The Seventh
International Proficiency Testing Conference

Oradea, Romania
10th – 13th September 2019

A VIRTUAL PROFICIENCY TESTING IN FOOD MICROBIOLOGY

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Abstract

REQUASUD has been organizing food microbiology proficiency testing (PT) since 1989. Traditional PT provide few information on the causes of unsatisfactory results, as the analytical process in the laboratories is a “black box” whose details are not transmitted to the PT provider. To identify more precisely the causes of false analytical results, an *in silico* PT scheme was organized in February 2019, involving 16 laboratories from Belgium and Luxembourg. In this virtual PT, pictures of Petri dishes and confirmation tubes, obtained from real analyses, were sent to the laboratories to assess the post-analytical steps.

For this virtual PT, REQUASUD selected analytical methods used by most participants in routine: enumeration of total aerobic flora, lactic bacteria and *Clostridium perfringens*.

The laboratories were asked to return the following results:
- Number of colonies counted on each Petri dish picture;
- Interpretation of the confirmation test, based on the pictures of five tubes;
- Calculation of the final result;
- Assessment of the conformity of the sample with respect to legislation.

In each of the 16 laboratories, one to three operators took part to the exercise, which enabled the PT organizer to assess intra- and inter-laboratory variability.

The results enabled to assess the contribution of four post-analytical steps (enumeration, confirmation, calculation and conclusions on conformity) to the global measurement uncertainty of the food microbiological analyses. For these steps, some

differences in practices between laboratories were highlighted, which can result in additionnal variability of the final analytical results.

This study also showed the usefulness of virtual PT schemes, which represent a privileged observation post to highlight and quantify individual sources of errors during the analytical and post-analytical process.

Keywords

Virtual Proficiency Testing, Food Microbiology, Sources of errors, Enumeration, Confirmation, Calculation

1 INTRODUCTION

REQUASUD has been organizing proficiency testing (PT) since 1989, in five analytical fields: minerals in soils or foodstuffs, near infra-red spectroscopy, nitrates in soils and food microbiology. It is a policy of REQUASUD to distribute PT samples as close as possible to real-life samples (e.g. naturally contaminated food samples). In these PT, the samples are sent to 5 to 26 laboratories, which return their results to REQUASUD for the evaluation of their analytical performances. However, these traditional PT provide limited information on the potential causes of unsatisfactory results, as the details of the analytical processes are not transmitted to the PT provider.

A virtual PT consists in sending *in silico* items (e.g. pictures, numbers) to the participant laboratories, instead of real samples. Although not applicable to any field of activity, virtual PT display numerous advantages: i) no transportation of the samples, as the virtual PT items are generally sent online; ii) no stability or homogeneity issue; iii) reduced costs; and iv) no limitations of the number of participants. Another advantage of virtual PT is the possibility to focus on a specific step of the analysis, *e.g*. assess only the counting or the calculation step.

Virtual PT have successfully been organized in the clinical field, for instance to assess the ability of laboratories to differentiate blood cells on the basis of images of microscope slides [1] but, to our knowledge, no true and complete virtual PT has been described in the field of food microbiology.

2 OBJECTIVES

Since 1989, the organisation of inter-laboratory comparisons enabled REQUASUD to highlight several issues linked to food microbiological analyses [2]. However, the information about the precise origin of these errors remained inaccessible with the traditional design of PT.

The purpose of this virtual PT was to quantify the contribution of four post-analytical steps (colony counting, confirmation test, calculation and interpretation of the results) on the total error of analytical results. The influence of all the other steps of the analyses (*e.g*. sample preparation, dilution, incubation) on the results was discarded.

3 EXPERIMENTAL

3.1 Methods

A virtual PT scheme was organized by REQUASUD in February 2019, involving 16 laboratories from Belgium and Luxembourg. For this *in silico* PT, pictures of Petri dishes and confirmation tubes, obtained from real analyses, were sent to the laboratories to assess the post-analytical steps.

Analytical methods used by most participants in routine were selected, to ensure that the laboratories were familiar with the culture media and the characteristics of the expected colonies. Three enumeration analyses were proposed in this first virtual PT: total aerobic flora on Plate Count Agar (PCA), lactic acid bacteria on Man-Rogosa-Sharpe agar (MRS) and *Clostridium perfringens* on Tryptose Sulphite Cycloserine agar (TSC) with confirmation in Lactose-Sulphite tubes (LS).

