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PRACTITIONER'S REPORT

Enumeration of lactic acid bacteria: lacuna and improvement areas highlighted by proficiency testing

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Abstract
During the 2011 to 2018 food microbiology proficiency testing (PT) schemes organized by REQUASUD, a systematic analytical issue was detected. For the enumeration of the lactic acid bacteria (LAB), laboratories carrying out the same analytical method (ISO 15214) yielded completely incoherent results while analysing naturally contaminated samples (raw milk, smoked salmon and ham sausage), leading to an atypically large distribution of the PT results. A comprehensive study of these atypical PT results provided insight into the source of systematic errors affecting the LAB enumeration. The main sources of analytical variability turned out to be linked to the ISO 15214:1998 standard. To better harmonize the analytical results, practical improvements can be proposed for the revision of this standard, mainly: the addition of precise criteria for the colony count, a restriction of the allowed incubation conditions and the use of a more selective culture medium (e.g. supplemented with sorbate) to limit the growth of interfering colonies. PT is a privileged observation post to highlight such analytical issues, which would otherwise remain unnoticed. Whenever an unusual distribution of PT results is observed (e.g. high dispersion or non-normal distribution of the values), it is the responsibility of the PT provider to try to identify the causes of non-normality, in a perspective of method improvement. The statistical analysis of atypical PT results requires specific attention: ignoring the non-normality of PT results when it occurs can lead to set an erroneous assigned value, unfairly penalizing participants.

Keywords Proficiency testing · Food microbiology · Analytical method · Lactic acid bacteria · ISO 15214 · Non-normal distribution

Introduction

The Belgian non-profit organization REQUASUD has been providing food microbiology proficiency testing (PT) since 1989, at a rate of 2 to 3 PT schemes a year. One specificity of this PT is the use of authentic, naturally contaminated food matrices as PT samples.

During the 2011 to 2018 PT schemes, involving 16 to 23 participants, one analytical parameter, the enumeration of lactic acid bacteria (LAB), raised systematic issues for the participants. Although 90 % of the laboratories were using the same analytical method, ISO 15214:1998 [1], a very high rate of unsatisfactory results was observed (21.7 %), together with an atypically large dispersion of the results. These problems were observed only in naturally contaminated food samples (raw milk, smoked salmon and ham sausage), the analysis appearing completely under control while analysing sterile and artificially inoculated samples (sterile whole milk or soy milk), as illustrated in Fig. 1.

Methods

To try to identify the causes of such variability of the analytical results, REQUASUD conducted a comprehensive study on the enumeration of LAB from 2011 to 2018. Any potential issue linked to the PT items was discarded, as the homogeneity of every sample was validated and the stability of the microbial contamination was ensured by the fact

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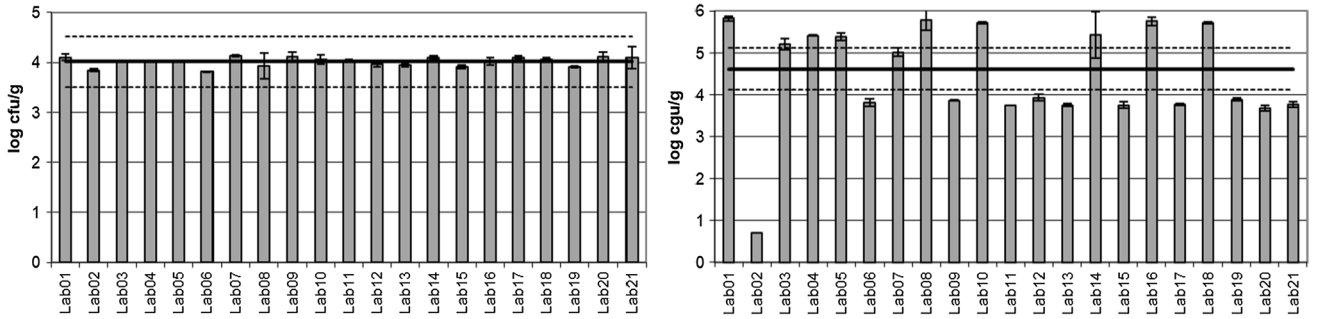

Fig. 1 Results of the enumeration of LAB in blind double samples during PT 2018-2: results obtained while analysing 2 sterile and artificially inoculated milk samples (left) versus results obtained while analysing 2 naturally and artificially contaminated salmon samples (right). The results are expressed in colony-forming units (cfu) per gram of sample. The means of the duplicate results are represented by grey bars, the assigned value by (—) and the tolerance limits by (---). The interlaboratory standard deviations (s_R) observed for the analysis of sterile milk and raw milk were 0.13 and 0.58, respectively

that all the participants analysed the PT items the same day (imposed date). Different complementary approaches were used to investigate the origin of the results incoherencies.

