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RESEARCH

Proficiency tests for the detection of *Cronobacter* spp. and *Salmonella* spp. in milk powder

Colin Howell[1] · Romain Le Neve[1] · Anne Tirard[1] · Sandrine Nguyen[1] · Abdelkader Boubetra[1]

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Abstract

The contamination of milk powder by pathogens such as *Cronobacter* spp. and *Salmonella* spp. represents a major public health risk, particularly as the primary consumers of milk powder, infants, have heightened vulnerability to bacterial infection. In the face of this danger, analytical laboratories should implement and practice suitable methods for the detection of these microorganisms with high specificity and sensitivity. For this reason, BIPEA (Bureau Interprofessionnel d'Études Analytiques) launched a new proficiency testing program for the detection of *Cronobacter* spp. in samples of milk powder in 2019, and detection of *Salmonella* spp. in the same samples was added to these tests in 2023. This paper presents the design and implementation of the program, as well as a detailed analysis of laboratory results and systems for the evaluation of qualitative proficiency testing performances. Results from these tests are encouraging, as the majority of laboratories are able to correctly identify both contaminated and uncontaminated samples. Participation in proficiency testing programs is an important quality control tool for analytical laboratories to assess and demonstrate their competence to carry out these microbiological analyses, which are critical for public health, and one of the ways to monitor their performance as per the requirements of the ISO/IEC 17025:2017 standard.

Keywords Proficiency testing · Laboratory performance · Microbiology · Milk powder · *Salmonella* · *Cronobacter*

Introduction

With a market size of over $35 billion in 2024, milk powder plays an essential role in the food sector, particularly in the manufacturing of infant formulas, because of its combination of high nutritional value and stability over time. The low water content in milk powder and derived products makes contamination by pathogenic microorganisms more difficult, but risks continue to exist from certain resistant pathogens [1]. One such genus is *Cronobacter,* of the *Enterobacteriaceae* family, a group of Gram-negative and oxidase-negative bacteria that are rod-shaped and facultatively anaerobic [2]. While *Cronobacter* spp. rarely cause disease in adults, infants are extremely vulnerable; *Cronobacter* spp. primarily induce meningitis, and an eight-year study calculated a 42% mortality rate for infants infected with *Cronobacter* spp. meningitis based on analysis of over 100 cases over that period [3]. Additional studies have documented a clear link between the numerous cases that have been reported globally since 1960 and consumption of powdered infant formula [4], while a 2014 assessment of American milk powder facilities determined that *Cronobacter* spp. was present in the manufacturing areas of 69% of the 55 facilities tested [5].

The other pathogen most frequently responsible for outbreaks associated with milk powder consumption is *Salmonella* spp., also of the *Enterobacteriaceae* family and similarly capable of surviving for extended periods of time in foods with low moisture levels. While contamination of milk powder by *Salmonella* spp. is less prevalent than by *Cronobacter* spp., there are significant risks as *Salmonella* spp., which mainly causes gastroenteritis, is considered one of the pathogens inducing the highest rates of serious illness and hospitalization. Beginning in late 2017, France experienced a major *Salmonella* Agona outbreak that led to over 30 cases under six months old from consumption of infant milk products, including 18 hospitalizations, and similar outbreaks have been reported across the world with substantial economic and health consequences [6].

✉ Colin Howell
chowell@bipea.org

[1] Bureau Interprofessionnel d'Études Analytiques (BIPEA), 189 Rue d'Aubervilliers, 75018 Paris, France

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It is therefore essential for laboratories to detect bacterial contamination of milk powder by these two pathogens. The ISO 22964:2017 and ISO 6579–1:2017 standards describe, respectively, reference methods for the detection of *Cronobacter* spp. and *Salmonella* spp. in food products, and a number of alternative methods have been validated according to ISO 16140–2:2016 [7–9]. Quality control of all of these methods is critical to ensure consumer safety and confidence, and the ISO/IEC 17025:2017 standard [10] indicates that, where available and appropriate, laboratories should demonstrate the validity of their results through external controls, including proficiency tests (PTs). BIPEA, an accredited PT provider according to the requirements of the ISO/IEC 17043:2023 standard, developed and implemented new PTs in February 2019 dedicated to the detection of *Cronobacter* spp. in milk powder, following interest expressed in response to a survey circulated by BIPEA [11]. *Salmonella* spp. detection in the same samples was added to the tests in June 2023 to address laboratory demand. These tests are now organized on a regular annual basis, with two rounds per year, and are organized under ISO/IEC 17043:2023 accreditation; the statistical methods applied include general principles described in the standard ISO 13528:2022, as well as specific considerations for qualitative microbiological tests in the standard ISO 22117:2019 [12, 13].