3.2 Materials

Good-quality pictures of Petri dishes and tubes, obtained from real analyses, were taken. The optical conditions (light intensity and white, grey or black backgrounds) were optimized to reduce light reflection and enable appropriate contrast of the bacterial colonies.

An Excel form, containing real-size pictures, was sent to the participants. The laboratories were asked to submit this file to one to three operators and to return, within the 2 weeks, the following results for each operator:
- The number of characteristic colonies counted on each Petri dish picture;
- The interpretation of the confirmation test, based on the pictures of the tubes, for the presence of gas and precipitate;
- The calculation of the final result;
- An assessment of the sample conformity with respect to legislation.

The participants were asked to enumerate the colonies directly on the screen, to avoid any additional variability linked to printing quality in the different laboratories. They were also asked to analyse the pictures at their 100 % size, without zooming, to remain close to real-life conditions.

4 RESULTS

Some technical hurdles were linked with the use of pictures on a computer instead of real Petri dishes. Indeed, the operators are not used to enumerate the colonies on a screen and they do not have the possibility to move the Petri dishes, to better count the colonies on the edges, as they do in routine.

The results of the enumeration, confirmation, calculation and conformity assessment steps are presented separately below.

4.1 Enumeration of the colonies

The variability of the enumeration results among operators was assessed for each analytical parameter. A representative result is illustrated in Fig. 1.

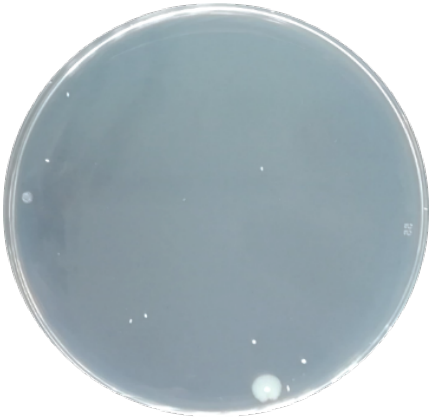

Fig. 1. A picture sent to the participants: Petri dish for the enumeration of total flora at the 10^{-5} dilution.

On average, the operators enumerated 11 colonies on this picture. The results of enumeration returned by each operator are presented in Fig. 2.

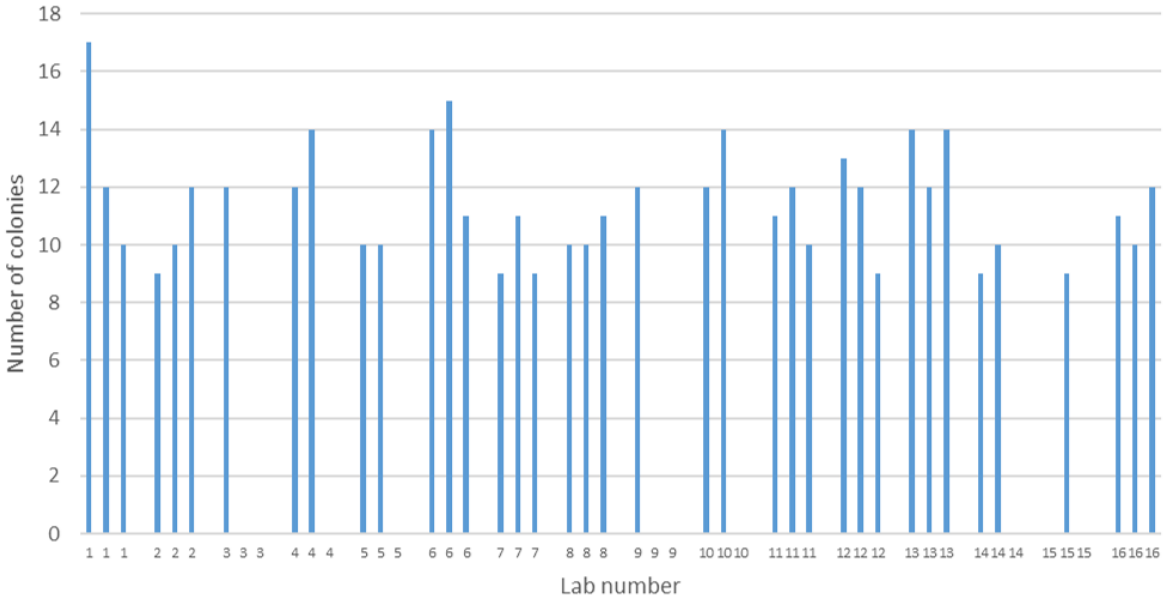

Fig. 2. Enumeration of colonies on the Petri dish displayed in Fig.1 by 16 laboratories and from one to three operators per laboratory.