First, enquiries were sent to the participant laboratories, to specify their analytical method for the enumeration of LAB (protocol, pH of the culture media used, incubation temperature) and, in parallel, to ask whether these laboratories observed small interfering colonies or not and whether they included them in their counts. The laboratories were also requested to provide photographs of their petri dishes after colony counting. The comparison of the laboratories outcomes obtained with different analytical protocols enabled to identify possible origins of the problem.

Eventually, the REQUASUD laboratory conducted internal investigations to assess the influence of the analytical method on the LAB enumeration results: the different protocols were compared internally, and 26 colonies of different shapes and sizes (on MRS agar) were identified by the 16S rDNA sequencing technique to assess whether they belonged to the LAB group or should be discarded from the counts.

Results

After a first enquiry among the participating laboratories, the poor coherence between the analytical results was rapidly attributed to differences of practices in the counting of the colonies, when tiny colonies were present on MRS agar (Man, Rogosa and Sharpe agar), the culture medium recommended in ISO 15214 [1]. Some laboratories enumerated only the large colonies while others enumerated all, including the tiny ones, leading to significantly higher results. This issue was not observed while analysing artificially inoculated samples, as the inoculum yielded only one colony type (Fig. 2).

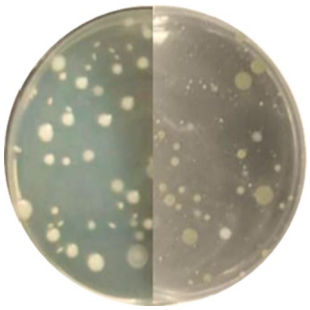

Fig. 2 Corresponding petri dishes on MRS agar (left: artificially inoculated sterile milk sample; right: naturally contaminated and inoculated raw milk sample)

During the analysis of an artificially inoculated milk, the laboratories observed easy to count colonies, presenting the same size and phenotype. Naturally contaminated samples, on the other hand, yielded various colony shapes and sizes, together with numerous interfering tiny colonies. These observations support the postulate that the colony-counting step can contribute significantly to the measurement uncertainty in food microbiology [2, 3].

Further investigations were conducted to better understand the parameters at the origin of the analytical variability observed during REQUASUD PT. It was assumed that the high dispersion of the PT results finds its origin in the fact that a wide variety of analytical protocols are used by the laboratories. Indeed, the enumeration of LAB following ISO 15214 allows not less than 8 different analytical protocols:

- MRS agar can be poured in single or double layer, with or without addition of sorbate,
- Incubation can be performed under aerobic, micro-aerobic or anaerobic conditions,

- A confirmation of the colonies can be performed by Gram or catalase test.

These differences in culture medium, incubation conditions and confirmation tests explain why some laboratories observed small interfering colonies while others did not. Moreover, ISO 15214 does not specify the size of the colonies to be counted on the petri dishes: this lack of criteria is at the origin of different enumeration practices (e.g. including or discarding the very small colonies) among the laboratories.

During PT 2016-2 and 2017-1, the small colonies occurring during the analysis of the naturally contaminated PT samples (ham sausage and smoked salmon) were identified by the 16S rDNA sequencing technique as being non-lactic bacteria. The tiny colonies identified were from various genera (*Staphylococcus*, *Escherichia*, yeasts) and should be discarded from the LAB counts.

During this same PT, a survey was carried out in collaboration with the participating laboratories, who sent to REQUASUD their observations and photographs of the MRS petri dishes with colonies.

In parallel, during PT 2017-1, the REQUASUD organizer laboratory investigated the source of variability of the ISO 15214 method. For this purpose, the analysis of the PT samples was carried out using the different protocols allowed by the standard: MRS agar was poured in single *vs* double layer, at a pH of 5.7 versus 6.2, with or without the addition of sorbate, with incubation at 30 °C versus 25 °C, under aerobic versus CO_2 incubation and finally with or without confirmation of the colonies by catalase test. The purpose of these investigations was to propose improvements for the ISO 15214:1998 standard.