Participation in these proficiency tests allows laboratories to detect and correct analytical problems, to demonstrate their performance for these analyses, and to compare results obtained by different protocols for the detection of these pathogens under operating conditions using real matrices. However, it is critical to note that PT participation should not be considered a discrete action, but one part of a broader quality strategy to ensure the validity of test results, which can include calibration records, duplicate testing, and positive and negative controls.

Methods

Proficiency tests involve the analysis by different laboratories of the same analytical parameters on identical samples. The implementation of a PT can be summarized in three principal steps: preparation of homogeneous and stable samples, analysis by participating laboratories, and statistical treatment of the data, which includes determination of assigned values and evaluation of laboratory performances. These steps are summarized visually as a flow chart in Fig. 1.

The first PT trial for *Cronobacter* spp. detection in milk powder, in 2019, gathered nine participants, and participation has steadily increased in the intervening years (Fig. 2) to reflect increased interest in the detection of these pathogens in food products, particularly milk powder infant formulas. Regular commission meetings are organized by

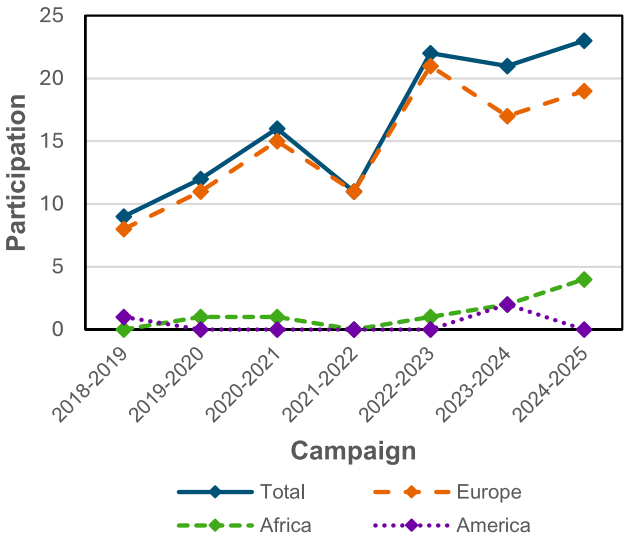

Fig. 2 Evolution of participation since implementation of the proficiency testing program, divided by geographic region

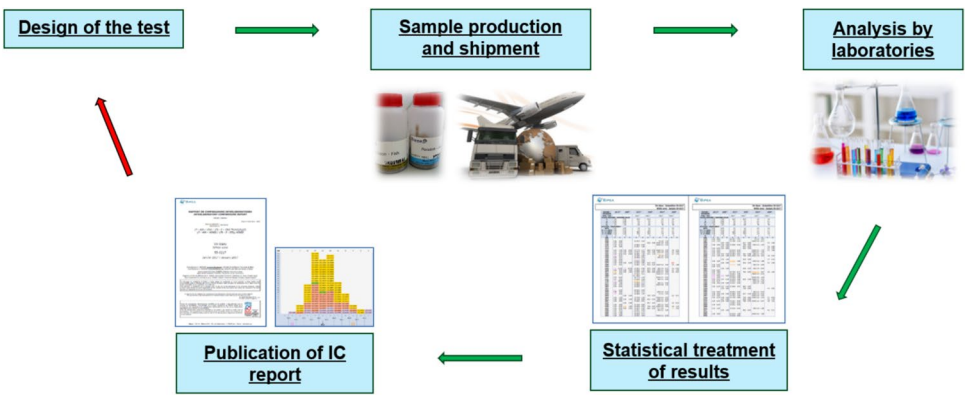

Fig. 1 Flow chart describing the main stages of PT program organization. The cyclical nature is highlighted, as results and participant feedback are used for continuous redesign

BIPEA to allow participants to discuss past results and potential technical evolutions for the program.

