



**Report of the second inter-laboratory study
on the enumeration of *E. coli*
in Live Bivalve Molluscs (PT29) –
2021**

Edited by:

Margherita Montalbano Di Filippo, Arianna Boni, Clarissa Ferreri, Silvia Arancia, Gianfranco Brambilla, Paola Chiani, Guendalina Fornari Luswergh, Federica Gigliucci, Arnold Knijn, Valeria Michelacci, Fabio Minelli, Rosangela Tozzoli, Stefano Morabito

1. INTRODUCTION AND OBJECTIVES OF THE STUDY

As an effect of Regulation (EU) 222/2018, the European Union Reference Laboratory for *Escherichia coli* (EURL-VTEC) has taken over the activities carried out by the former EU reference laboratory for monitoring the bacteriological contamination of bivalve molluscs as regards the analytical tests for *E. coli*. In this specific case, the EURL has the task (i) to assess the performance of the designated National Reference Laboratories (NRLs) for the microbiological contamination of bivalve molluscs in the EU and (ii) to assist the EFTA Member States, EU Candidate Countries and third countries in using the method for the enumeration of *E. coli* in live bivalve molluscan shellfish.

The EU reference method for enumeration of *E. coli* in live bivalve molluscs is represented by ISO 16649 -part 3 ***“Microbiology of the food chain - Horizontal method for the enumeration of β -glucuronidase-positive Escherichia coli Part 3: Detection and most probable number technique using 5-bromo-4-chloro-3-indolyl- β -D-glucuronide”***.

This scheme is intended to provide proficiency testing (PT) for laboratories performing the analysis of live bivalve molluscs, from production areas in accordance with Regulation (EC) N° 854/2004 and from throughout the production chain in accordance with Regulation (EC) N° 2073/2005.

The main objective of this study was to provide data on the proficiency of the laboratories and to identify possible areas of improvement in the application of the analytical procedure. The PT29, was carried out on commercial common live mussels, without any additional contamination. The molluscs were taken at a site designated as Area C, with no further treatments for depuration applied.

This document represents the evaluation report of the PT29. The study was conducted according to the International Standard ISO/IEC 17043:2010 “Conformity assessment – General requirements for proficiency testing”.

2. PARTICIPANTS

Thirty NRLs, representing 28 EU Member States plus two EFTA countries accepted the invitation to participate. Each NRL received its own individual laboratory numerical code, which was used to label the laboratories in the result tables.

The Laboratories participating in the study were:

- AGES, Institut für Lebensmittelsicherheit Wien, Gruppe Mikrobiologie, Wien, Austria

- Sciensano, Foodborne Pathogens, Brussels, Belgium
- NRL *Listeria*, *E. coli* and foodborne viruses, Sofia, Bulgaria
- Croatian Veterinary Institute, Veterinary Institute SPLIT (HVI-VZS), Laboratory for microbiology of food and animal feeding stuffs (S-2), Split, Croatia
- Croatian Veterinary Institute, Department Rijeka, Laboratory for food and feed microbiology, Rijeka, Croatia
- Laboratory for the Control of Food of Animal Origin (LCFAO), Cyprus Veterinary Services, Nicosia, Cyprus
- Microbiological Laboratory Ringsted, Ringsted, Denmark
- LNR Microbiologie des coquillages/LSEM, Nantes, France
- German Federal Institute for Risk Assessment, Unit Food Microbiology, Host-Pathogen-Interaction, Department Biological Safety, Berlin, Germany
- Department of Food Hygiene of Athens, Ministry of Rural Development and Food, Athens, Greece
- Veterinary Laboratory of Kavala, Ministry of Rural Development & Food, Kavala, Greece
- Icelandic Food and Biotech R&D Matis ohf., Iceland
- Marine Institute, Rinville, Oranmore, Co. Galway, Ireland
- Marine Institute, Complete Laboratory Solutions, Rosmuc, Ireland
- Southern Scientific Ireland Ltd, Dunrinc, Ireland
- Eurofins Food Testing Ireland, Waterford, Ireland
- Pelagia Feed Ireland Ltd. t/a AQUALAB, Donegal, Ireland
- Italy, Istituto Superiore di Sanità
- IZS Umbria e Marche "Togo Rosati", Sezione di Ancona, Italy
- BIOR, Riga, Latvia
- Institute of Marine Research, Laboratory for molecular biology, Bergen, Norway
- National Veterinary Research Institute (NVRI), Department of Hygiene of Food of Animal Origin, Pulawy, Poland
- IPMA/DMRM, Alges, Portugal
- Institute for Hygiene and Veterinary Public Health, Bucharest, Romania
- Department of Food Hygiene, Veterinary and food institute, Dolny Kubin, Slovakia
- UL, Veterinary faculty / National veterinary institute, Institute for food safety, feed and environment – Unit for food safety – NRL for *E. coli*, Ljubljana, Slovenia
- Microbiology Food Department, Agencia Española de Seguridad Alimentaria y