The main observations of these internal tests are summarized below:

- Pouring a double layer of MRS agar enabled to limit the growth of invading mould colonies, but did not avoid the growth of small interfering colonies.
- Incubation at 25 °C instead of 30 °C only slightly reduced de spreading of interfering moulds.
- MRS pH 5.7 reduced the amount of interfering colonies, compared to pH 6.2, but moulds still grew.
- Incubation under anaerobic conditions (CO_2) prevented the growth of moulds, but the small interfering colonies were still present.
- MRS supplemented with sorbate allowed to avoid most interfering colonies and moulds.
- Catalase confirmation enabled to discard most interfering colonies which were catalase positive (the LAB being, by definition, catalase negative).

These tests show that the use of MRS at pH 5.7 supplemented with sorbate could prevent the growth of interfering colonies. Pouring a double layer or incubating under anaerobic conditions or at 25 °C only slightly limits the mould growth. Eventually, suspect colonies can be confirmed by means of a simple catalase test to discard non-lactic colonies.

Discussion—responsibility of the PT provider

The statistical analysis of atypical PT results (e.g. high dispersion or non-normal distribution of the values) requires specific attention. Whenever such unusual distribution of the PT results is observed, it is the responsibility of the PT provider to try to identify the causes of non-normality. These investigations require collecting enough data from the participants to get insight into the analytical specificities of each laboratory.

The multi-modality of some PT results, i.e. the presence of two (or more) distinct clusters of results, is problematic because the statistical analysis of food microbiological PT results is based on the hypothesis that the data follow a Gaussian distribution after log transformation [4]. Ignoring the non-normality of PT results when it occurs can lead to set an erroneous assigned value, unfairly penalizing some participants, as illustrated in Fig. 3.

In the case presented in Fig. 3, calculating two assigned values as the consensual mean of each cluster represents a more accurate description of the results, although it raises the issue of setting two different assigned values for one identical PT sample.

In case of non-normality of the PT results, resulting in several possible assigned values, the main challenge for the organizer is to determine what the "true" value is. Two scenarios can be observed.

First, one of the two clusters may turn out to be erroneous, due, for instance, to the enumeration of the wrong colony phenotype or to an incomplete application of the analytical protocol. This cluster must then simply be discarded from the determination of the assigned value, which will naturally re-centre on the correct cluster. Another way to identify the "false" cluster of results would be to have an idea of what the PT samples' "true" value is. For instance, if the level of artificial inoculation of the sample is known, the PT provider can identify which cluster of results is the closest to this theoretical assigned value and discard the other one. For naturally contaminated samples, however, it is generally impossible to determine the true value with the currently existing microbiological techniques.

The second scenario, much tougher to interpret, is the case where none of the two clusters can be identified as

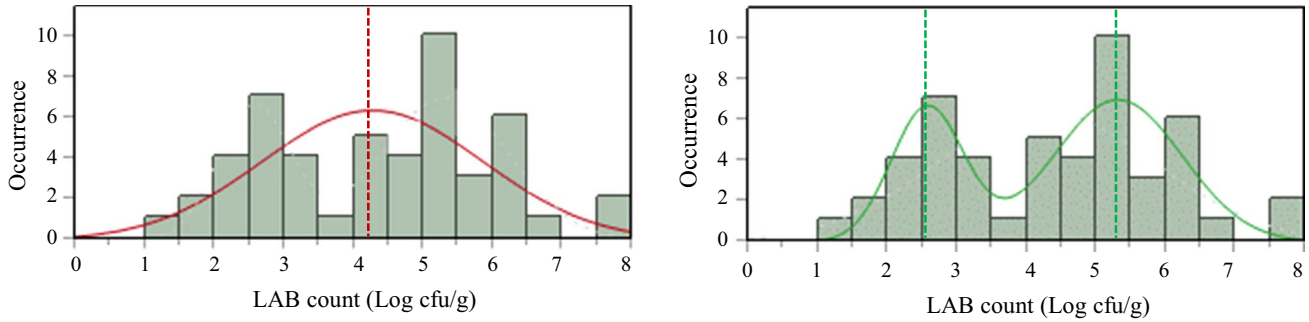

Fig. 3 Atypical distribution of PT results for the enumeration of LAB, and their possible statistical analyses. Left: the assigned value (---) is calculated as the consensual mean of the results, while erroneously assuming they follow a normal distribution (—), which can lead to a high number of outlying result. Right: the results are subdivided into two clusters following a bimodal distribution (—), and the two corresponding assigned values are represented by (---)

erroneous. This is observed, for instance, when the PT provider does not collect enough data from the laboratories to identify the feature (e.g. the pH of the culture medium) that led to two distinct groups of results. In this case, none of the two clusters can be invalidated and it is difficult to analyse these PT results in a fair and efficient way, as illustrated in Fig. 4.