We observed variability in the colony counts between laboratories (inter-laboratory variability) but also among operators (intra-laboratory variability). In the example presented above, the counts for the same picture ranged from 9 to 17 colonies. The standard deviations (SD) associated with the enumeration step, for the three different analyses, are summarized in Table 1.

Table 1. Average relative SD observed in the enumeration step for the three analyses.

	Total flora	Lactic bacteria	*Cl. perfringens*
Intra-laboratory s_R (%)	7,98	11,76	4,39
Inter-laboratory s_R (%)	10,53	13,06	4,39

For two of the three analyses, the variability of the enumeration results among operators turned out to be surprisingly high. The exception is the enumeration of *Cl. perfringens*, as the characteristic colonies form large black spots easy to distinguish.

This basic step of colony counting turned out to contribute greatly to the total error of the analytical result. This observation lends support to the postulate of Augustin and Carlier [2] that the step of colony counting contributes significantly to the measurement uncertainty in food microbiology.

4.2 Interpretation of the confirmation test

The participants received five pictures of lactose-sulphite (LS) tubes, for the confirmation of *Cl. perfringens* (Fig. 3).

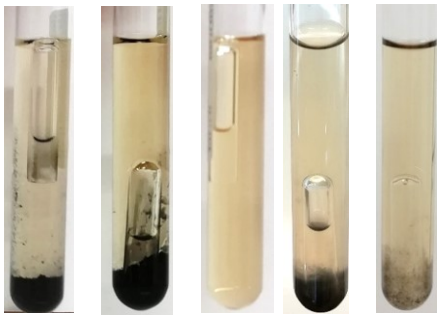

Fig. 3. The pictures of the five LS tubes, sent to the participants.

Each tube was interpreted as positive or negative by the operators, on the basis of the amount of gas (the minimum to be deemed positive is a quarter of the bell) and the presence of a black precipitate in each tube. The results are presented in Fig. 4.

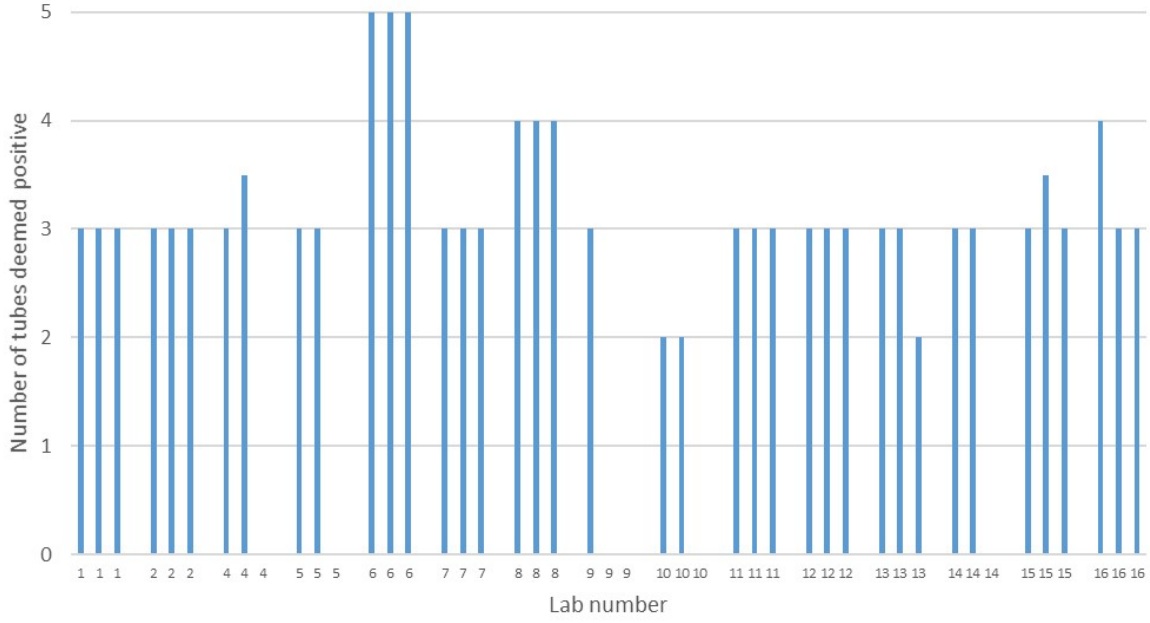

Fig. 4. Interpretation of the five confirmation tubes (LS).