Nutrición (AESAN), Spanish Food Safety and Nutrition Agency, National Center for Food -
Centro Nacional de Alimentación (CNA), Madrid, Spain
- Swedish Food Agency, the Biology department, Uppsala, Sweden
- RIVM, Bilthoven, The Netherlands
- Laboratory Food and Feed Safety, Netherlands Food and Consumer Product Safety
Authority, Wageningen, The Netherlands

3. MATERIALS AND METHODS

3.1. Sample preparation

A single batch of 23,5 kg common mussels (*Mytilus gallusprovincialis*) were collected from a harvesting area class C in Italy, on 28/09/2021. Prior to packing, the shellfish were placed in a large, disinfected container and thoroughly mixed. Randomly selected samples of approximately 500 g of mussels were formed and packed, and the parcels were shipped refrigerated to the participating laboratories by courier.

3.2. Sample distribution and examination

Each individual sample was packed in a polystyrene refrigerate box containing one plastic pocket with a stomacher bag containing the common mussels. A total of 30 boxes were shipped and 30 samples were despatched on 28/09/2021 to the participants. Participants were requested to analyse the samples as soon as possible after receipt and within 24 hours of arrivals, following the part 3 of the method ISO 16649.

3.3. Collection and Elaboration of the NRLs Results

The results were submitted through an on-line service of the EURL for *E. coli*. The instruction on how to report the results and the link to access the form was sent by E-mail to all the participants' laboratories. In particular, the participants were requested to fill in both (i) the Evaluation form (arrival date/time/condition/temperature, storage temperature, analysis' start date/time and notes field in order to specify any problem with the samples delivery/packaging) and (ii) the Sample Results section. The participants were also requested to upload the MPN calculation sheet and the data downloaded from the portable datalogger included in the parcel to monitor the sample's temperature

3.4. Analysis of the NRLs' results

Out of the 30 precipitating laboratories, 29 returned the results and have been included in the present report.

3.4.1 Parameters used for the assignment of the scores

A scoring system is used to assess the participant's performance (Table 1).

Table 1: *E. coli* MPN scores; dark green boxes represent the maximum value' score (12), the dark red ones the minimum (0) (graduation colour bar).

Results	Returning of results	Score allocated		Score
		Replicate 1	Replicate 2	
Both replicates MPN results are within the expected range*	2	5	5	12
One replicate MPN result reported is outside the expected range and falls between the median \pm 3 SD and the median \pm 5 SD value	2	5	2	9
Both replicates MPN results reported are outside the expected range and fall between the median \pm 3SD and the median \pm 5 SD value	2	2	2	6
One replicate MPN result reported is outside the median \pm 5 SD value	2	5	0	7
Both replicates MPN results reported are outside the median \pm 5 SD value	2	0	0	2
Single MPN result reported only	2	5	0	7
Tube combination inconsistent with MPN reported (only one replicate)	2	7		9
Tube combination inconsistent with MPN reported (both replicates)	2	5		7
Sample not examined or results returned late, or no explanation received	0	0	0	0
High censored result (<i>i.e.</i> MPN => 18000 per 100g)		<u>Score not assigned</u>		

***expected range:** Participants' Median \pm 3SD – SD stands for Theoretical Standard Deviation = 0,24
The expected range values are reported in detail in Table 3 (Results Section).

4. RESULTS

4.1. Reference results

Ten randomly selected samples from the initial batch of mussels, consisting in approximately 100 g of mussels each, were analyzed in duplicate on 29/09/2021 following the ISO 16649 part 3 method. The reference results are reported in Table 2.

Table 2: *E. coli* MPN/100 g reference results.

SDT stands for Theoretical Standard Deviation = 0,24

Note: 4,49E+02 stands for $4,49 \times 10^2$ which is 4,49 times 10 (E) to the 2nd power (+02)

Sample Number - Type	Range (<i>E.coli</i> MPN/100g)		Median	Median±3SDT*		Median±5SDT*	
	Minimum Value	Maximum Value					
Sample 1 - Mussels	0	330	85,5	4,49E+02	16,29	1,36E+03	5,39

4.2. Participants' results

Performance assessment was carried out according to the scoring parameters reported in Table 1 – Section Materials and Methods. Participant's results and scores are shown in Table 3, 4, 5 and Figure 1.

