	0	0	<i>E. coli</i> <i>S. marcescens</i>	11	0	0

- no target organism or no value; (*microorganism*) 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. The strain of *S. xylosum* was present in a somewhat higher concentration than *B. cereus* and *P. acidilactici*. Five low and three high outliers were reported for the analysis at 30 °C. No outliers or false negative results were reported for the analysis at 20 °C.

Sample B

All strains in the sample were target organisms. *K. rhizophila*, *K. pneumoniae* and *E. hirae* were present in similar concentrations. Five low and nine high outliers were reported for the analysis at 30 °C. No outliers or false negative results were reported for the analysis at 20 °C.

Sample C

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*. Five low and two high outliers were reported for the analysis at 30 °C. No outliers or false negative results were reported for the analysis at 20 °C.

General remarks

As a whole, the analyses were without problem for the laboratories. No differences in the results attributed to the use of a specific method or medium could be identified, neither at 30 °C nor at 20 °C.

A relatively high number of outliers were reported at 30 °C, but no outliers were reported at 20 °C. In total, 17 laboratories reported at least one outlier at 30 °C. Of these, eight reported at least two outliers at 30 °C, and six of these further reported false results or outliers for other analyses in this proficiency testing round.

Similar methods and media were used at both temperatures. At 30 °C the most common methods were NMKL 86:2013 (25 %), 3M Petrifilm (22 %) and ISO 4833-1:2013 (20 %). The older methods NMKL 86:2006 and ISO 4833:2003 were still used by 9 % and 5 % of the laboratories respectively. The different methods are however very similar. All are also based on incubation on plate count agar (PCA) or milk plate count agar (MPCA). Laboratories that use Petrifilm AC may however use different times/temperatures, depending on which method 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.

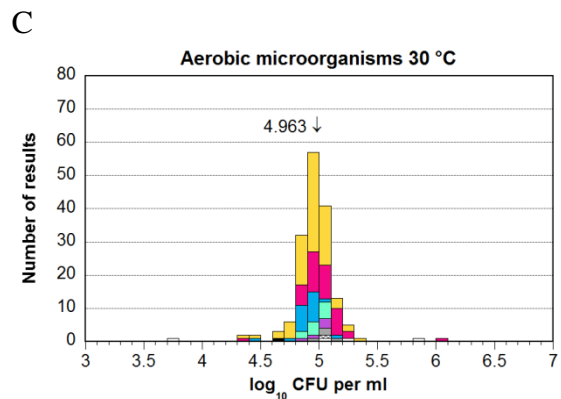
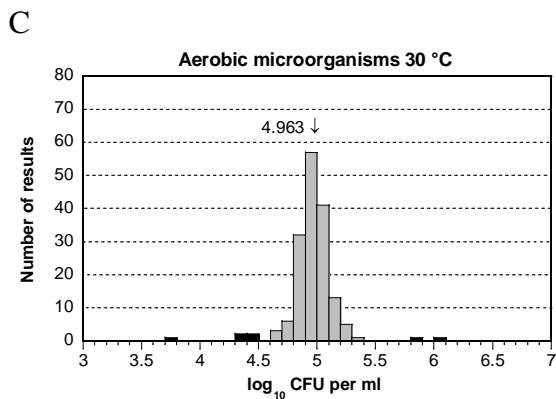
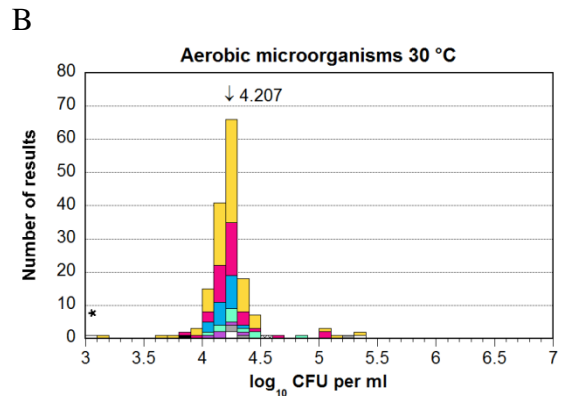
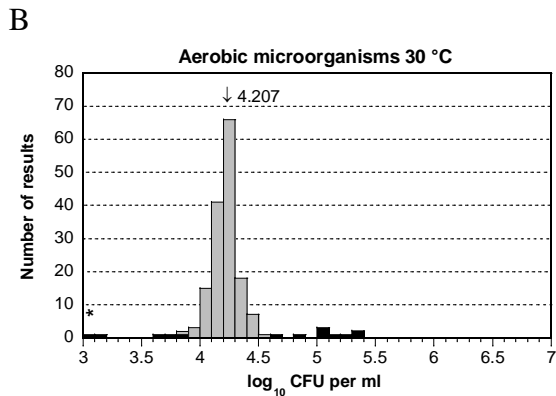
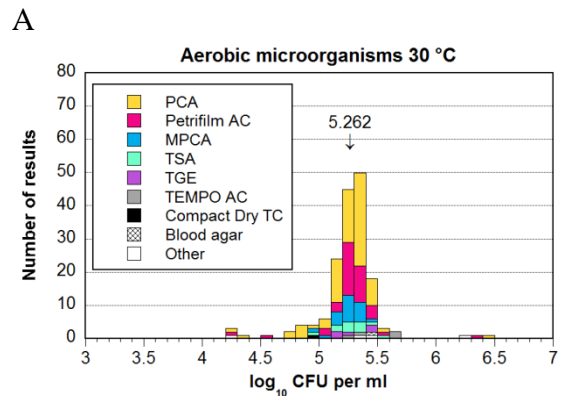
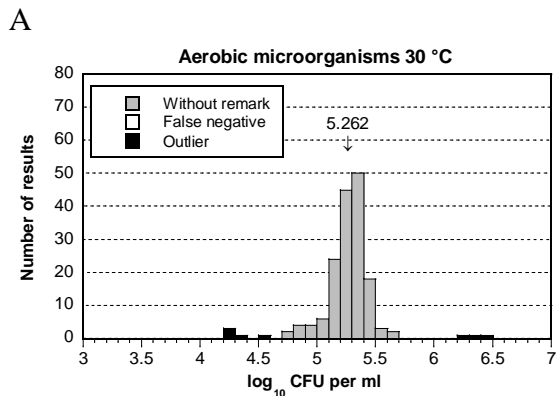
As in previous proficiency testing rounds, MPCA was mainly used by laboratories in the dairy industry. Incubation on tryptone soya agar (TSA) was mainly attributed to the use of a company-specific method. The results for both media were similar to PCA and Petrifilm AC.

At 30 °C, 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 wells with different volumes. A substrate in the wells emits fluorescence when hydrolysed by the microorganisms. The concentration is determined by the number and size of the fluorescent wells.

At 20 °C, three laboratories followed NMKL 184, which is a method for enumeration of aerobic microorganisms and specific spoilage organisms in fish and fish products. With this method, incubation is done on iron agar (IA).

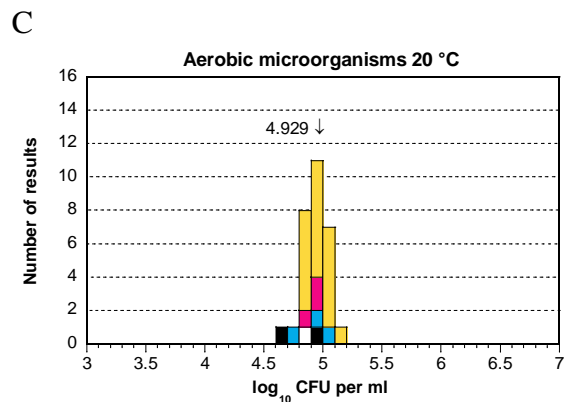
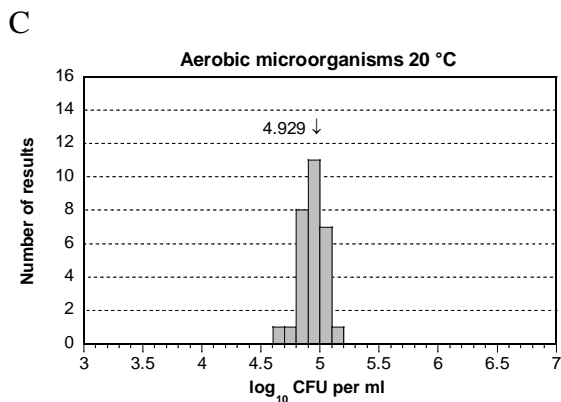
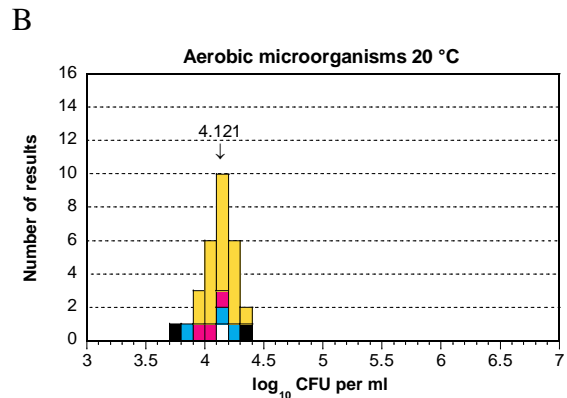
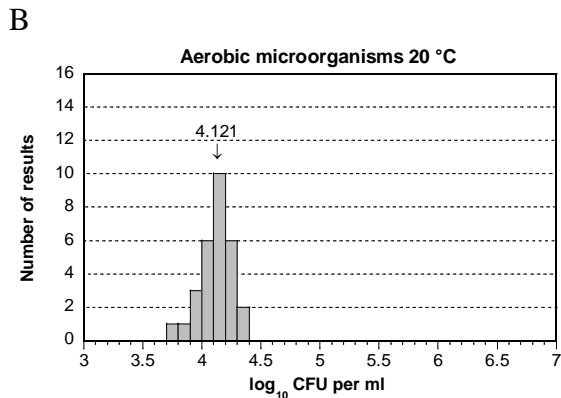
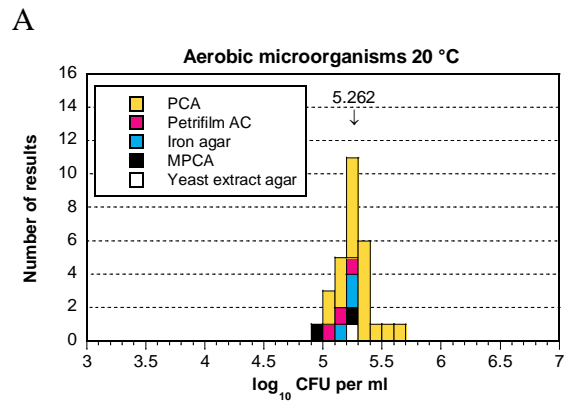
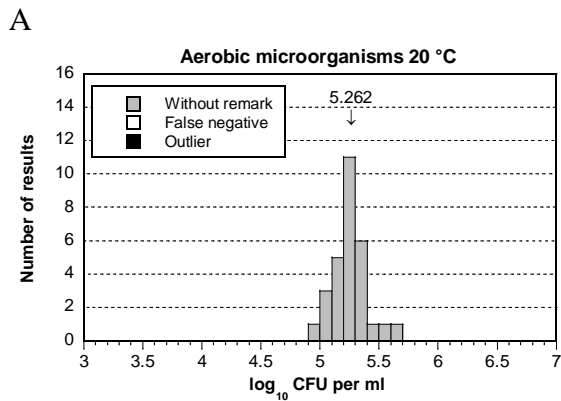
Results from analysis of aerobic microorganisms, 30 °C

Medium	N	Sample A					Sample B					Sample C				
		n	m	s	F	< >	n	m	s	F	< >	n	m	s	F	< >
All results	166	158	5.262	0.156	0	5 3	152	4.207	0.107	0	5 9	158	4.963	0.116	0	5 2
PCA	79	76	5.243	0.173	0	2 1	73	4.207	0.103	0	3 3	76	4.943	0.121	0	2 0
Petrifilm AC	40	37	5.277	0.108	0	2 1	36	4.200	0.101	0	1 3	38	5.011	0.107	0	1 1
MPCA	21	21	5.245	0.113	0	0 0	21	4.190	0.077	0	0 0	20	4.916	0.073	0	1 0
TSA	11	11	5.279	0.157	0	0 0	10	4.257	0.116	0	0 1	11	4.966	0.085	0	0 0
TGE	5	5	5.274	0.155	0	0 0	5	4.192	0.107	0	0 0	5	4.970	0.104	0	0 0
TEMPO AC	4	4	-	-	0	0 0	3	-	-	0	0 1	4	-	-	0	0 0
Compact Dry TC	1	1	-	-	0	0 0	1	-	-	0	0 0	1	-	-	0	0 0
Blood agar	1	1	-	-	0	0 0	1	-	-	0	0 0	1	-	-	0	0 0
Other	4	2	-	-	0	1 1	2	-	-	0	1 1	2	-	-	0	1 1