Design

For each trial of this program, each laboratory receives both positive (contaminated) and negative (uncontaminated) samples, according to a contamination scheme unknown to them. These samples are ready for analysis as received; laboratories do not contaminate the samples themselves. The objective of the trial is to correctly detect or not detect the target pathogens in each of these samples.

Sample production and shipment

For proficiency tests to be effective, it is crucial that homogeneous and stable samples be produced. For this PT, a batch of milk powder is first analyzed to detect the possible presence of pathogens, before being contaminated with calibrated suspensions of *Cronobacter sakazakii* and/or *Salmonella* Enteritidis. The *Cronobacter sakazakii* is a collection strain isolated from milk powder, while the *Salmonella* Enteritidis is a wild strain. The target initial concentrations at this stage immediately after contamination are 10^2–10^4 CFU (colony-forming units) per sample for each microorganism. This is to ensure that final concentrations upon analysis, which can decrease significantly due to the dehydration step and in response to bacterial stress, remain significantly superior to each method's LOD (Limit of Detection): 1.1 CFU/sample for ISO 22964 and between 2.2 and 6.0 CFU/sample for ISO 6579–1. For proficiency testing, as opposed to method validation, it is important that a laboratory correctly performing the analysis conclude correctly for each sample and be scored based on their performance, rather than on the characteristics of the sample, and concentrations close to the LOD could allow participants to incorrectly identify positive samples despite adequate application of the method. The batch is then homogenized and divided into a series of samples of 60g each. While both target pathogens are frequently present in the same samples, there is currently no contamination with non-target organisms; this is a possible future evolution for these tests.

To demonstrate stability, a batch of samples was produced and stored at room temperature, and a different set of three of these samples were analyzed for the two target microorganisms after zero, four, eleven and fourteen days; detection of both *Cronobacter* spp. and *Salmonella* spp. in each of these analyses confirmed the stability of the samples for the course of the test period. Moreover, for positive samples, the homogeneity of the batch is additionally verified by an experimental study on ten samples, taken randomly across the batch and analyzed in random order by an accredited subcontracting laboratory, according to the requirements of ISO 13528. Detection analyses of *Cronobacter* spp. and *Salmonella* spp. are conducted using the reference methods ISO 22964 and ISO 6579–1, respectively, and the set of samples is considered homogeneous if the microorganisms are detected in all analyzed samples. Qualitative measurements are favored for homogeneity and stability testing as no validated quantitative method for the analysis of *Cronobacter* spp. currently exists.

Three samples are then shipped to each participating laboratory at room temperature. Potential transport effects, which can be divided into transport time and transport temperature, are surveilled through examination of participant results, and any link between these factors and deviations from the expected conclusions can be studied. No such effect has been identified.

Analysis by laboratories

Laboratories can perform the analyses using either the reference methods or alternative methods and are additionally asked to indicate the date of analysis. They then submit their results to BIPEA via online reply forms. The reply forms also recommend a storage temperature of 4 °C and instruct participants to treat the samples for proficiency testing in the same manner as those they usually process.

Considering the inherently unstable nature of microbiological samples, participants are recommended to analyze the samples as soon as possible after reception, although they are allowed three weeks from the shipment date to complete and submit their reply forms.

Statistical treatment

The results from these tests are qualitative (detected/not detected), and the assigned value for each parameter is therefore a known value determined by the production process of the samples, as defined by ISO/IEC 17043. This leads to an evaluation of results as follows:

If the target microorganism is detected when the sample was contaminated with the strain, the result is satisfactory.

If the target microorganism is not detected when the sample was not contaminated with the strain, the result is satisfactory.

If a false negative or a false positive is obtained, the result is considered unacceptable and should be interpreted as an action signal.