Table 3: Summary statistics of participants' results (total results received: 29 laboratories).

<i>E. coli</i> MPN – summary statistics'	Sample 1
Participants reporting duplicate results for <i>E. coli</i> MPN	29
Participants reporting a single MPN result	0
Participants reporting both replicate MPN results within expected range*	25/29
Participants reporting both replicate MPN results outside expected range	3/29
Participants reporting one replicate MPN result outside expected range	1/29
Participants reporting one replicate MPN results as censored results	0
Participants reporting both replicate MPN results as censored results	0
Participants reporting tube combination and/or MPN results inconsistent with ISO 7218*	0

****expected range:** Participants' Median ±3SD – SD stands for Theoretical Standard Deviation = 0,24

****points deducted from participants returning results with incorrect tube combinations and/or inconsistent with ISO 7218.**

Table 4: *E. coli* MPN/100 g participants' results.

Sample Number - Type	Range (<i>E. coli</i> MPN/100g)		Median	Median±3SDT*		Median±5SDT*	
	Minimum Value	Maximum Value					
Sample 1 - Mussels	0	3,3E+02	78	4,09E+02	15	1,2E+03	4,9E+00

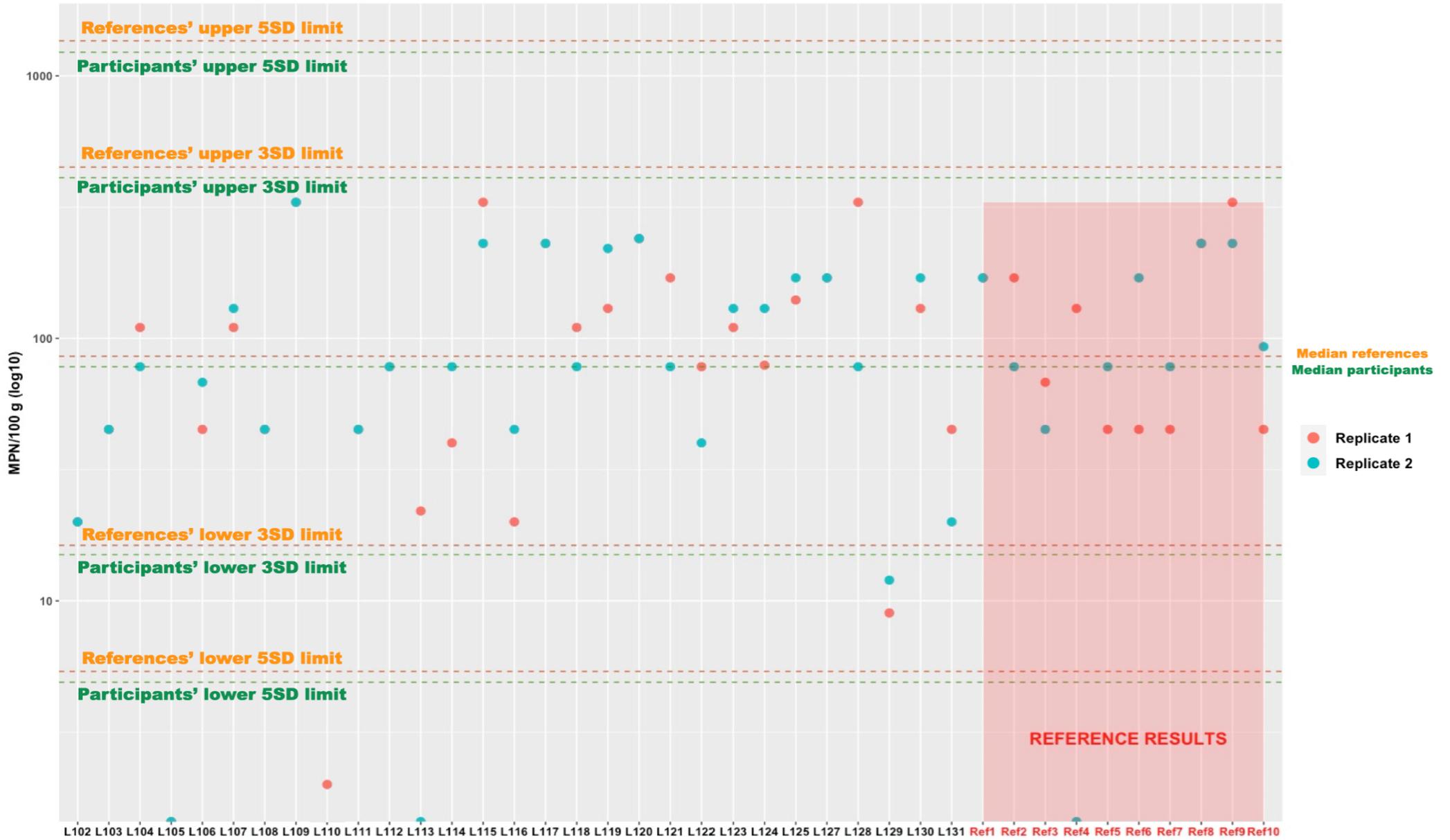
Note: The median and upper and lower limits (± 3 SD and ± 5 SD) were calculated from participants' results. SDT calculations were based on the inherent variability of the 5 x 3 MPN method ($0.24 \log_{10}$). **Reference values were excluded from the calculation of the participants' median.**