Results from analysis of aerobic microorganisms, 20 °C

Medium	N	Sample A					Sample B					Sample C				
		n	m	s	F	< >	n	m	s	F	< >	n	m	s	F	< >
All results	29	29	5.262	0.140	0	0 0 0	29	4.121	0.127	0	0 0 0	29	4.929	0.105	0	0 0 0
PCA	20	20	5.289	0.147	0	0 0 0	20	4.141	0.100	0	0 0 0	20	4.951	0.097	0	0 0 0
Petrifilm AC	3	3	-	-	0	0 0 0	3	-	-	0	0 0 0	3	-	-	0	0 0 0
IA	3	3	-	-	0	0 0 0	3	-	-	0	0 0 0	3	-	-	0	0 0 0
MPCA	2	2	-	-	0	0 0 0	2	-	-	0	0 0 0	2	-	-	0	0 0 0
Yeast extract agar	1	1	-	-	0	0 0 0	1	-	-	0	0 0 0	1	-	-	0	0 0 0



Contaminating microorganisms in dairy products

Sample A

All microorganisms in the sample can form colonies on sugar-free agar (SFA). The strain of *S. xylosus* was however present in a higher concentration than *B. cereus* and *P. acidilactici*. The strain of *P. acidilactici* has in previous proficiency testing rounds (October 2013) formed very small (pin-point) colonies on SFA. According to ISO 13559:2002 / IDF 153:2002, such colonies shall be excluded in the enumeration of colonies. One of the results deviated clearly from the median, and was considered as a low outlier.

Sample B

All microorganisms in the sample can form colonies on SFA. *K. rhizophila*, *K. pneumoniae* and *E. hirae* were also present in similar concentrations. *E. hirae* is catalase negative, and may therefore have been excluded if a confirmation test was performed. Despite this, no low results were reported. One of the results was however clearly higher than the median, and was considered as a high outlier.

Sample C

All microorganisms in the sample can form colonies on SFA. The strain of *E. durans* is catalase negative and formed small white colonies at the National Food Agency. It may therefore have been excluded during confirmation. At the same time, *E. durans* was present in a low concentration in the sample, and exclusion of this strain should only have had a marginal effect on the result. The two results that were clearly lower than the median were therefore considered as low outliers.

General remarks

Only 18 laboratories performed the analysis, and the results were therefore difficult to evaluate statistically. Deciding which results that are outliers has therefore been done by a manual inspection. This included consideration of the species and concentrations present in the sample (Table 3), the median of the laboratories' results, as well as the distribution of results that is normally seen in this analysis.

Ten of the 18 laboratories (56 %) followed ISO 13559:2002 / IDF 153:2002. One laboratory stated the older IDF 153:1999. The remaining laboratories followed either internal methods, or did not specify further what method they used. All laboratories incubated on SFA.

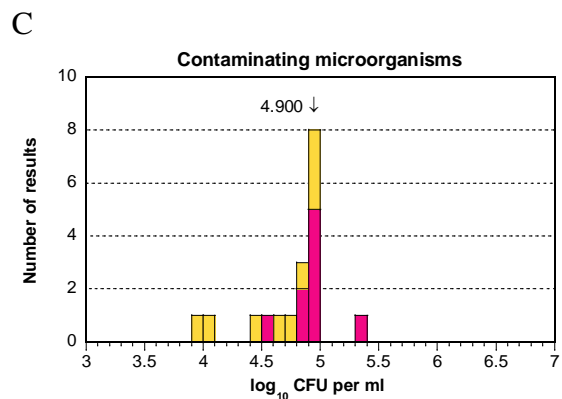
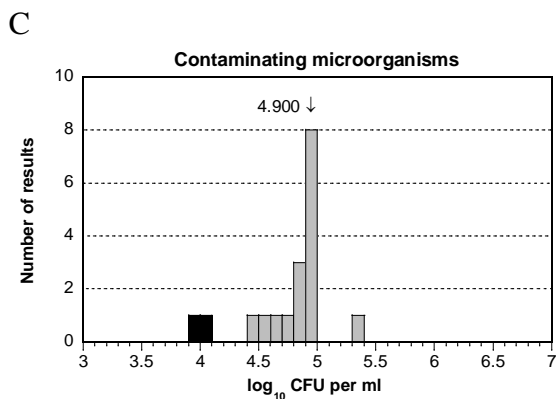
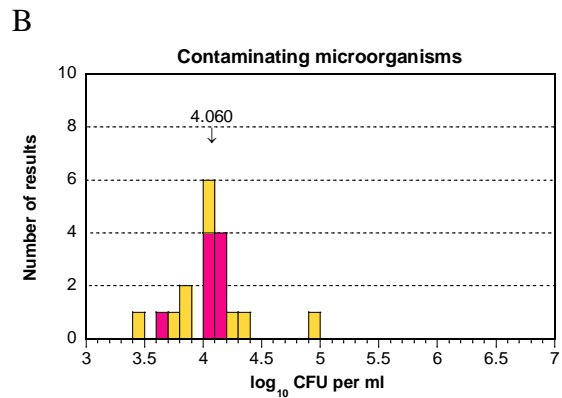
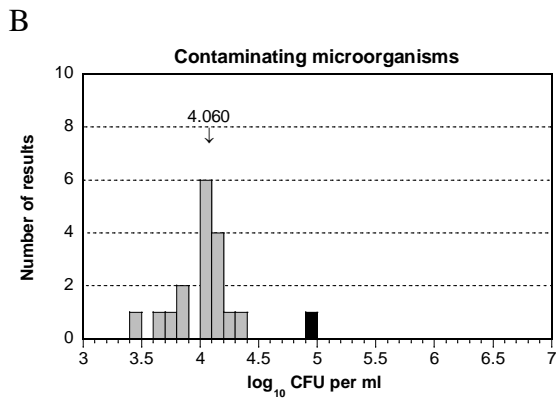
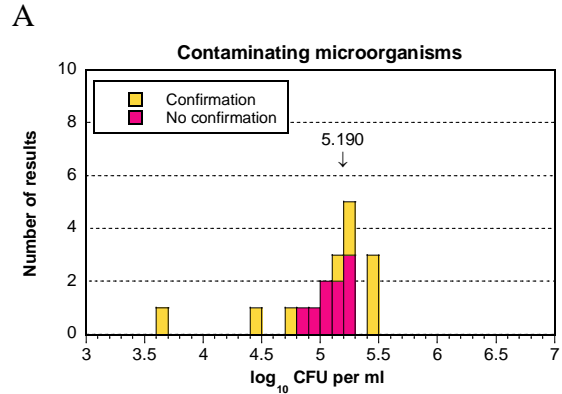
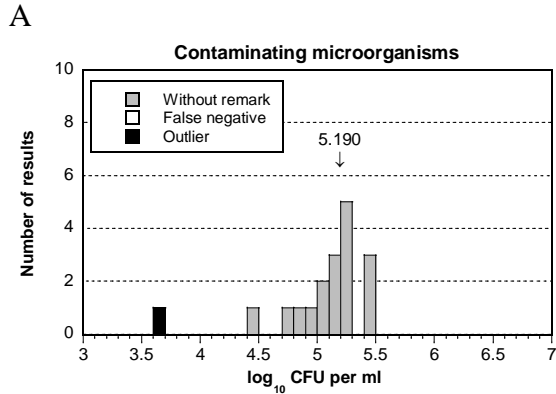
The goal of the analysis is to identify potential contaminating microorganisms in dairy products. According to ISO 13559:2002 / IDF 153:2002, lactic acid bacteria are in this sense not classified as contaminating microorganisms. Lactic acid bacteria are catalase negative, and several laboratories therefore perform a catalase test as confirmation. Such a test is however not included in ISO 13559:2002 / IDF 153:2002, which only specifies the enumeration of colonies that are "characteristic contaminating microorganisms". In total, nine of the laboratories (50 %) reported performing a confirmation test, in most cases a catalase test. No obvious difference could be seen between results from laboratories that performed a confirmation and those that did not.

Results from analysis of contaminating microorganisms

Method	N	Sample A					Sample B					Sample C							
		n	Med*	s	F	<	>	n	Med*	s	F	<	>	n	Med*	s	F	<	>
All results	18	17	5.190	0.261	0	1	0	17	4.060	0.223	0	0	1	16	4.900	0.206	0	2	0
Confirmation**	9	8	5.245	0.367	0	1	0	8	3.965	0.279	0	0	1	7	4.880	0.179	0	2	0
No confirmation	9	9	5.190	0.134	0	0	0	9	4.090	0.162	0	0	0	9	4.910	0.217	0	0	0

* Med: median.

** "Confirmation" includes three laboratories for which the method of confirmation is not clearly specified.



Enterobacteriaceae

Sample A

No target organism was present in the sample. No false positive results were reported.

Sample B

The strain of *K. pneumoniae* was target organism. Four low and two high outliers were reported, as well as one false negative result.

Sample C

The strains of *E. coli* and *S. marcescens* were target organisms. At the National Food Agency, two types of colonies were observed on violet red bile glucose agar (VRBG). Both were red, and surrounded by typical precipitation zones. They were also oxidase negative upon confirmation. The results were distributed around a distinct peak, but with a tail of low values. Six low and one high outlier were reported.

General remarks

Similar to previous proficiency testing rounds, most laboratories followed NMKL 144:2005 (48 %), or used Petrifilm EB (22 %). The different ISO methods (various editions) were in total used by 20 % of the laboratories. Comparable numbers of laboratories used the new ISO 21528-2:2017 and the older ISO 21528-2:2004 (7 % and 8 % respectively). The new ISO 21528-1:2017 was however only used by two laboratories (1 %). ISO 21528-2:2017 is based on colony-count, whereas ISO 21528-1:2017 is based on MPN (Most Probable Number). The MPN method is recommended when the expected concentration of Enterobacteriaceae is lower than 100 cfu g⁻¹. The mean values for the different ISO methods were however very similar, for all three samples.

As in the analysis of aerobic microorganisms, a smaller number of laboratories used a method based on detection of fluorescence (TEMPO[®] Enterobacteriaceae). With this method, there was a tendency towards higher results, mainly in sample C, but also in sample B. No outliers were however reported by users of this method. Such somewhat higher results – but within the acceptance limits – have been seen in several previous proficiency testing rounds. The number of users of this method is however small, which makes it difficult to evaluate this observation further.

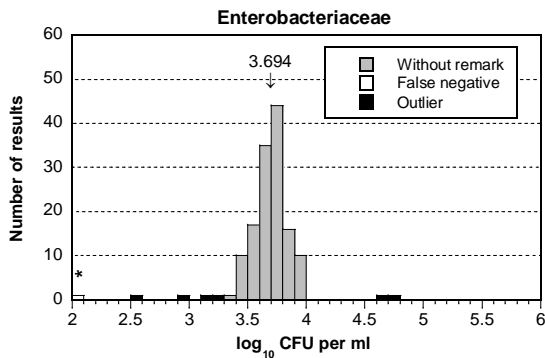
Enterobacteriaceae are Gram-negative and oxidase negative bacteria, that ferment glucose with the production of acid by-products. With both NMKL 144 and ISO 21528-2, they are enumerated on VRBG. On this medium, Enterobacteriaceae form pink/red colonies, with or without a bile precipitation zone. The appearance is similar on Petrifilm EB, which also includes a colour indicator for acid by-products and a plastic film for detection of gas production.

According to NMKL 144:2005, presumptive colonies on VRBG shall be confirmed with an oxidase test. With ISO 21528 2:2017, confirmation is done with both an oxidase test and a test for glucose fermentation. Oxidase-negative bacteria that also ferment glucose in glucose oxidation/fermentation (OF) medium are considered as Enterobacteriaceae. In total, 66 % of the laboratories reported performing some kind of confirmation test. The most common was an oxidase test, but the use of a glucose fermentation test was also fairly frequent. Other methods used for confirmation included API 20 E and Maldi-Tof. Performing or not performing a confirmation test does not appear to have had an effect on the overall performance of the laboratories.