The standard deviations linked to the confirmation step are summarized in Table 2. Globally, inside a same laboratory, the operators follow coherent decision rules. However, between laboratories, the instructions seem to differ greatly, as the number of tubes (out of five tubes) deemed positive varies from two to five, leading to an inter-laboratory SD of 21,82 % for the reading of the confirmation test.

Table 2. Relative standard deviations obtained for the LS confirmation.

	Confirmation (LS)
Intra-laboratory s_R (%)	8,12
Inter-laboratory s_R (%)	21,82

4.3 Calculation and final result

The final results were calculated by the participants, based on the enumeration result of each Petri dish and, for *Cl. perfringens*, also on the number of positive tubes for the confirmation.

An example of the variability of the final results is shown in Fig. 5.

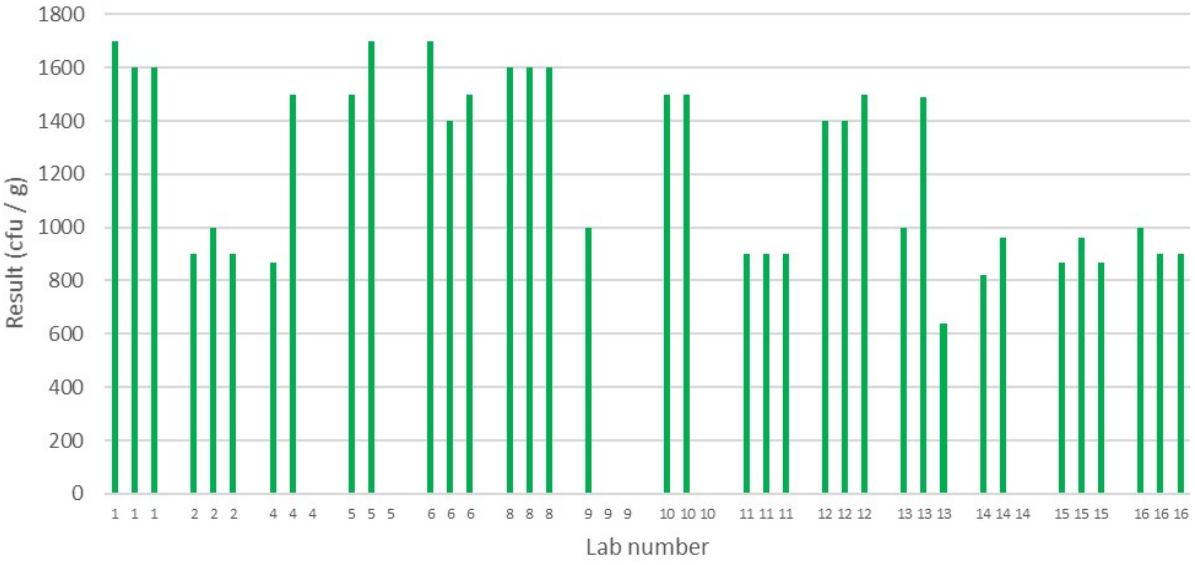

Fig. 5. Enumeration results of *Cl. perfringens*, expressed in Colony-Forming Units (CFU) per gram of sample.

The standard deviations of the final results are summarized in Table 3. The global variability of the final results can be considered as the consequence of the differences in counting, test interpretation and calculation.

Table 3. Average relative standard deviations of the final results for the 3 analyses.

	Total flora	**Lactic bacteria**	***Cl. perfringens***
Intra-laboratory s_R (%)	22,86	8,05	14,26

Inter-laboratory s_R (%)	38,17	8,34	27,99

4.4 Conformity assessment

Based on their analytical results, the laboratories were asked to conclude on the conformity or non-conformity of the sample, with respect to the regulatory limits (Table 4). The sample was described by the PT organizer as a “rice and shrimp salad”.

Table 4. Range of results returned by the participants; legal criteria used for interpretation; and conclusion on the sample conformity for the three analyses.

