Analytical issues linked to ISO 15214 and ISO 21528-2 identified through Proficiency Testing

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INTRODUCTION

REQUASUD's Food microbiology **Proficiency Testing** (PT) schemes involve up to 29 laboratories. Between 2018 and 2025, some results for the enumeration of Lactic Acid Bacteria (LAB) and *Enterobacteriaceae* (EB) on naturally contaminated food matrices raised issues: a **bimodal** distribution was observed, despite most laboratories following the same ISO methods. The **two clusters** of results were attributed to **methodological discrepancies.**

INVESTIGATION OF BIMODAL PT RESULTS

ISO 15214 (LAB enumeration) lacks guidance on **colony size**: labs counting only large colonies reported lower results than those including small colonies.

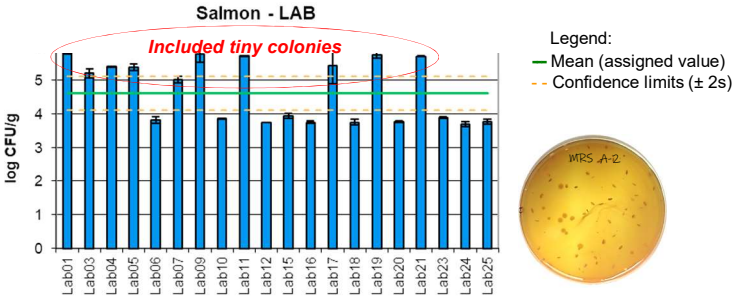

Fig 1. Results of the enumeration of LAB in blind double salmon samples (PT2018). The corresponding Petri dish on the right (salmon 10-2 on MRS) shows various colony shapes and sizes, with numerous tiny colonies that can be included or not in the count.

ISO 21582-2 (EB enumeration), as well as the EB alternative methods, permit incubation at either **37°C** – general case – or **30°C** – to include psychrotrophs.

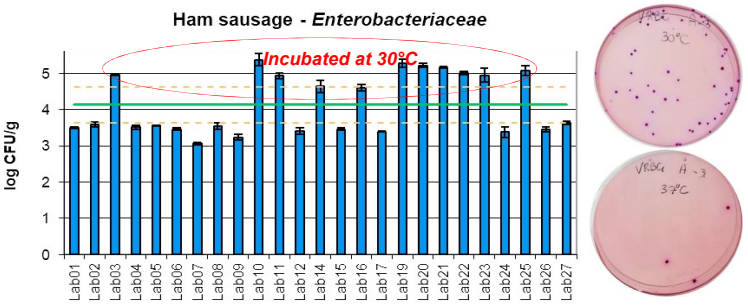

Fig. 2. Results of the enumeration of EB in blind double ham samples (PT2025). The corresponding Petri dishes on the right (ham 10-3 on VRBG, incubated at 30 vs 37°C) show that a 37°C incubation prevented the growth of most EB (the psychrotrophic ones).

ISO 15214 allows up to 8 protocol variations (single/double layer, sorbate addition, (an-)aerobic incubation, Gram stain or Catalase confirmation). In this case, 16S rDNA sequencing revealed small colonies on MRS agar were not LAB but *Staphylococcus hominis* and yeasts, naturally present in the salmon.

The artificial inocula (*E. coli* and *Enterobacter aerogenes,* ~ 3000 CFU/g) grew at both 30 and 37°C on VRBG agar. However, psychrotrophic EB naturally present in the ham samples (~100 000 CFU/g) grew at 30°C but not at 37°C: they were identified as *Rahnella inusitata* and *Serratia proteamaculans*.

STATISTICAL ANALYSIS OF BIMODAL RESULTS

Standard statistics, based on a general mean as assigned value, fail to accommodate bimodal results, placing many laboratory values outside confidence intervals (as shown in Fig. 1 and 2). ISO 13528 recommends segmenting data by methodology and assigning separate reference values for each subgroup (see Fig. 3).

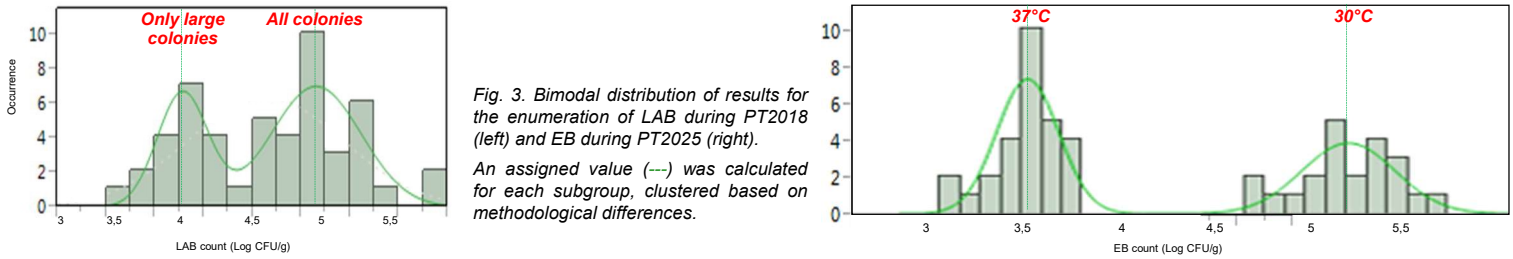

Fig. 3. Bimodal distribution of results for the enumeration of LAB during PT2018 (left) and EB during PT2025 (right).

An assigned value (---) was calculated for each subgroup, clustered based on methodological differences.

CONCLUSIONS – HARMONIZING ISO STANDARDS

Laboratories carrying out the same ISO method can reach different results and opposite conclusions on compliance of samples towards official criteria. This bimodality could be identified while analyzing naturally contaminated PT samples, containing heterogenous, psychrotrophic and stressed strains.

The observed bimodal distributions highlight discrepancies in ISO 15214 and ISO 21528-2. To improve consistency, standardization committees should refine these methods taking into account the technical improvements proposed below.

ISO 15214 (LAB)

- Limit protocol variations (*e.g.* MRS in single/double layer, with/without sorbate, aerobic/anaerobic incubation, Gram/Catalase confirmation).
- Define minimum colony size and characteristics for counting.

ISO 21528-2 (*Enterobacteriaceae*)

- Harmonize the incubation temperature, with a preference for 30°C (instead of 35 or 37°C) to enable the growth of both psychrotrophic and pathogenic *Enterobaceriaceae*.

Avec le soutien de la Wallonie