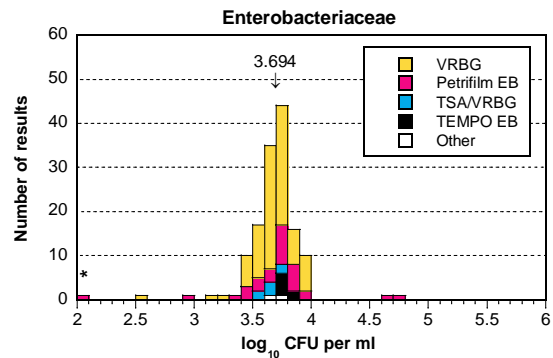
Results from analysis of Enterobacteriaceae

Medium	N	Sample A					Sample B					Sample C							
		n	m	s	F	<	>	n	m	s	F	<	>	n	m	s	F	<	>
All results	137	137	-	-	0	-	-	133	3.694	0.132	1	4	2	133	4.541	0.172	0	6	1
VRBG	91	91	-	-	0	-	-	90	3.692	0.129	0	3	0	89	4.561	0.154	0	4	0
Petrifilm EB	30	30	-	-	0	-	-	27	3.690	0.168	1	1	2	28	4.485	0.188	0	2	1
TSA/VRBG	7	7	-	-	0	-	-	7	3.657	0.076	0	0	0	7	4.426	0.256	0	0	0
TEMPO EB	7	7	-	-	0	-	-	7	3.757	0.063	0	0	0	7	4.680	0.075	0	0	0
Other	2	2	-	-	0	-	-	2	-	-	0	0	0	2	-	-	0	0	0

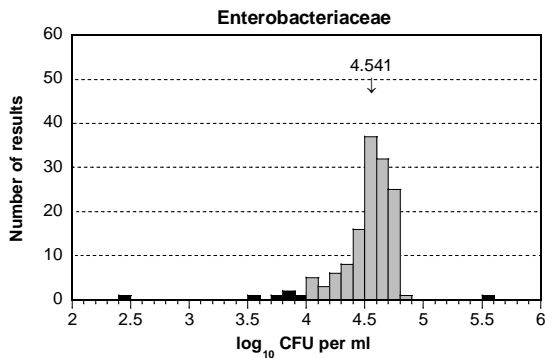
B



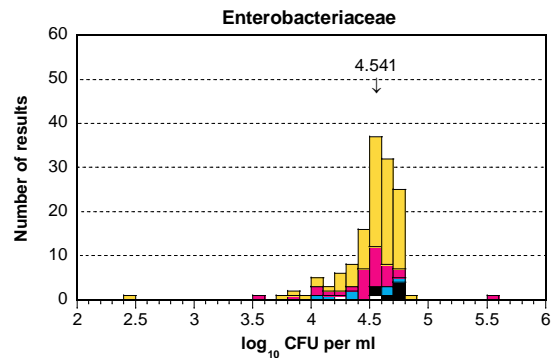
B



C



C



Coliform bacteria, 30 °C and 37 °C

Sample A

No target organism was present in the sample. One false positive result was reported at both 30 °C and at 37 °C.

Sample B

The strain of *K. pneumoniae* was target organism. It forms typical red colonies on violet red bile agar (VRB) and produces gas in brilliant green lactose bile broth (BGLB). At the National Food Agency, the production of gas was observed to be lesser at 30 °C than at 37 °C.

At 30 °C, two low and two high outliers were reported. At 37 °C, one low and one high outlier were reported, as well as three false negative results.

Sample C

The strain of *E. coli* was target organism. At the National Food Agency, two types of colonies were observed on VRB. Large red colonies with a precipitation zone, and a smaller number of colonies with a less prominent zone. Both colony types were oxidase negative, but only the larger colonies produced gas in BGLB. In addition to *E. coli*, the sample contained *S. aureus*, *E. durans* and *S. marcescens*. *S. aureus* and *E. durans* are Gram-positive and should normally not form colonies on VRB, as they are inhibited by the presence of bile salts and crystal violet in VRB. The smaller colonies can therefore be assumed to be *S. marcescens*, which is a weak fermenter of lactose and thus capable of forming small colonies on VRB.

The results at both 30 °C and 37 °C were distributed with two overlapping peaks, one at approximately \log_{10} 4.0 and one at \log_{10} 4.5 cfu ml⁻¹. The two peaks could not be separated statistically, but the low and high peaks correspond very well to the concentrations of *E. coli* and *E. coli* + *S. marcescens* in the sample respectively.

It is difficult to attribute the low and high results to the use of a specific method or medium, partly since several methods/media were used by a smaller number of laboratories. Performing a confirmation test was also reported in by comparable numbers of laboratories in both peaks. Possibly, users of Petrifilm appear to have reported relatively more results in the lower peak compared to users of other media. Users of TSA/VRB also appear to have somewhat more results reported in the higher peak. Different results can also have been obtained due to variations in the definition of coliform bacteria by the different methods.

At 30 °C, one low and one high outlier were reported. At 37 °C, one high outlier was reported, as well as two false negative results.

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. They are normally surrounded by a red/pink zone of precipitation, which is formed due to the precipitation of bile salts when the pH decreases.

The most commonly used methods at both temperatures 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 in BGLB, whereas ISO 4832:2006 states that 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 tryptone soya agar (TSA). Such a pre-incubation could also contribute to higher results. Petrifilm CC and Petrifilm EC/CC are also based on VRB, and have a plastic film that facilitates detection of gas production.

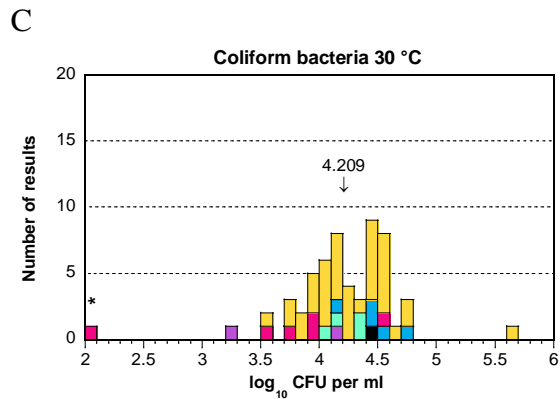
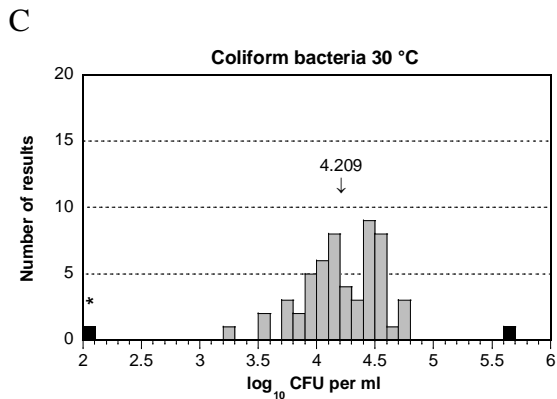
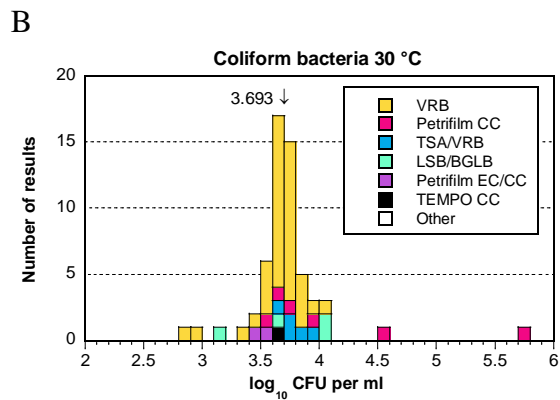
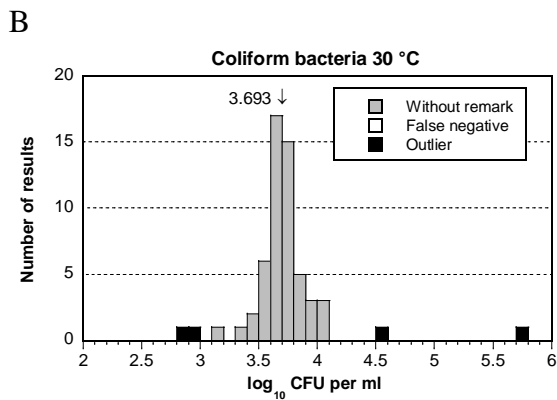
Lauryl sulphate broth (LSB) in combination with BGLB was used by laboratories that followed ISO 4831 and NMKL 96 (various editions). ISO 4831:2006 is based on MPN (Most Probable Number) and is adapted for use when the expected concentration of coliform bacteria is lower than or equal to 100 cfu g⁻¹. 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⁻¹. In some previous proficiency testing rounds, users of these methods have had problems with correctly determining higher concentrations – such as those in samples B and C. However in the current proficiency testing round, all results were without remark.

For the analysis at 37 °C, four laboratories used RAPID'E. coli 2 agar, which is a chromogenic medium that detects β-glucuronidase and β-galactosidase activity. On this medium, coliform bacteria (Gal+/Gluc-) form blue/green colonies, while *E. coli* (Gal+/Gluc+) form pink/purple colonies.

Results from analysis of coliform bacteria, 30 °C

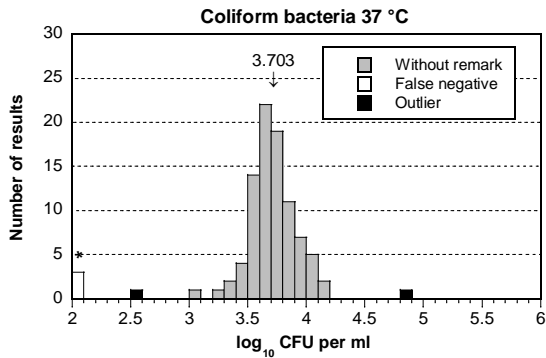
Medium	N	Sample A					Sample B					Sample C							
		n	m	s	F	<	>	n	m	s	F	<	>	n	m	s	F	<	>
All results	57	56	-	-	1	-	-	53	3.693	0.161	0	2	2	55	4.209	0.331	0	1	1
VRB	39	38	-	-	1	-	-	37	3.687	0.122	0	2	0	38	4.228	0.303	0	0	1
Petrifilm CC	6	6	-	-	0	-	-	4	3.713	0.189	0	0	2	5	3.968	0.390	0	1	0
TSA/VRB	5	5	-	-	0	-	-	5	3.788	0.095	0	0	0	5	4.440	0.220	0	0	0
LSB/BGLB	4	4	-	-	0	-	-	4	-	-	0	0	0	4	-	-	0	0	0
Petrifilm EC/CC	2	2	-	-	0	-	-	2	-	-	0	0	0	2	-	-	0	0	0
TEMPO CC	1	1	-	-	0	-	-	1	-	-	0	0	0	1	-	-	0	0	0



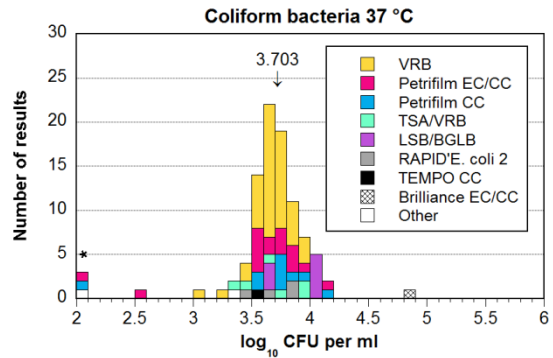
Results from analysis of coliform bacteria, 37 °C

Medium	N	Sample A						Sample B						Sample C					
		n	m	s	F	<	>	n	m	s	F	<	>	n	m	s	F	<	>
All results	94	93	-	-	1	-	-	88	3.703	0.191	3	1	1	89	4.232	0.305	2	0	1
VRB	44	44	-	-	0	-	-	44	3.663	0.164	0	0	0	43	4.269	0.315	0	0	0
Petrifilm EC/CC	17	16	-	-	1	-	-	15	3.710	0.187	1	1	0	16	4.115	0.208	1	0	0
Petrifilm CC	10	10	-	-	0	-	-	9	3.768	0.175	1	0	0	9	4.012	0.373	0	0	1
TSA/VRB	6	6	-	-	0	-	-	6	3.707	0.241	0	0	0	6	4.347	0.229	0	0	0
LSB/BGLB	9	9	-	-	0	-	-	8	3.894	0.202	0	0	0	8	4.238	0.286	0	0	0
Rapid'E.coli2	4	4	-	-	0	-	-	4	-	-	0	0	0	3	-	-	1	0	0
TEMPO CC	1	1	-	-	0	-	-	1	-	-	0	0	0	1	-	-	0	0	0
Brilliance EC/CC	1	1	-	-	0	-	-	0	-	-	0	0	1	1	-	-	0	0	0
Other	2	2	-	-	0	-	-	1	-	-	1	0	0	2	-	-	0	0	0