In addition, BIPEA has chosen to present an overall assessment of each laboratory's ability to correctly identify negative and positive samples by calculating relative specificity (r_{SP}), relative sensitivity (r_{SE}), and relative accuracy (r_{AC}) as described in the ISO 22117 standard, defined as follows:

r_{SP} (%): Number of true negatives divided by the total number of expected negative samples. Relative specificity measures a laboratory's ability to correctly identify samples as being free of the target microorganism.

r_{SE} (%): Number of true positives divided by the total number of expected positive samples. Relative sensitivity measures a laboratory's ability to detect the target microorganism when it is present.

r_{AC} (%): Number of true results divided by the total number of samples. Relative accuracy measures a laboratory's overall ability to correctly conclude on the presence or absence of the target microorganism.

For this PT, each laboratory's overall performance is considered acceptable if their relative specificity and relative sensitivity are 100%, and therefore if their relative accuracy is 100% as well.

Results and discussion

Since January 2020, BIPEA has organized two regular proficiency tests per year for these analyses. The results of the four most recent tests, since *Salmonella* spp. was added to the samples, are summarized in Table 1 (*Cronobacter* spp. detection) and Table 2 (*Salmonella* spp. detection). For each of the trials presented here, the homogeneity tests detected the relevant pathogens in all analyzed positive samples. It should be noted that discrepancies in the number of results received for different samples of the same trial are possible, as laboratories are under no obligation to analyze all three samples they receive.

Approximately 75% of laboratories used the reference method ISO 22964 [7] for *Cronobacter* spp. detection and approximately 50% of laboratories used the reference method ISO 6579–1 [8] for *Salmonella* spp. detection. For both pathogens, results are generally very satisfactory: for 16 of the 24 sets of samples studied here, all laboratories concluded correctly, and for each of the remaining samples only between 5 and 15% of laboratories reported results that deviated from the expected conclusion. In addition, performance in these tests has remained relatively stable over time, demonstrating that most laboratories master these detection analyses. It is also important to note that the rates of false positives are either similar, in the case of *Salmonella* spp., or greater, in the case of *Cronobacter* spp., than the rates of false negatives. This is reassuring, as the consequences of

Table 1 Summary of *Cronobacter* spp. detection results for four trials. Unacceptable results are indicated in italics

		Sample 1	Sample 2	Sample 3
Trial 1	Contamination scheme	*Not spiked*	*Spiked*	*Spiked*
	Laboratory results	Detected: 0 Not detected: 21	Detected: 21 Not detected: 0	Detected: 21 Not detected: 0
Trial 2	Contamination scheme	*Spiked*	*Not spiked*	*Not spiked*
	Laboratory results	Detected: 19 Not detected: 0	Detected: 0 Not detected: 18	Detected: 0 Not detected: 19
Trial 3	Contamination scheme	*Not spiked*	*Not spiked*	*Spiked*
	Laboratory results	Detected: *1* Not detected: 19	Detected: *2* Not detected: 18	Detected: 17 Not detected: *3*
Trial 4	Contamination scheme	*Spiked*	*Not spiked*	*Not spiked*
	Laboratory results	Detected: 20 Not detected: 0	Detected: *1* Not detected: 17	Detected: 0 Not detected: 19

Table 2 Summary of *Salmonella* spp. detection results for four trials. Unacceptable results are indicated in italics

		Sample 1	Sample 2	Sample 3
Trial 1	Contamination scheme	*Not spiked*	*Spiked*	*Not spiked*
	Laboratory results	Detected: 0 Not detected: 6	Detected: 6 Not detected: 0	Detected: 0 Not detected: 6
Trial 2	Contamination scheme	*Spiked*	*Spiked*	*Not spiked*
	Laboratory results	Detected: 15 Not detected: 0	Detected: 14 Not detected: 0	Detected: *1* Not detected: 14
Trial 3	Contamination scheme	*Not spiked*	*Not spiked*	*Spiked*
	Laboratory results	Detected: 0 Not detected: 15	Detected: *1* Not detected: 14	Detected: 14 Not detected: *1*
Trial 4	Contamination scheme	*Spiked*	*Spiked*	*Not spiked*
	Laboratory results	Detected: 16 Not detected: *1*	Detected: 18 Not detected: 0	Detected: 0 Not detected: 18

false positives are primarily economic, such as unnecessary product recalls, while false negatives can lead to outbreaks and have serious impacts on public health, including the death of contaminated persons.