	Range (CFU/g)	**Criteria (CFU/g)**	**Conformity**
Total flora	220 000 - 580 000	1 000 000	95%
Lactic bacteria	240 000 - 380 000	1 000 000	100%
Cl. perfringens	900 - 1700	1000	50%

The conformity assessment was very coherent among the laboratories. The only exception was for the *Cl. perfringens* parameter, which could be interpreted as conform or not-conform in function of the result calculated by the operator.

5 DISCUSSION

5.1 Differences in practices

The results of this first virtual PT organized by REQUASUD were discussed with the participants, for educational purposes. A first observation is that the practices for colony-enumeration and interpretation of confirmation tests vary between the laboratories. Globally, the operators inside a same laboratory sometimes apply different rules for the enumeration of the colonies, and the laboratories have individual rules for the interpretation of the lactose-sulphite test.

Different practices were identified for the following steps:

- For total flora, some operators enumerate all the colonies, while others consider a Petri dish uncountable (“> 300 colonies”) if the next decimal dilution contains more than 30 colonies. This decision rule induced a high variability of the enumeration and final results in the present virtual PT.

- The lactic bacteria colonies can be either lenticular, or circular if they grow on the bottom of the plate. Some operators enumerated only the lenticular colonies, yielding significantly lower results than the others. Also, some operators counted only the large colonies, while others took also the tiny colonies into account.

- For *Cl. perfringens*, when a Petri dish is completely blackened by the colonies, most laboratories do not count it but some laboratories place those dishes one night in the fridge to remove the black halo and count the colonies.

- For the confirmation of *Cl. perfringens*, when a LS tube is doubtful (no precipitate or little gas), some laboratories consider it negative, whereas others consider it positive or re-incubate it for 24 hours.

All these differences in practices result in a high variability of the final analytical results, as shown with this virtual PT.

5.2 Contribution of each step to the global standard deviation

Considering that the four steps of the post-analytical process can introduce variability in the final result, the main purpose of this virtual PT was to quantify the contribution of each of these steps to the measurement uncertainty. Table 5 summarizes the average standard deviations observed when comparing (all analytical parameters together) the results of the colony counts, confirmation test and final result.

Table 5. Average variability (quadratic mean of the relative SD) observed at each post-analytical step.

Step	s_R intralab (%)	s_R interlab (%)
Colony counting	9,72	11,37
Confirmation test	8,12	21,82
Final result	16,24	27,75

The variation in the colony count results seems to be mainly linked to the operator, as the intra-laboratory variability (9,72 %) is almost as high as the inter-laboratory variability (11,37 %). On the other hand, the interpretation of the confirmation test (LS tubes) is highly dependent on the laboratory instructions, as we observe 21,82 % of variability between the laboratories.

Eventually, the variability of the final results can be seen as the conjunction of the variability linked to the enumeration, confirmation and calculation steps. The contribution of the latter (s_R due to calculation) could not be quantified with the design of this virtual PT.

5.3 Contribution of the calculation step

The calculation of the final result is a critical step where the laboratories can have different practices: the formula used and the selection of the dilutions to retain for the calculation can have a huge impact on the final result. Therefore, to better assess the contribution of the calculation step to the global error of the result, a specific virtual PT will be conducted by REQUASUD in 2022, in which the results of colony counts will be provided and the participants will only have to calculate the final results.

5.4 Conformity statements

Despite the high variability of some final results, at the end, no big differences in the conformity statements provided by the laboratories were noticed. The results variability had only an impact on the conformity statement for the parameter *Cl. perfringens*, for which half of the laboratories concluded that the sample conformed to

the microbiological criteria, whereas the other laboratories deemed the sample non-conform.

The very clear instructions provided by the Belgian Federal Agency for the Safety of the Food Chain (FASFC) can explain these very coherent results: the legal criteria for food microbiology are compiled in a detailed Excel table called “Action limits for the microbiological contaminants in foodstuffs” which is transmitted by FASFC to the Belgian laboratories.

6 CONCLUSIONS

This virtual PT enabled to quantify the individual contribution of four post-analytical steps to the global measurement uncertainty of the food microbiology analyses. This trial demonstrated that the enumeration, interpretation and calculation errors contribute to the analytical uncertainty, whereas the statements of conformity were globally harmonized among the 16 participating laboratories from Belgium and Luxemburg.

This study shows the usefulness of virtual PT schemes, which represent a privileged observation post to highlight and quantify the individual sources of errors during the analytical and post-analytical process.

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