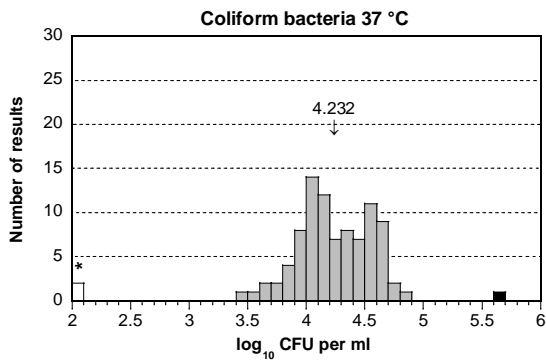
B



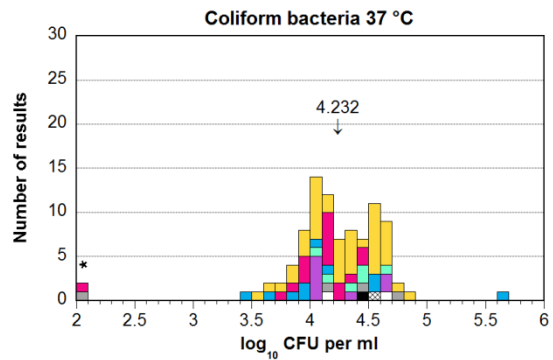
B



C



C



Thermotolerant coliform bacteria and *Escherichia coli*

Sample A

No target organism was present in the sample. No false results were reported, neither for thermotolerant coliform bacteria nor for *E. coli*.

Sample B

The strain of *K. pneumoniae* was target organism for the analysis of thermotolerant coliform bacteria, but not for *E. coli*. The strain produces gas but not indole in lactose tryptone lauryl sulphate broth (LTLSB).

One low outlier was reported for thermotolerant coliform bacteria. Four false positive results were reported for *E. coli*.

Sample C

The strain of *E. coli* was target organism for both analyses. At the National Food Agency, it produced both gas and indole in LTLSB. It was also positive for β -glucuronidase.

One low outlier was reported for thermotolerant coliform bacteria, as well as one false negative result. Three low and one high outlier were reported for *E. coli*, as well as two false negative results.

General remarks

As a whole, the analyses were without problem for the laboratories. No obvious differences in the results from the methods and media that were used could be identified. However similar to previous proficiency testing rounds (October 2016, 2017), the group “Other” was fairly large for the analysis of *E. coli*. This was mainly due to unclear/ambiguous method information from some laboratories.

NMKL 125:2005 was the most commonly used method for the analysis of thermotolerant coliform bacteria. 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 *E. coli* broth (EC) or in LTLSB. 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 LTLSB or in tryptone broth are considered 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 β -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 β -glucuronidase activity. The method uses tryptone bile X-glucuronide agar (TBX), on which *E. coli* form typical blue colonies after 18-24 h at 44 °C. No further confirmation of β -glucuronidase positive colonies is required according to ISO 16649-2:2001.

Confirmation of some kind was performed by 81 % of the laboratories in the analysis of thermotolerant coliform bacteria and by 63 % in the analysis of *E. coli*. Confirmation of *E. coli* was less often reported by laboratories that used Petrifilm or that followed

ISO 16649-2:2001, which is reasonable since confirmation is not required by those methods. No obvious difference in the results could however be seen between laboratories that performed a confirmation and those that did not. It should here be mentioned that NMKL 125 is currently being revised, and the new version will likely be more similar to ISO 16649-2. Among other things, changing the confirmation by replacing the use of Kovac's reagent is considered.

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. A few laboratories also used methods based on the detection of fluorescence (TEMPO[®] *E. coli*).

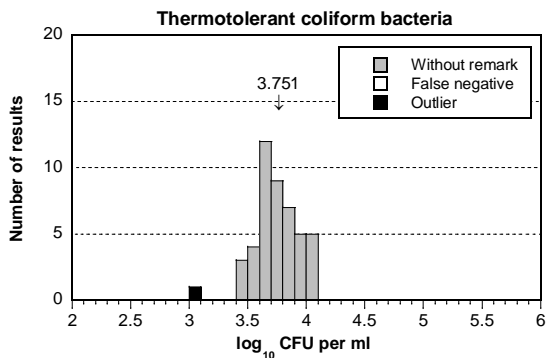
As in previous proficiency testing rounds the results for *E. coli* were somewhat lower for TBX, and higher for TSA/VRB, compared to other media. A possible explanation could be if a pre-incubation has been performed or not. For samples that are suspected to contain stressed microorganisms, ISO 16649-2:2001 prescribes a pre-incubation at 37 °C for 4 h, prior to the final incubation at 44 °C. In comparison, with NMKL 125:2005 a similar pre-incubation is routinely carried out (1-2 h on TSA at 20-25 °C). As in previous proficiency testing rounds, the differences were however small.

Results from analysis of thermotolerant coliform bacteria

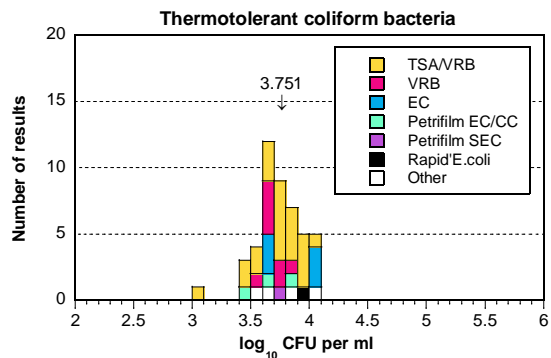
Medium	N	Sample A						Sample B						Sample C					
		n	m	s	F	<	>	n	m	s	F	<	>	n	m	s	F	<	>
All results	48	48	-	-	0	-	-	45	3.751	0.163	0	1	0	46	4.168	0.187	1	1	0
TSA/VRB	24	24	-	-	0	-	-	22	3.754	0.159	0	1	0	22	4.168	0.116	1	1	0
VRB	8	8	-	-	0	-	-	8	3.682	0.089	0	0	0	8	4.135	0.165	0	0	0
EC*	6	6	-	-	0	-	-	6	3.845	0.214	0	0	0	6	4.303	0.306	0	0	0
Petrifilm EC/CC	4	4	-	-	0	-	-	3	-	-	0	0	0	4	-	-	0	0	0
Petrifilm SEC	1	1	-	-	0	-	-	1	-	-	0	0	0	1	-	-	0	0	0
Rapid'E.coli	1	1	-	-	0	-	-	1	-	-	0	0	0	1	-	-	0	0	0
Other	4	4	-	-	0	-	-	4	-	-	0	0	0	4	-	-	0	0	0

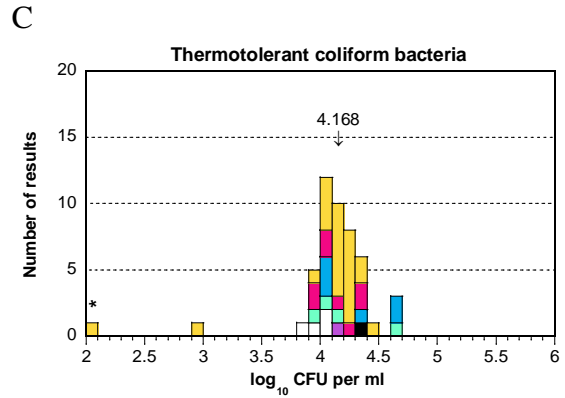
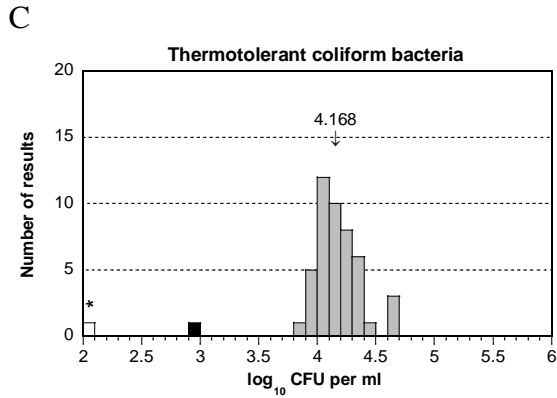
* *E. coli* broth (EC) was mostly reported by laboratories that used MPN-based methods, where EC is normally used after a first enrichment in LSB.

B



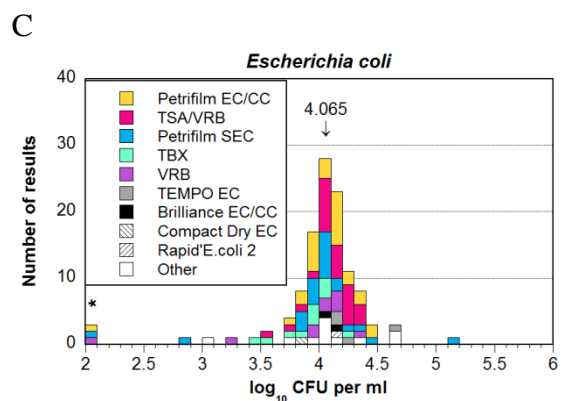
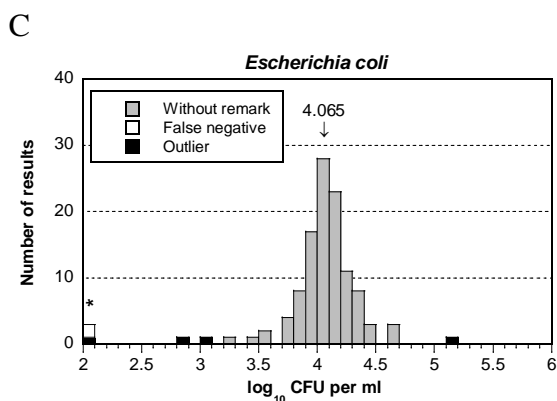
B





Results from analysis of *Escherichia coli*

Medium	N	Sample A						Sample B						Sample C					
		n	m	s	F	<	>	n	m	s	F	<	>	n	m	s	F	<	>
All results	119	119	-	-	0	-	-	115	-	-	4	-	-	109	4.065	0.221	2	3	1
Petrifilm EC/CC	27	27	-	-	0	-	-	26	-	-	1	-	-	26	4.082	0.190	1	0	0
TSA/VRB	26	26	-	-	0	-	-	26	-	-	0	-	-	26	4.095	0.176	0	0	0
Petrifilm SEC	22	22	-	-	0	-	-	21	-	-	1	-	-	19	4.036	0.157	1	1	1
TBX	12	12	-	-	0	-	-	12	-	-	0	-	-	11	3.879	0.227	0	0	0
VRB	10	10	-	-	0	-	-	9	-	-	1	-	-	9	4.001	0.300	0	1	0
TEMPO EC	4	4	-	-	0	-	-	4	-	-	0	-	-	4	-	-	0	0	0
Brilliance EC/CC	3	3	-	-	0	-	-	3	-	-	0	-	-	2	-	-	0	0	0
Compact Dry EC	1	1	-	-	0	-	-	1	-	-	0	-	-	1	-	-	0	0	0
Rapid'E.coli 2	1	1	-	-	0	-	-	1	-	-	0	-	-	1	-	-	0	0	0
Other	13	13	-	-	0	-	-	12	-	-	1	-	-	10	4.168	0.309	0	1	0



Presumptive *Bacillus cereus*

Sample A

The strain of *B. cereus* was target organism. In the homogeneity analysis at the National Food Agency, the strain formed typical grey colonies surrounded by a zone of haemolysis on blood agar (BA). On the same medium, two other types of colonies were observed. Both were atypical shiny colonies, without a zone of haemolysis. Further, only the colonies of *B. cereus* formed typical blue colonies with a zone of precipitation upon confirmation on *Bacillus cereus* selective agar (BcsA).

Three low and two high outliers were reported, as well as one false negative result.

Sample B

No target organism was present in the sample. One false positive result was reported.

Sample C

No target organism was present in the sample. Two false positive results were reported. Possibly, these may be due to detection of *S. marcescens* or *S. aureus*, which may sometimes grow on BcsA. At the National Food Agency, small atypical colonies were observed on BA. Upon confirmation, they formed atypical colonies without blue colour on BcsA.