In recent years, several propositions have been published for numerical scoring systems, designed to allow for easy interpretation of participant performances, for qualitative proficiency testing data. The objective of these systems, which include the L-score [14], the a-score [15], and the S-score [16], is to mimic the widely accepted z-score used for quantitative data and give participants a simpler way to evaluate their results.

Each of these systems is a useful contribution to the assessment of qualitative PT data, making it easier to compare between tests and examine laboratory performance over time. However, each also has certain limitations. The L-score requires at least 10 participants, five different parameters where failure has been recorded, and specific statistical modeling software; in addition, it is fundamentally a relative evaluation rather than an absolute one, as the most satisfactory scores are impossible for a laboratory to achieve unless other laboratories perform poorly. For this reason, identical results can be judged differently on different tests, making continued assessment over time complicated. The a-score remedies several of these difficulties but needs a minimum of 20 participants to be implemented. The S-score removes this barrier but uses a more complex system that requires PT providers to define a priori the difficulty of each analysis, which leads to numerical scores with less transparent interpretations when compared with the simplicity of the z-score. Replicates are also necessary in some cases.

BIPEA applies the specificity and sensitivity criteria indicated in ISO 22117 and considers the use of relative specificity, sensitivity, and accuracy to be the system best adapted to evaluating laboratory performance for its qualitative proficiency tests. The relative accuracy is adapted from the standard ISO 16140–2 to provide an easy-to-interpret assessment of the overall ability of the laboratory to complete the analyses studied, while relative specificity and sensitivity allow the essential distinction to be made between difficulty detecting positive samples and incorrectly identifying negative samples, which are errors necessitating significantly different corrective actions—just as large positive and large negative z-scores clearly indicate different kinds of analytical problems. These three indicators can be calculated for a given laboratory regardless of the overall number of participants or analyses and are calculated independently for each parameter so as to provide an absolute evaluation of laboratory competence to perform the detection analysis in question. It is also simple to calculate these rates. In addition, if a laboratory participates in multiple rounds of such a PT, it is straightforward enough to monitor evolution in performance by graphing relative accuracy against time, as described by Chabirand et al. [17]. Figure 3 provides an example of such a graph, monitoring the relative accuracy for *Cronobacter* spp. detection of the nine laboratories that participated in all four trials presented here.

By examining in detail the results of the four trials previously presented for the detection of *Cronobacter* spp. (Table 3) and *Salmonella* spp. (Table 4) in milk powder, each laboratory's global performance can be evaluated using these assessment parameters. For each pathogen, all but three laboratories achieved relative accuracy scores of 100%, and therefore 100% relative specificity and sensitivity as well. The overall performance on these tests can thus be considered highly satisfactory. Participants in this program are provided with their relative specificity, sensitivity, and accuracy for each trial for which they submit results and can easily calculate these three scores over a period of multiple trials if desired, as demonstrated here.

If one of the primary goals of proficiency testing is to enable laboratories to demonstrate their competence for given analyses, there is a final factor to be considered. While it is clear that a laboratory with 100% relative accuracy has demonstrated greater ability than one with 33% relative accuracy and that a laboratory that consistently achieves scores of 100% masters the analyses to a greater extent than one that oscillates between scores of 100% and 50%, frequency of participation must also be taken into account. For example, by studying multiple trials collectively as in Tables 3 and 4, it is possible to observe for each participant not only the rates of relative specificity, sensitivity, and accuracy, but also a final rate, the rate of participation (r_P), which corresponds to the number of samples analyzed divided by the total number of samples proposed over a given time period. For an analytical laboratory, achieving a relative accuracy of 100% while participating in all four trials can be a way to signal greater expertise than obtaining the same rate while