None of the two solutions illustrated in Fig. 4 are totally satisfactory. Using the global mean as assigned value penalizes all the participants, except if broader tolerance limits are adopted (right graph of Fig. 4), which will then result in penalizing nobody—even the possible outliers.

Clustering the results (left graph of Fig. 4) is less penalizing for the laboratories, but it will provide two different assigned values. The question remains: which assigned value is the true one? This methodology is counterproductive, as it implies that the laboratory customers (e.g. food operators) may receive two different analytical results, which runs contrary to the harmonization purpose of PT.

Conclusions

PT is a privileged observation post to highlight analytical issues, which would otherwise remain unnoticed. During REQUASUD's 2011 to 2018 PT schemes, laboratories carrying out the same analytical method (ISO 15214) yielded completely incoherent results. A comprehensive study of these atypical PT results, in close collaboration with the participating laboratories, provided insight into the sources of systematic errors affecting the LAB enumeration.

The main source of analytical variability turned out to be linked to the ISO 15214 standard, which allows 8 different analytical protocols and does not specify criteria for the size of the colonies to be enumerated. The pertinence of having so many possible protocols in one standard, making differences in the laboratory results much likely to arise, is highly questionable.

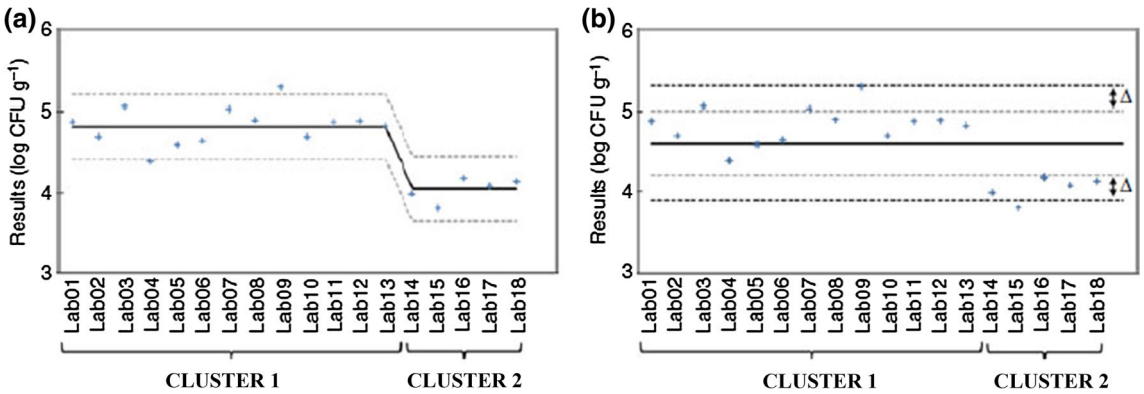

Fig. 4 Analysis of PT results when two groups of results are observed. The participants results are illustrated by (+), the assigned value by (—) and the tolerance limits by (---). In the left graph, the results are clustered and two assigned values are fixed. In the right graph, the results are analysed without clustering and the tolerance limits are extended by an additional uncertainty on the assigned value (Δ). (Reprinted and modified from Abdelmassih et al. [5])

To better harmonize the analytical results for the enumeration of LAB, practical improvements can be proposed for the revision of ISO 15214, mainly: the addition of size criteria for the colony count, the restriction of the allowed incubation conditions and the use of a more selective culture medium (e.g. MRS agar supplemented with sorbate) to limit the growth of interfering colonies.

More generally, every remarkable situation occurring during PT should be reported and investigated by the organizer, to try to identify the causes of divergences and emit suggestions for the improvement of the analyses. Atypical PT results should never be ignored by PT providers as they require at least a thorough investigation of the causes and a suitable statistical analysis.

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