General remarks

As in previous proficiency testing rounds most laboratories followed either NMKL 67:2010 (57 %) or ISO 7932:2004 (20 %). NMKL 67:2010 is based on 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 (a chromogenic medium). On BcsA presumptive *B. cereus* form bluish colonies that are surrounded by a 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 comparison, ISO 7932:2004 prescribes plating onto mannitol egg yolk polymyxin agar (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, Oxoid Brilliance™ *Bacillus cereus* agar (CBC) was used by a group of eight laboratories. CBC is a chromogenic medium, and cleavage of X-Gluc present in CBC by *B. cereus* β -glucuronidase results in white colonies with a blue/green centre. Two laboratories reported using BACARA®, which is another chromogenic medium used for detecting *B. cereus*. Another two laboratories used the fluorescence-based TEMPO® *Bacillus cereus* (TEMPO BC).

As in previous proficiency testing rounds the reporting of method data was in several cases unclear. For example, several laboratories reported that the same medium was used in both steps in the analysis. Other laboratories reported combinations of method and media that were incompatible. As a general rule, the tables and figures below are based on the methods/media stated by the laboratories, regardless if these are compatible or not. Laboratories that have only stated “chromogenic medium” are

included in the group “Other”. Despite these uncertainties the results and mean values for the different methods and media are very similar.

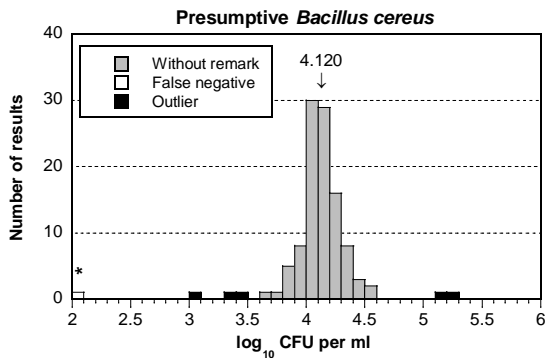
Confirmation was carried out by 59 % of the laboratories. No obvious difference in the results could be seen between laboratories that confirmed and those that did not.

Results from analysis of presumptive B. cereus

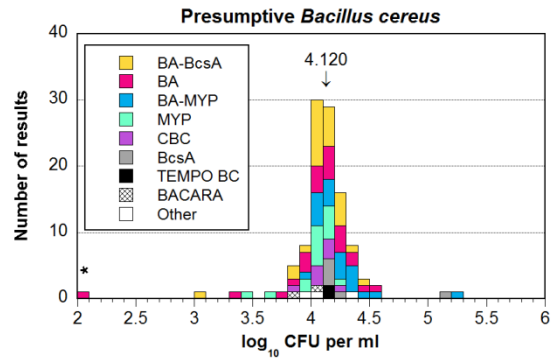
Medium	N	Sample A						Sample B						Sample C					
		n	m	s	F	<	>	n	m	s	F	<	>	n	m	s	F	<	>
All results	109	103	4.120	0.156	1	3	2	108	-	-	1	-	-	107	-	-	2	-	-
BA-BcsA*	27	26	4.122	0.139	0	1	0	27	-	-	0	-	-	27	-	-	0	-	-
BA	24	22	4.125	0.183	1	1	0	23	-	-	0	-	-	22	-	-	1	-	-
BA-MYP	21	20	4.190	0.169	0	0	1	19	-	-	1	-	-	19	-	-	1	-	-
MYP	16	15	4.047	0.145	0	1	0	16	-	-	0	-	-	16	-	-	0	-	-
CBC	8	8	4.069	0.111	0	0	0	8	-	-	0	-	-	8	-	-	0	-	-
BcsA	6	5	4.165	0.073	0	0	1	7	-	-	0	-	-	7	-	-	0	-	-
TEMPO BC	2	2	-	-	0	0	0	2	-	-	0	-	-	2	-	-	0	-	-
BACARA	2	2	-	-	0	0	0	2	-	-	0	-	-	2	-	-	0	-	-
Other	3	3	-	-	0	0	0	4	-	-	0	-	-	4	-	-	0	-	-

* Use of PEMBA has been interpreted as BcsA and is therefore included in this group.

A



A



Coagulase-positive staphylococci

Sample A

No target organism was present in the sample. Thirteen false positive results were reported, most likely due to the detection of *S. xylosus* that was present in the sample. The strain can form colonies on for example Baird-Parker agar (BP) with characteristic grey, but coagulase-negative, colonies.

Sample B

No target organism was present. Three false positive results were reported.

Sample C

The strain of *S. aureus* was target organism. At the National Food Agency, this formed typical grey colonies with a precipitation zone on BP with rabbit plasma fibrinogen (RPFA). Two low and three high outliers were reported, as well as four false negative results.

General remarks

Similar to previous proficiency testing rounds most laboratories (45 %) followed NMKL 66:2009. Other commonly used methods were ISO 6888-1:1999 (13 %), ISO 6888-2:1999 (11 %) and 3M™ Petrifilm™ Staph Express (14 %). 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. 3M™ Petrifilm™ Staph Express (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.

As a whole, the results were very similar for the most commonly used media BP, RPFA and Petrifilm Staph, in all of the samples. At the same time, the reported concentrations were somewhat lower for Petrifilm Staph in all three samples. Similar lower results have however been seen for Petrifilm Staph in several previous proficiency testing rounds (latest April 2018), and could in that sense be regarded as normal.

Several media were used only by a small number of laboratories, which makes them difficult to evaluate. As an example, both laboratories that used Compact Dry X-SA failed the analysis of sample A. The method is validated for enumeration of *S. aureus* against the reference method ISO 6888-1:1999 (NordVal 042).

Traditionally, coagulase-positive staphylococci are confirmed 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. With Petrifilm Staph, confirmation is instead often carried out with 3M™ Petrifilm™ Staph Express Disk (Petrifilm Disk). This test detects 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.

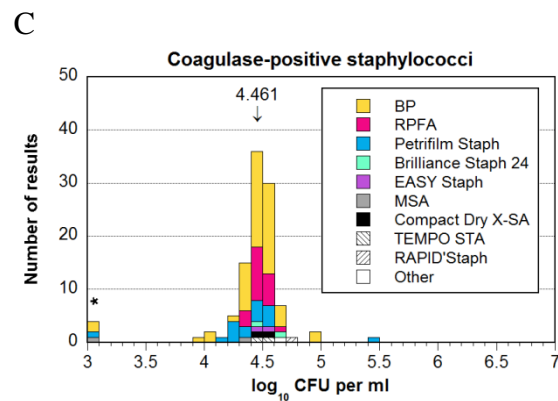
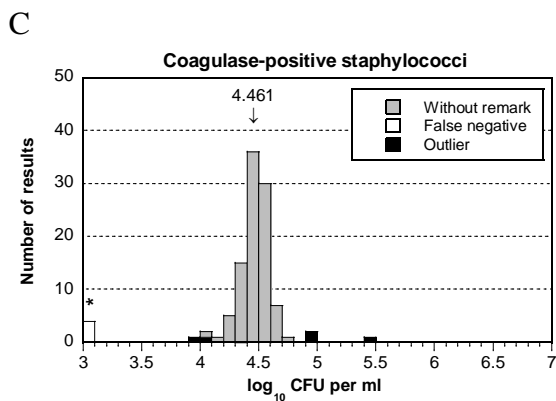
Confirmation of some kind was carried out by 73 % of the laboratories as a whole, and by 95 % of the laboratories that incubated on BP. The most common method for the confirmation was a tube coagulase test, followed by latex agglutination test and Petrifilm Disk. No clear difference could however be seen between laboratories that confirmed and those that did not.

Results from analysis of coagulase-positive staphylococci

Medium	N	Sample A						Sample B						Sample C					
		n	m	s	F	<	>	n	m	s	F	<	>	n	m	s	F	<	>
All results	105	92	-	-	13	-	-	102	-	-	3	-	-	96	4.461	0.114	4	2	3
BP	56	49	-	-	7	-	-	54	-	-	2	-	-	50	4.467	0.109	2	2	2
RPFA	20	20	-	-	0	-	-	20	-	-	0	-	-	20	4.467	0.074	0	0	0
Petrifilm Staph	17	14	-	-	3	-	-	16	-	-	1	-	-	15	4.381	0.129	1	0	1
Brilliance Staph 24	2	2	-	-	0	-	-	2	-	-	0	-	-	2	-	-	0	0	0
EASY Staph	2	2	-	-	0	-	-	2	-	-	0	-	-	2	-	-	0	0	0
MSA*	2	2	-	-	0	-	-	2	-	-	0	-	-	1	-	-	1	0	0
Compact Dry X-SA	2	0	-	-	2	-	-	2	-	-	0	-	-	2	-	-	0	0	0
TEMPO STA	2	1	-	-	1	-	-	2	-	-	0	-	-	2	-	-	0	0	0
RAPID'Staph**	1	1	-	-	0	-	-	1	-	-	0	-	-	1	-	-	0	0	0
Other	1	1	-	-	0	-	-	1	-	-	0	-	-	1	-	-	0	0	0

* MSA: Mannitol salt agar

** RAPID'Staph (BIO-RAD)



Enterococci

Sample A

No target organism was present in the sample. Twenty-one false positive results were reported, likely due to detection of *P. acidilactici* in the sample. The strain forms atypical, faint pink colonies on Slanetz & Bartley *Enterococcus*-agar (ENT). In subsequent confirmation on bile esculin agar (BEA) these normally do not cause any blackening of the medium after 2 hours, but a faint blackening may be seen after 24 hours. Since the various methods have different incubation times on BEA, and since the interpretation of blackening can differ, positive results are also considered correct. The analysis is therefore not evaluated and no z-scores have been calculated. The results are also not included in the tables under the box plots.

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 brain heart infusion broth (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.

Sample B

The strain of *E. hirae* was target organism. One low and two high outliers were reported.

Sample C

The strain of *E. durans* was target organisms. Three low and two high outliers were reported, as well as one false negative result.

General remarks

A clear majority of the laboratories (70 %) reported following NMKL 68:2011. Less frequently used methods were IDF 149A:1997 (6 %), the older NMKL 68:2004 (3 %) and the water method ISO 7899-2:2000 (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.

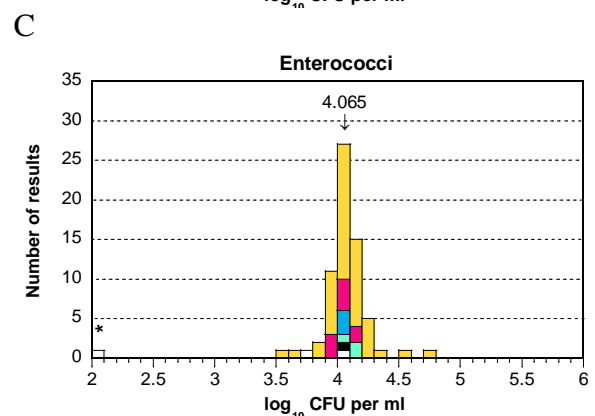
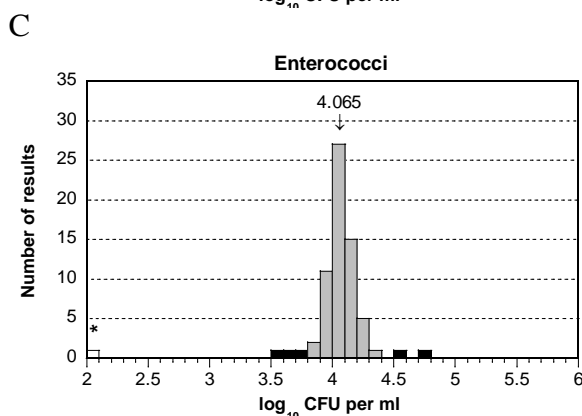
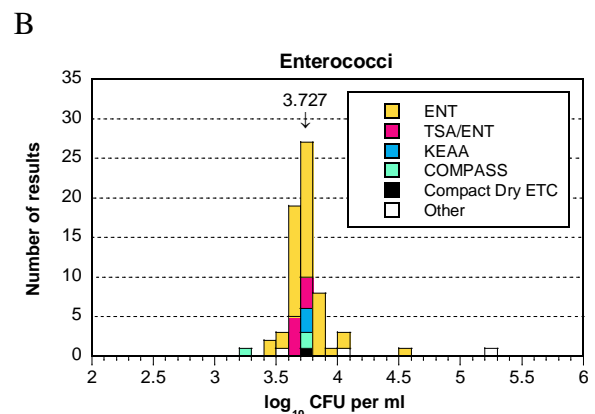
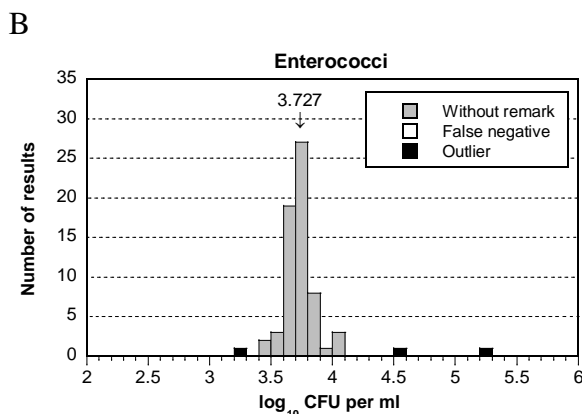
With NMKL 68:2011, enterococci are defined as Gram-positive, coagulase negative and oval cocci that hydrolyse esculin at 44 °C. Incubation is done on Slanetz & Bartley *Enterococcus*-agar (ENT) at 44 °C. On ENT, enterococci reduce the colourless substrate 2,3,5-trifenylnitroimidazolium chloride to red 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 bile esculin agar (BEA). On BEA the substrate esculin is hydrolysed by β -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 blackening of 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. Confirmation is, as in the NMKL method, by sub-culture on BEA (possibly with the addition of azide), but incubation is 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 A.