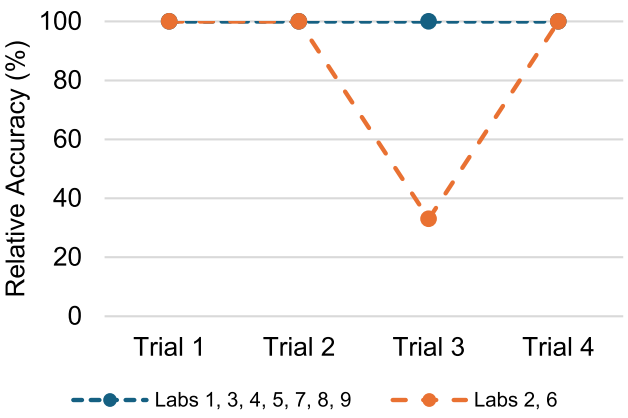

Fig. 3 Relative accuracy (r_{AC}) for *Cronobacter* spp. detection over time for the nine laboratories that participated in the four trials presented here

Table 3 Detailed results of four trials for the detection of *Cronobacter* spp. in milk powder, including evaluation and rate of participation (r_P) for each laboratory

	Trial 1			Trial 2			Trial 3			Trial 4			Evaluation			
Lab	0	1	1	1	0	0	0	0	1	1	0	0	r_{SP} (%)	r_{SE} (%)	r_{AC} (%)	r_P (%)
1	0	1	1	1	0	0	0	0	1	1	0	0	100	100	100	100
2	0	1	1	1	0	0	0	*1*	*0*	1	0	0	86	80	83	100
3	0	1	1	1	0	0	0	0	1	1	0	0	100	100	100	100
4	0	1	1	1	0	0	0	0	1	1	0	0	100	100	100	100
5	0	1	1	1	0	0	0	0	1	1	0	0	100	100	100	100
6	0	1	1	1	0	0	*1*	0	*0*	1	0	0	86	80	83	100
7	0	1	1	1	0	0	0	0	1	1	0	0	100	100	100	100
8	0	1	1	1	0	0	0	0	1	1	0	0	100	100	100	100
9	0	1	1	1	0	0	0	0	1	1	0	0	100	100	100	100
10	0	1	1	1	0	0	0	0	1	1		0	100	100	100	92
11	0	1	1	1	0	0	0	0	1	1			100	100	100	83
12	0	1	1	1	0	0	0	0	1				100	100	100	75
13				1	0	0	0	0	1	1	0	0	100	100	100	75
14				1	0	0	0	0	1	1	0	0	100	100	100	75
15				1	0	0	0	0	1	1	0	0	100	100	100	75
16				1	0	0	0	0	1	1	0	0	100	100	100	75
17				1	0	0	0	*1*	*0*	1	*1*	0	67	67	67	75
18	0	1	1	1		0	0	0	1				100	100	100	67
19	0	1	1							1	0	0	100	100	100	50
20	0	1	1							1	0	0	100	100	100	50
21				1	0	0	0	0	1				100	100	100	50
22	0	1	1										100	100	100	25
23	0	1	1										100	100	100	25
24	0	1	1										100	100	100	25
25	0	1	1										100	100	100	25
26	0	1	1										100	100	100	25
27	0	1	1										100	100	100	25
28							0	0	1				100	100	100	25
29										1	0	0	100	100	100	25
30										1	0	0	100	100	100	25

The contamination scheme is displayed in the table header, 0 and 1 correspond to "Non-detected" and "Detected," respectively, and unacceptable results are indicated in italics

participating in a single test, and such performance can be extremely valuable for earning and maintaining consumer trust.

Conclusion

Outbreaks involving *Cronobacter* spp. and *Salmonella* spp. in milk powder or infant formulas have been reported in many countries and can have enormous consequences, including serious illness, hospitalization, and death, and the global nature of food supply chains can allow contamination to easily propagate nationally and internationally. The health risks associated with non-detection of contaminated samples of milk powder are particularly high because the principal consumers are infants, who have underdeveloped immune systems. Furthermore, the rates of reported *Cronobacter* spp. infections in infants have risen significantly in recent decades, although it is not clear whether this is due to a true increase in cases or simply reflects increased awareness and interest [18].