Table 4. Details of the analysis performed by the Laboratories and scores obtained; dark green boxes represent the maximum value' score (12), the dark red ones the minimum (0) (graduation colour bar)



Lcode	Shipment Date	Start Date	<i>E. coli</i> MPN/100g						Score
			Replicate 1	Rarity Category	Rarity Index	Replicate 2	Rarity Category	Rarity Index	
L102	28/09/2021	29/09/2021	20	1	1	20	1	1	12
L103	28/09/2021	30/09/2021	45	1	1	45	1	1	12
L104	28/09/2021	30/09/2021	110	1	0,565	78	1	1	12
L105	28/09/2021	29/09/2021	0	1	1	0	1	1	2
L106	28/09/2021	29/09/2021	45	1	1	68	1	0,354	12
L107	28/09/2021	29/09/2021	110	1	0,564	130	1	1	12
L108	28/09/2021	29/09/2021	45	1	1	45	1	1	12
L109	28/09/2021	29/09/2021	330	1	1	330	1	1	12
L110	28/09/2021	29/09/2021	2	1	1	2,2	3	0,009	2
L111	28/09/2021	29/09/2021	45	1	1	45	1	1	12
L112	28/09/2021	29/09/2021	78	1	1	78	1	1	12
L113	28/09/2021	29/09/2021	22	1	1	0	1	1	7
L114	28/09/2021	29/09/2021	40	1	0,205	78	1	1	12
L115	28/09/2021	29/09/2021	330	1	1	230	1	0,772	12
L116	28/09/2021	29/09/2021	20	1	1	45	1	1	12
L117	28/09/2021	29/09/2021	230	1	0,772	230	1	0,772	12
L118	28/09/2021	30/09/2021	110	1	0,564	78	1	1	12
L119	28/09/2021	29/09/2021	130	1	1	220	1	0,315	12
L120	28/09/2021	29/09/2021	240	1	0,744	240	1	0,744	12
L121	28/09/2021	29/09/2021	170	1	0,981	78	1	1	12
L122	28/09/2021	30/09/2021	78	1	1	40	1	0,205	12
L123	28/09/2021	29/09/2021	110	1	0,564	130	1	1	12
L124	28/09/2021	30/09/2021	79	1	1	130	1	1	12
L125	28/09/2021	29/09/2021	140	1	0,147	170	1	0,083	12
L127	28/09/2021	30/09/2021	170	2	0,013	170	2	0,013	12
L128	28/09/2021	29/09/2021	330	1	1	78	1	1	12
L129	28/09/2021	30/09/2021	9	1	1	12	1	0,051	6
L130	28/09/2021	30/09/2021	130	1	1	170	1	0,918	12
L131	28/09/2021	29/09/2021	45	1	1	20	1	1	12

Results' Dot Graph - Sample 1 Mussels



5. CONCLUDING REMARKS

1. Thirty laboratories participated in the study, and 29 of them returned the results. One Laboratory (L126) did not perform the analysis.
2. Twenty-five of the 29 participants (86%) obtained the highest score. Out of the remaining four laboratories two reported results falling outside the acceptability result of the median \pm 5 standard deviations for both the replicates.
3. Twenty-seven of the 29 laboratories that returned the results (93%) performed satisfactorily in the PT29 configuring a good overall performance of the network.