In total, 86 % of the laboratories incubated either on ENT or on TSA/ENT. In addition to these media, a smaller number of laboratories used kanamycin esculin azide agar (KEAA) or COMPASS® Enterococcus Agar (COMPASS). Another laboratory used 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. No clear difference in the results could be seen between the different media that were used.

Confirmation of some kind was performed by 76 % of the laboratories. Performing or not performing a confirmation does not appear to have had an effect on the outcome as a whole. Of the 21 laboratories that reported a false positive result for sample A, 14 stated that they performed a confirmation test.

Results from analysis of enterococci.

Medium	N	Sample A					Sample B					Sample C							
		n	m	s	F	<	>	n	m	s	F	<	>	n	m	s	F	<	>
All results	66	45	-	-	21	-	-	63	3.727	0.116	0	1	2	61	4.065	0.096	1	3	2
ENT	48	31	-	-	17	-	-	46	3.729	0.123	0	0	1	44	4.073	0.107	0	2	2
TSA/ENT	9	5	-	-	4	-	-	9	3.691	0.041	0	0	0	9	4.021	0.064	0	0	0
KEAA	3	3	-	-	0	-	-	3	-	-	0	0	0	3	-	-	0	0	0
COMPASS	2	2	-	-	0	-	-	2	-	-	0	1	0	3	-	-	0	0	0
Compact Dry ETC	1	1	-	-	0	-	-	1	-	-	0	0	0	1	-	-	0	0	0
Other	3	3	-	-	0	-	-	2	-	-	0	0	1	1	-	-	1	1	0



Gram-negative bacteria in pasteurized dairy products

Sample A

No Gram-negative bacteria were present in the sample. All laboratories reported a correct negative result.

Sample B

The strain of *K. pneumoniae* was target organism. All laboratories reported a correct positive result.

Sample C

The strains of *E. coli* and *S. marcescens* were target organisms. All laboratories reported a correct positive result.

General remarks

The analysis was without problem for the laboratories. All eleven laboratories stated the use of violet red bile glucose agar (VRBG). Nine laboratories followed NMKL 192:2011. The remaining two laboratories followed a company-specific method.

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 pasteurisation (HTST), where the temperature is raised to 72 °C for at least 15 seconds. Presence of Gram-negative bacteria therefore indicates recontamination, something which may limit the shelf-life of the product. With the method, the unopened package of milk/cream is incubated at 25 °C for 24 hours, followed by plating of 10 µl onto VRBG. As an alternative, 100 µl can be plated after incubation at room temperature for 28 hours. No matter which incubation that is used, 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.

Results from analysis of Gram-negative bacteria in dairy products

Method	N	Sample A		Sample B		Sample C	
		n	F	n	F	n	F
All results	11	11	0	11	0	11	0
NMKL 192:2011	9	9	0	9	0	9	0
Other	2	2	0	2	0	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 only 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

Z-scores, box plots and deviating results

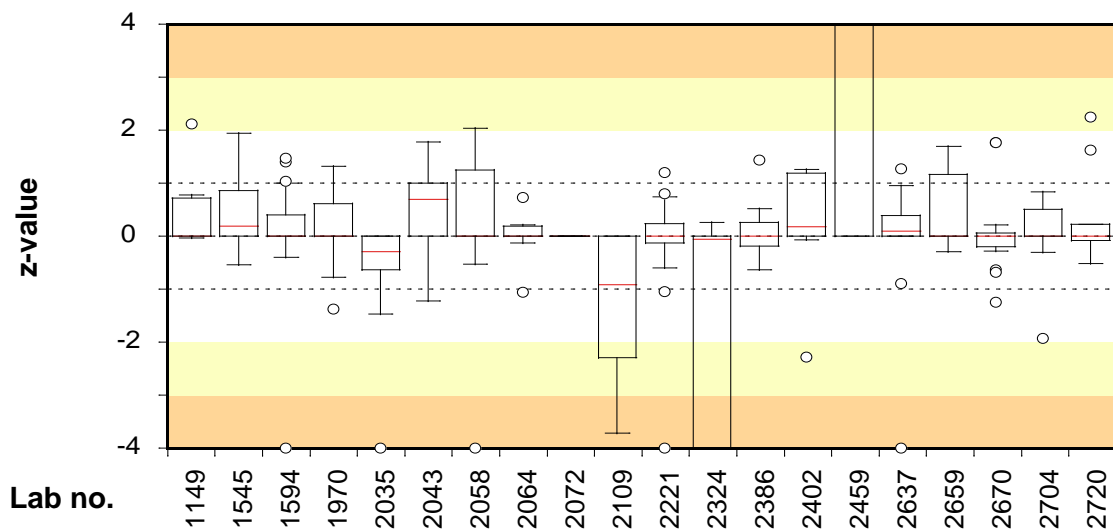
In order to allow comparison of the results from different analyses and samples, all results are transformed into standard values (z-scores). For quantitative analyses, a z-score is either positive or negative, depending on whether the individual result is higher or lower than the mean value calculated 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. 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. A small box, centred around zero, indicates the results of that individual laboratory, with false results excluded, are close to the general mean values calculated for all laboratory results. For each laboratory, the number of false results and outliers are also listed in the tables below the box plots.

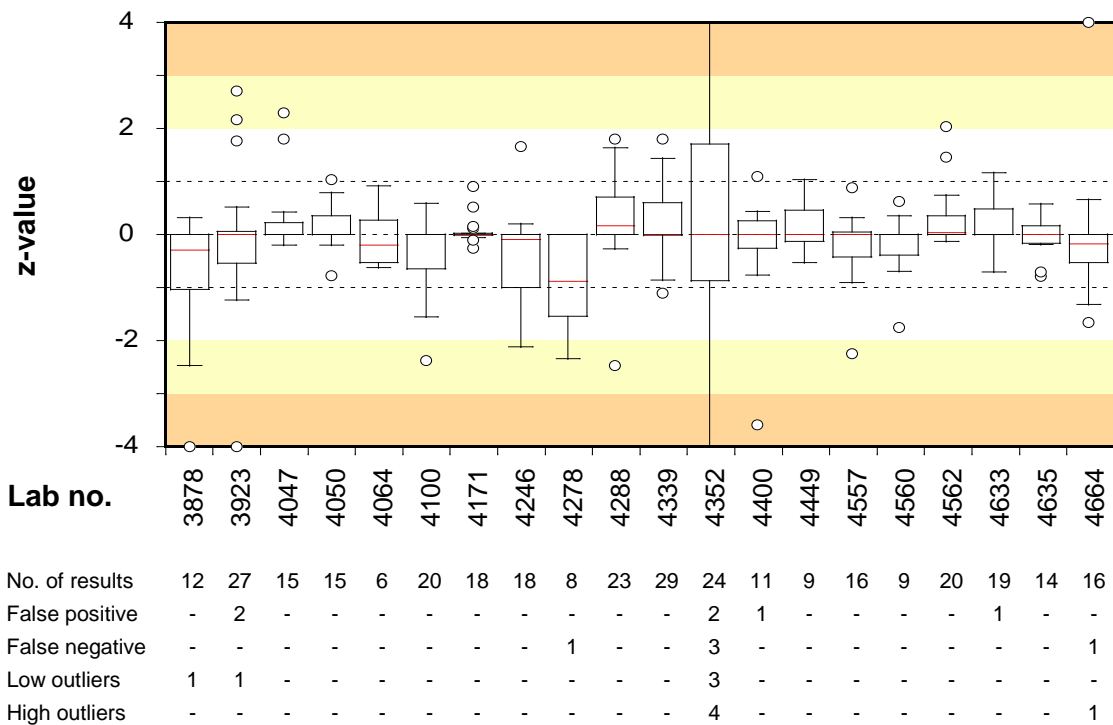
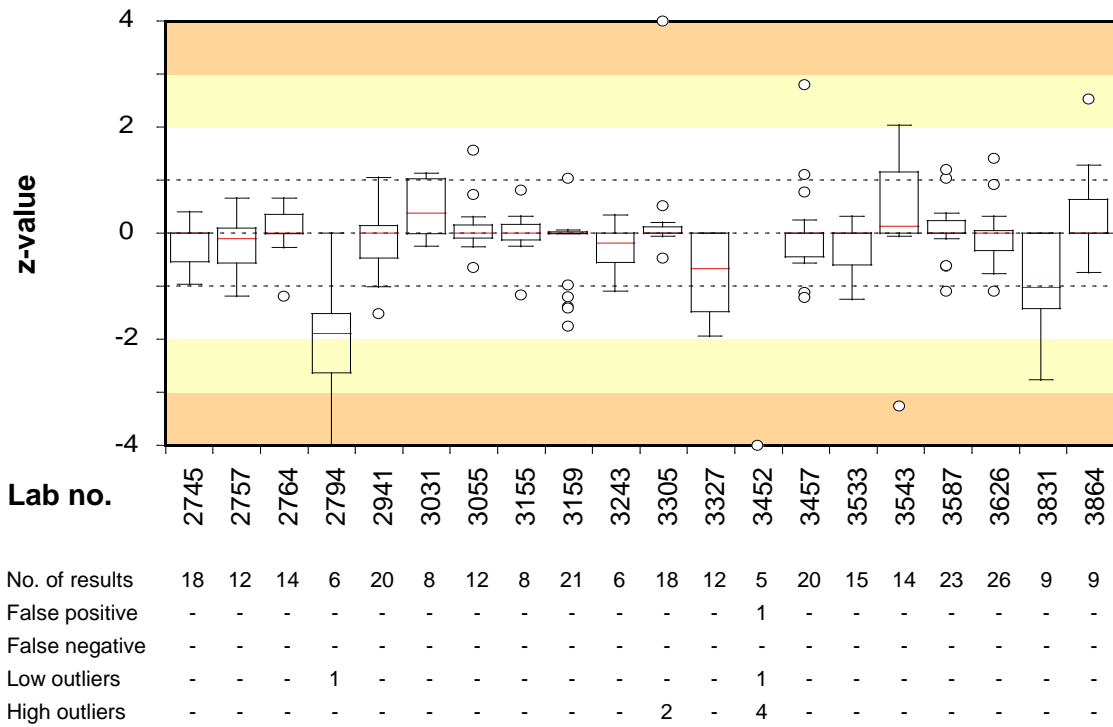
Box plots and numbers of deviating results for each laboratory