The proficiency tests presented here have been developed to provide laboratories with a quality tool to assess their ability to detect these pathogens in milk powder. The PTs are offered regularly, with two rounds per year, and the performances of participating laboratories are highly satisfactory, with relative accuracy equal to 100% for both pathogens for most laboratories. When laboratories do obtain unsatisfactory results, they are encouraged to contact BIPEA to try to collectively consider possible causes for the deviation. While a proficiency testing provider cannot easily identify specific errors in the application of an

Table 4 Detailed results of four trials for the detection of *Salmonella* spp. in milk powder, including evaluation and rate of participation (r_P) for each laboratory

	Trial 1			Trial 2			Trial 3			Trial 4			Evaluation			
Lab	0	1	0	1	1	0	0	0	1	1	1	0	r_{SP} (%)	r_{SE} (%)	r_{AC} (%)	r_P (%)
1	0	1	0	1	1	0	0	0	1	1	1	0	100	100	100	100
2	0	1	0	1	1	0	0	0	1		1	0	100	100	100	92
3				1	1	0	0	*1*	*0*	1	1	0	75	80	78	75
4				1	1	*1*	0	0	1	1	1	0	75	100	89	75
5	0	1	0	1	1	0				1	1	0	100	100	100	75
6				1	1	0	0	0	1	1	1	0	100	100	100	75
7				1	1	0	0	0	1	1	1	0	100	100	100	75
8				1	1	0	0	0	1	1	1	0	100	100	100	75
9				1	1	0	0	0	1	1	1	0	100	100	100	75
10				1	1	0	0	0	1	1	1	0	100	100	100	75
11				1	1	0	0	0	1	1	1	0	100	100	100	75
12				1	1	0	0	0	1	1	1	0	100	100	100	75
13	0	1	0	1		0	0	0	1				100	100	100	67
14				1	1	0				1	1	0	100	100	100	50
15	0	1	0							1	1	0	100	100	100	50
16				1	1	0	0	0	1				100	100	100	50
17										*0*	1	0	100	50	67	25
18	0	1	0										100	100	100	25
19							0	0	1				100	100	100	25
20							0	0	1				100	100	100	25
21										1	1	0	100	100	100	25
22										1	1	0	100	100	100	25
23										1	1	0	100	100	100	25

The contamination scheme is displayed in the table header, 0 and 1 correspond to "Non-detected" and "Detected," respectively, and unacceptable results are indicated in italics

analytical method by a participant, having access to large amounts of testing data can allow potential sources of error to be suggested, including storage conditions, choice of method, temperature of analysis, and delay before analysis. As the results are generally very good, there may also be interest in BIPEA increasing the difficulty of the tests so that laboratories can further evaluate the capacity of their analyses. This could include decreasing the inoculation levels and adding non-target organisms to the samples; such modifications will be discussed with participating laboratories. In addition, samples that are contaminated by batch and homogenized may not reflect the distribution of pathogens in naturally contaminated samples and therefore do not test laboratories on all aspects of the sampling process. BIPEA is currently preparing for a PT program for in situ sampling in the food microbiology sector, which will allow participants to evaluate this vital skill.

By participating in proficiency testing, laboratories can verify the reliability and stability of their results, as well as obtain recognition of their analytical procedures by customers and accreditation bodies according to ISO/IEC 17025. These initial results are encouraging and reassuring for consumers and public organizations, as an indicator that laboratories master these essential detection analyses.

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Author contributions Conceptualization was done by Colin Howell and Abdelkader Boubetra; methodology was done by Anne Tirard and Abdelkader Boubetra; formal analysis and investigation were done by Anne Tirard and Sandrine Nguyen; writing—original draft was done by Colin Howell; writing—review and editing was done by Anne Tirard, Sandrine Nguyen, and Abdelkader Boubetra; project administration was done by Colin Howell; and supervision was done by Romain Le Neve and Abdelkader Boubetra.

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Data availability Data will be made available upon request, as will examples of reply forms and proficiency testing reports.

Declarations

Conflict of interest All authors certify that they have no competing interests to declare that are relevant to the content of the article.

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