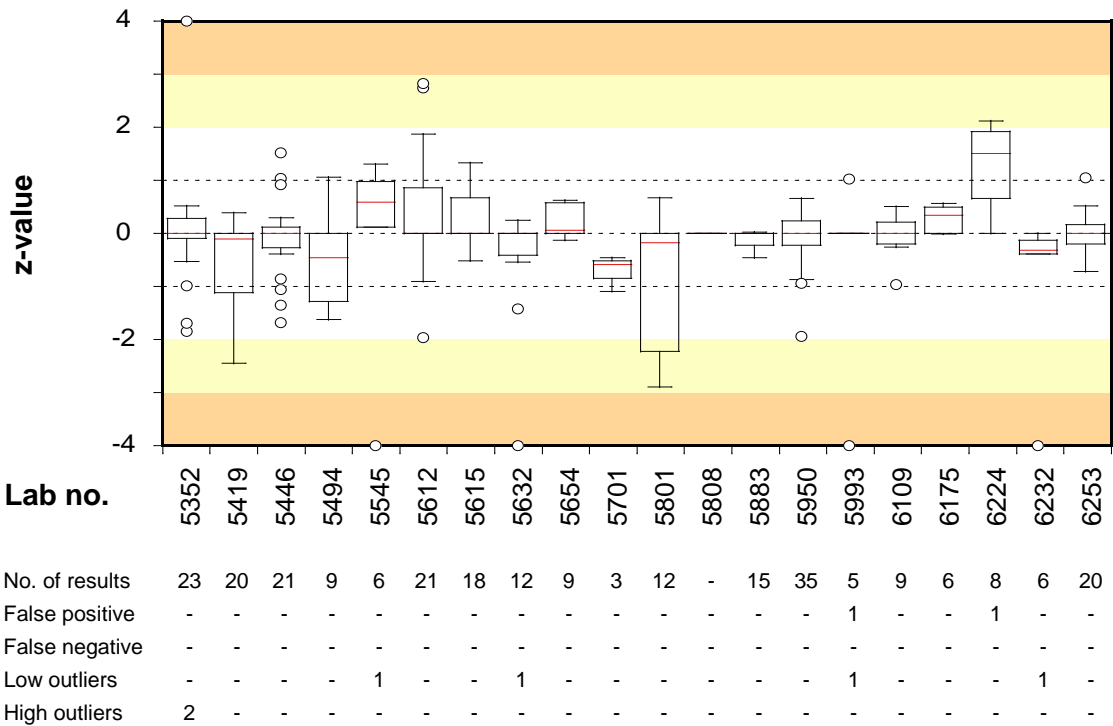
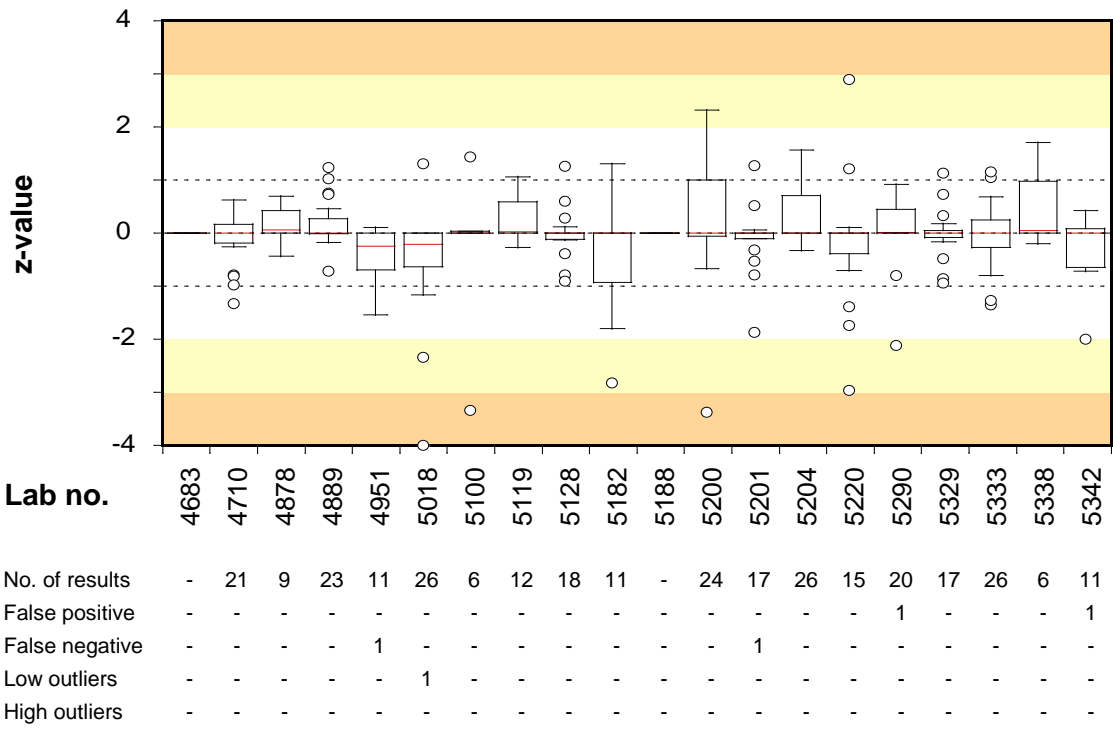
- Z-scores are calculated according to the formula: $z = (x-m)/s$, where x is the result of the individual laboratory, m is the mean of the results of all participating laboratories, and s is the standard deviation of the participating laboratories, after removing outliers and false results.
- Outliers 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 red 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.

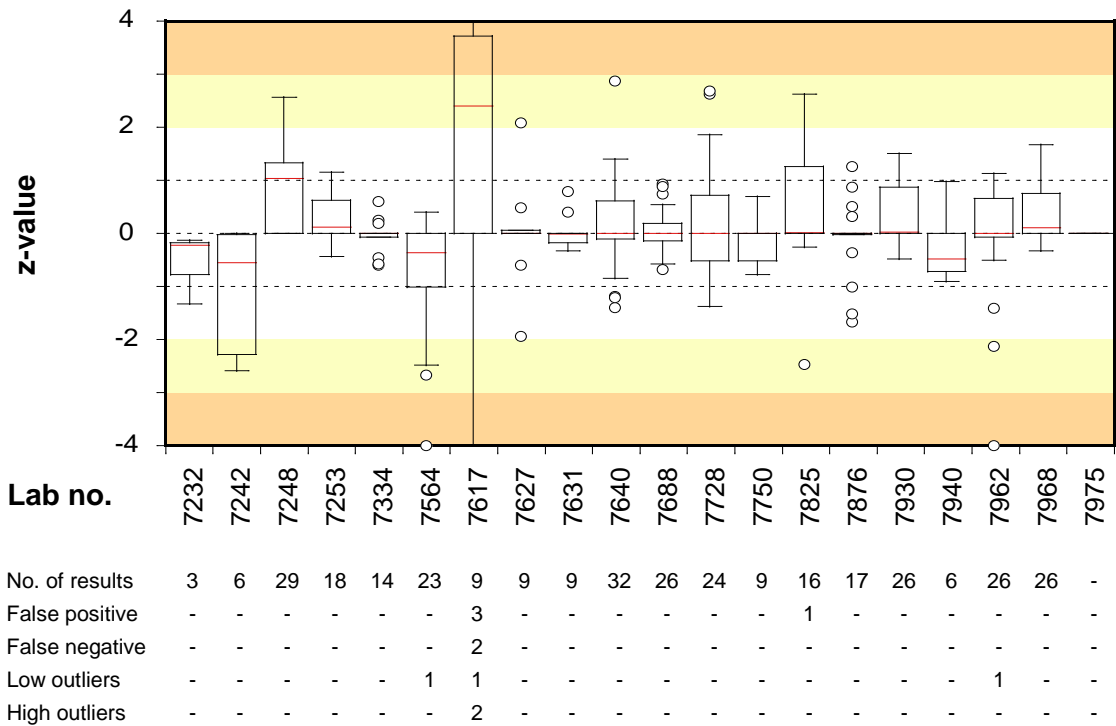
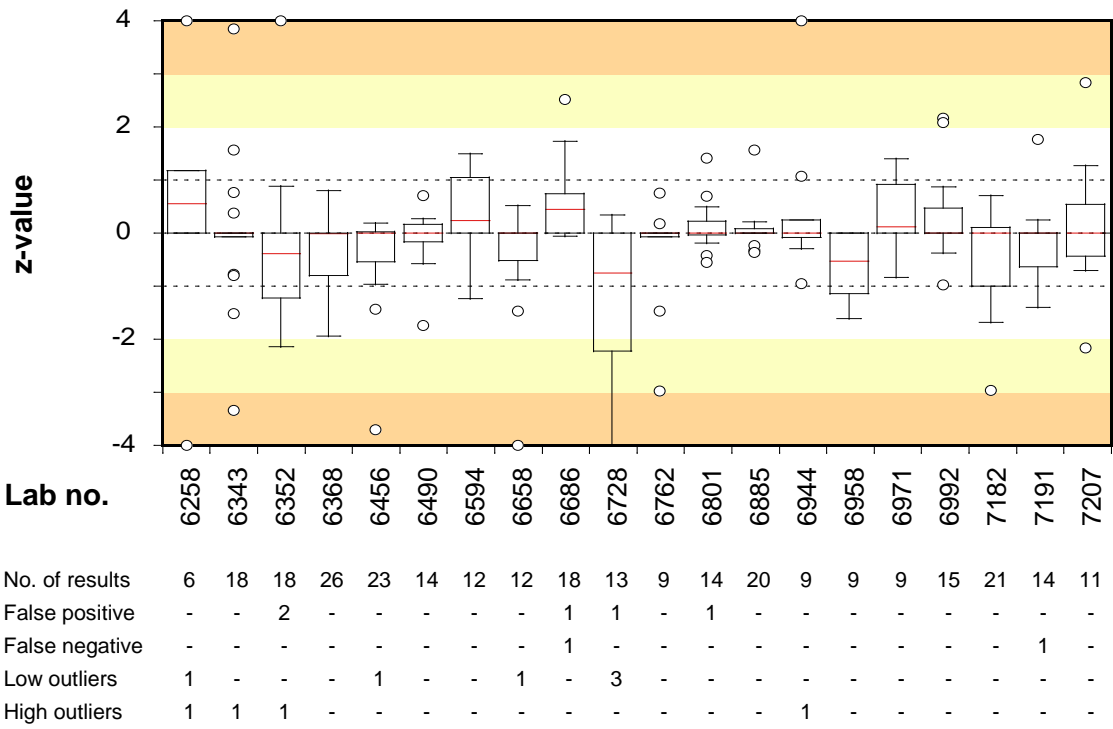
* $< [lowest\ value\ in\ the\ box - 1,5 \times (highest\ value\ in\ the\ box - lowest\ value\ in\ the\ box)]$ or
 $> [highest\ value\ in\ the\ box + 1,5 \times (highest\ value\ in\ the\ box - lowest\ value\ in\ the\ box)]$.

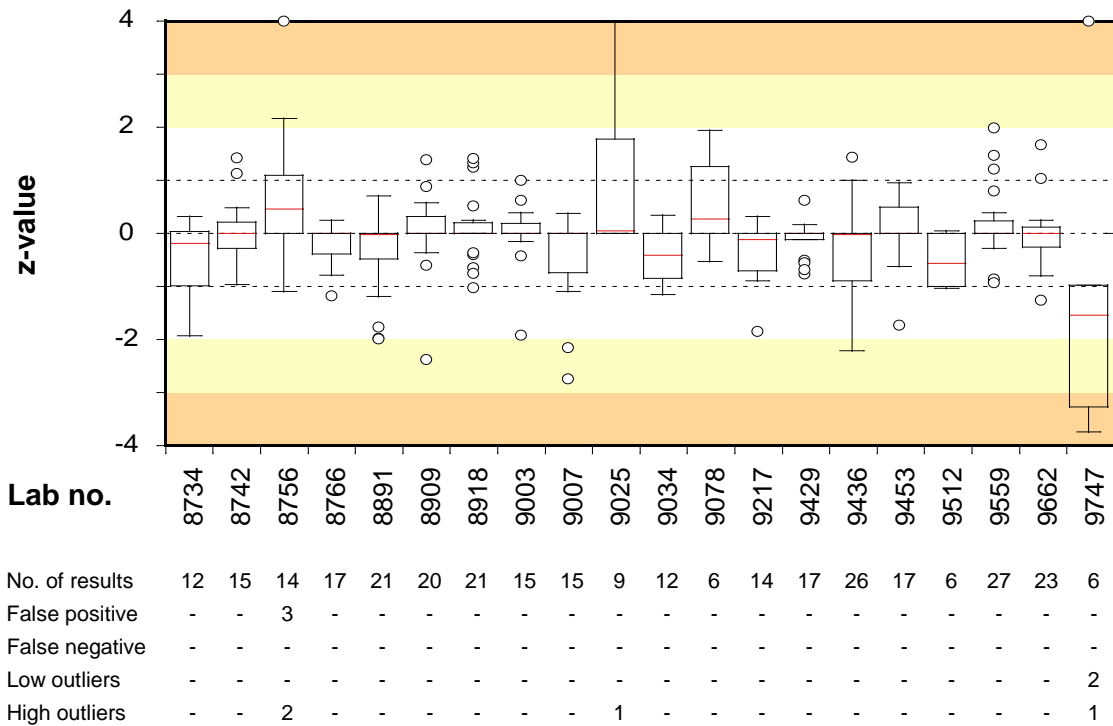
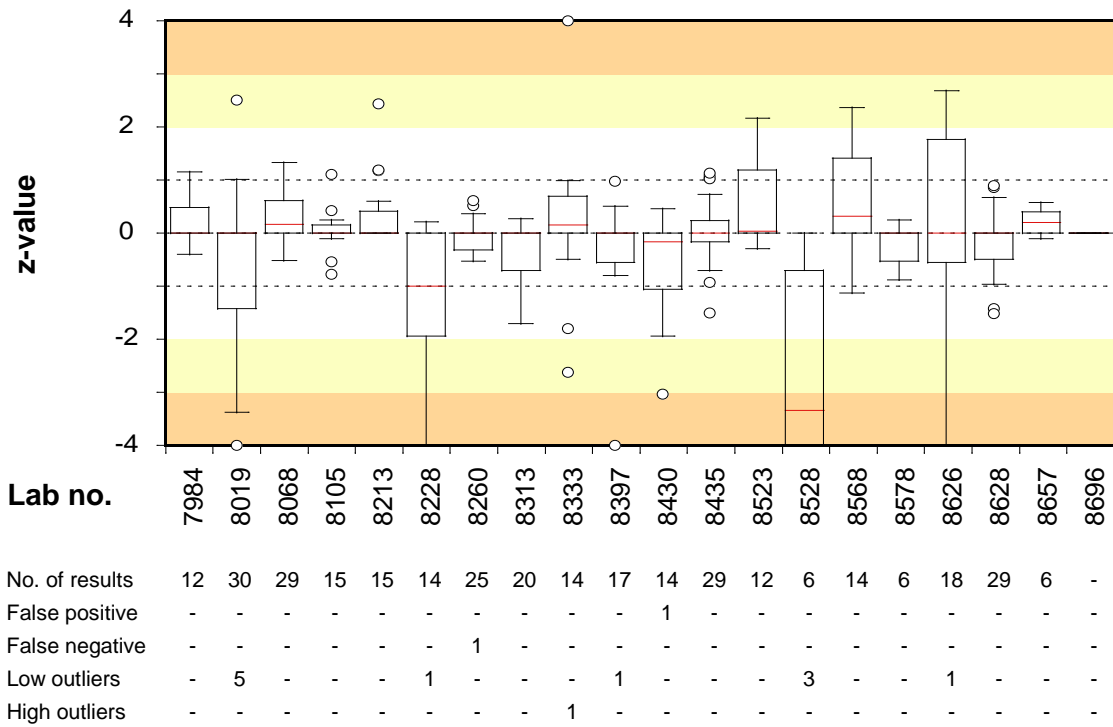


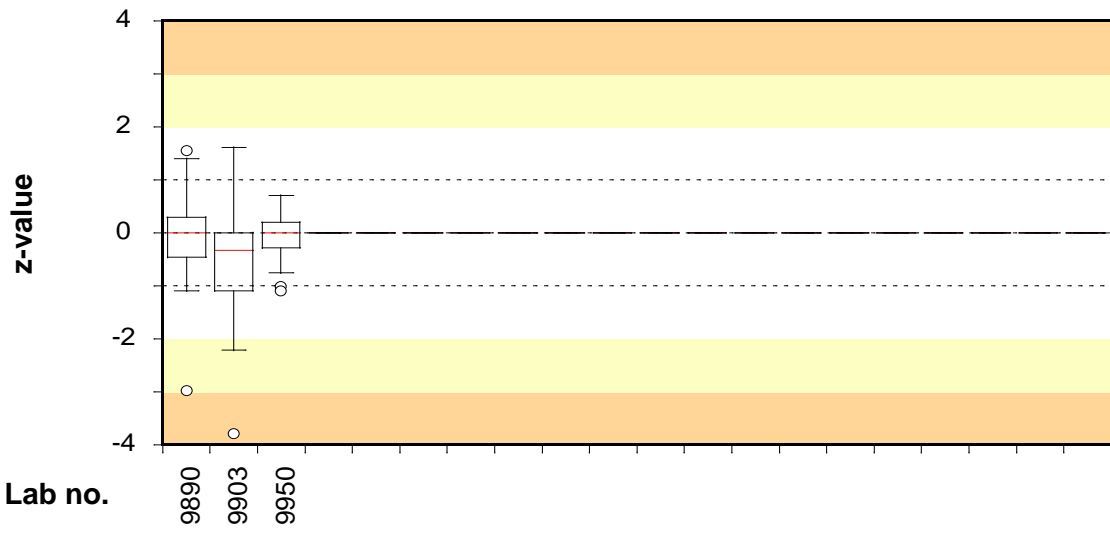
No. of results	15	20	29	29	9	6	9	9	-	8	26	15	12	12	17	20	18	15	18	9
False positive	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-
False negative	-	-	-	-	-	-	-	-	-	1	-	1	-	-	1	-	-	-	-	-
Low outliers	-	-	1	-	1	-	1	-	-	-	1	6	-	-	-	1	-	-	-	-
High outliers	-	-	-	-	-	-	-	-	-	-	-	-	-	-	9	-	-	-	-	-











Lab no.	9890	9903	9950
No. of results	21	17	15
False positive	-	-	-
False negative	-	-	-
Low outliers	-	1	-
High outliers	-	-	-

Test material and quality control

Test material

Each laboratory received three manufactured freeze-dried microbial samples, 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 had to be dissolved in 254 ml of sterile diluent. The organisms present in the sample mixtures are listed in Table 2.

Table 2. *Microorganisms present in samples A-C.*

Sample ¹	Microorganism	Strain	
		SLV no. ²	Reference ³
A	<i>Bacillus cereus</i>	SLV-518	CCUG 44741
	<i>Pediococcus acidilactici</i>	SLV-213	CCUG 45146
	<i>Staphylococcus xylosum</i>	SLV-283	Cheese, 1989
B	<i>Enterococcus hirae</i>	SLV-536	CCUG 46536
	<i>Kocuria rhizophila</i>	SLV-055	CCUG 35073
	<i>Klebsiella pneumoniae</i>	SLV-186	CCUG 45102
C	<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

¹ The links between the samples and the randomised sample numbers are shown in Annex 1.

² Internal strain identification no. at the National Food Agency.

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

Quality control of the samples

In order to allow comparison of the freeze-dried samples, it is essential to have equal volumes of a homogeneous sample mixture in all vials. Quality control is therefore performed on 10 randomly chosen vials in conjunction with the manufacture, 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 samples; *m* is expressed in \log_{10} cfu (colony forming units) per ml of sample.

Analysis and method	A ¹			B ¹			C ²		
	m	T	I_2	m	T	I_2	m	T	I_2
Aerobic microorganisms, 30 °C NMKL method no. 86:2013	5.382	1.37	2.803	4.186	1.16	0.896	4.998	1.32	1.821
Aerobic microorganisms, 20 °C NMKL method no. 86:2013	5.317	1.08	0.153	4.147	1.23	1.565	4.980	1.20	0.830
Contaminating microorganisms ISO method no. 13559:2002 IDF method no. 153:2002	5.363	1.41	3.599	4.279	1.52	0.825	5.011	1.20	0.842
Enterobacteriaceae NMKL method no. 144:2005	-	-	-	3.744	1.73	4.581	4.693	1.39	1.372
Coliform bacteria 30, °C NMKL method no. 44:2007	-	-	-	3.645	1.48	1.584	4.011	2.03	1.333
Coliform bacteria, 37 °C NMKL method no. 44:2007	-	-	-	3.646	1.21	0.381	4.104	1.74	1.074
Thermotolerant coliform bacteria NMKL method no. 125:2005	-	-	-	3.737	1.36	1.310	4.127	1.45	4.214
<i>Escherichia coli</i> NMKL method no. 125:2005	-	-	-	-	-	-	4.127	1.45	4.214
Presumptive <i>Bacillus cereus</i> NMKL method no. 67:2010	4.235	1.71	1.535	-	-	-	-	-	-
Coagulase-positive staphylococci NMKL method no. 66:2009	-	-	-	-	-	-	4.541	1.44	1.179
Enterococci NMKL method no. 68:2011	-	-	-	3.771	1.29	0.972	4.073	1.32	1.170
Gram-negative bacteria in pasteurized milk and cream. Detection of recontamination. NMKL method no. 192:2011	Neg.	-	-	Pos.	-	-	Pos.	-	-

- No target organism and therefore no value

¹ n = 5 vials analysed in duplicate

² n = 10 vials analysed in duplicate

References

1. Kelly, K. 1990. Outlier detection in collaborative studies. *J. Assoc. Off. Anal. Chem.* 73:58-64.
2. Anonymous, 2015. Protocol, Microbiology. Drinking Water & Food, The National Food Agency, Sweden.
3. Peterz, M., Steneryd. A.C. 1993. Freeze-dried mixed cultures as reference samples in quantitative and qualitative microbiological examinations of food. *J. Appl. Bacteriol.* 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			<i>Escherichia coli</i>			Presumptive <i>Bacillus cereus</i>			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	A	B	C	
N		166	166	165	29	29	29	18	18	18	137	140	140	57	57	57	94	93	92	48	46	48	119	119	115	109	109	109	105	105	105	66	66	67	11	11	11	N
Min		4.28	2.98	3.73	4.99	3.78	4.65	3.65	3.45	3.95	0	0	2.48	0	2.86	1.61	0	0	0	0	3.02	0	0	0	0	0	0	0	0	0	0	0	3.2	0	-	-	-	Min
Max		6.41	5.33	6.01	5.62	4.30	5.18	5.46	4.96	5.38	0	4.75	5.54	1.30	5.70	5.68	3.75	4.83	5.64	0	4.04	4.66	0	4.16	5.19	5.23	3.46	4.08	5.39	4.20	5.40	4.45	5.20	4.76	-	-	-	Max
Med		5.28	4.21	4.96	5.28	4.15	4.94	5.19	4.06	4.90	0	3.70	4.57	0	3.69	4.23	0	3.70	4.20	0	3.75	4.12	0	0	4.05	4.11	0	0	0	0	4.48	0	3.72	4.07	-	-	-	Med
m		5.262	4.207	4.963	5.262	4.121	4.929	5.125	4.004	4.864	0	3.694	4.541	0	3.693	4.209	0	3.703	4.232	0	3.751	4.168	0	0	4.065	4.120	0	0	0	0	4.461	0	3.727	4.065	neg	pos	pos	m
s		0.156	0.107	0.116	0.140	0.127	0.105	0.261	0.223	0.206	0	0.132	0.172	0	0.161	0.331	0	0.191	0.305	0	0.163	0.187	0	0	0.221	0.156	0	0	0	0	0.114	0	0.116	0.096	-	-	-	s
F+		0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	4	0	0	1	2	13	3	0	21	0	0	0	0	0	F+
F-		0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	3	2	0	0	1	0	0	2	1	0	0	0	0	4	0	0	1	0	0	0	F-
<		5	5	5	0	0	0	1	0	2	0	4	6	0	2	1	0	1	0	0	1	1	0	0	3	3	0	0	0	0	2	0	1	3	-	-	-	<
>		3	9	2	0	0	0	0	1	0	0	2	1	0	2	1	0	1	1	0	0	0	0	0	1	2	0	0	0	0	3	0	2	2	-	-	-	>
< OK		4.73	3.81	4.64	4.99	3.78	4.65	4.43	3.45	4.45	0	3.30	4.02	0	3.15	3.23	0	3.06	3.48	0	3.43	3.84	0	0	3.27	3.60	0	0	0	0	4.09	0	3.47	3.82	-	-	-	< OK
> OK		5.69	4.51	5.40	5.62	4.30	5.18	5.46	4.30	5.38	0	3.99	4.81	0	4.05	4.76	0	4.17	4.80	0	4.05	4.66	0	0	4.69	4.57	0	0	0	0	4.78	0	4.08	4.34	-	-	-	> OK

N = number of analyses performed
Min = lowest reported result

Max = highest reported result
Median = 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

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 National 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 National Food Agency's reference material

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

More information is available on our website: www.livsmedelsverket.se/en/rm-micro