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1           **An international inter-laboratory study on *Nosema* spp. spore detection and**  
2           **quantification through microscopic examination of crushed honey bee abdomens**

3  
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53

54 Keywords: diagnosis, interlaboratory comparison, microscopic counting, microsporidia, *Nosema* spp.

55

## 56 **Abstract**

57 Nosemosis is a microsporidian disease causing mortality and weakening of honey bee colonies,  
58 especially in the event of co-exposure to other sources of stress. As a result, the disease is regulated in  
59 some countries. Reliable and harmonised diagnosis is crucial to ensure the quality of surveillance and  
60 research results. For this reason, the first European Interlaboratory Comparison (ILC) was organised in  
61 2017 in order to assess both the methods and the results obtained by National Reference Laboratories  
62 (NRLs) in counting *Nosema* spp. spores by microscopy. Implementing their own routine conditions of  
63 analysis, the 23 participants were asked to perform an assay on a panel of ten positive and negative  
64 samples of crushed honey bee abdomens. They were asked to report results from a qualitative and  
65 quantitative standpoint. The assessment covered specificity, sensitivity, trueness and precision.  
66 Quantitative results were analysed in compliance with international standards NF ISO 13528 (2015)  
67 and NF ISO 5725-2 (1994). Three results showed a lack of precision and five a lack of trueness.  
68 However, overall results indicated a global specificity of 98% and a global sensitivity of 100%, thus  
69 demonstrating the advanced performance of the microscopic methods applied to *Nosema* spores by the  
70 NRLs. Therefore, the study concluded that using microscopy to detect and quantify spores of *Nosema*  
71 spp. was reliable and valid.

72

## 73 **1. Introduction**

74 Nosemosis is a global disease of adult honey bees. It is caused by a spore-forming unicellular parasite  
75 of the Microsporidia group *Nosema*. However, a recent study based on a molecular comparison of the  
76 SSU rRNA gene proposed a new definition of the *Nosema* clade (Tokarev et al., 2020). The two main  
77 species of *Nosema* causing disorders in honey bees worldwide are *Nosema apis* (Zander, 1909) and  
78 *Nosema ceranae* (Fries et al., 1996). Another species, *Nosema neumannii*, has been found in honey  
79 bees in Uganda (Chemurot et al., 2017) but the implications of infections with *N. neumannii* still have  
80 to be studied. *Nosema apis* and *N. ceranae* multiply in the epithelial cells of the posterior ventricle

81 region. The spores present in the lumen of the digestive tract germinate and release polar filaments  
82 that mechanically perforate epithelial cells and through which the sporoplasm enters the cell to  
83 multiply. The cell is damaged as a large number of spores is generated. Two types of spores are  
84 produced: the primary spores that are capable of transmitting infection to adjacent cells, and the  
85 mature environmental spores that may be voided with the faeces or stay in the gut to start a new  
86 multiplication cycle (Fries, 1988, Goblirsch, 2018, Higes et al., 2007). *Nosema apis* is a parasite of the  
87 European honey bee (*Apis mellifera*) while *N. ceranae*, originally described in the Asian honey bee  
88 (*Apis cerana*) but also detected in *A. mellifera* populations in a number of geographically distant  
89 regions: Europe (Higes et al., 2006), South and North America (Calderón et al., 2008, Chen et al.,  
90 2008), Africa and Asia (Chen et al., 2009). *Nosema* species (spp.) spores have an ovoid morphology.  
91 *Nosema ceranae* spores measure approximately  $4.7 \times 2.7 \mu\text{m}$ , making them smaller on average than *N.*  
92 *apis* spores, which measure approximately  $6 \times 3 \mu\text{m}$  (Fries et al., 2013, Fries et al., 1996, Zander and  
93 Böttcher, 1984). However, this slight difference in size is not sufficient for a differential diagnosis in  
94 routine microscopy analyses. Molecular methods (PCR) are therefore required to identify *Nosema* spp.  
95 *Nosema* infection between adult bees is spread by the exchange of spores during feeding (trophallaxis)  
96 or comb-cleaning. Contaminated beekeeping equipment, honey stores and water also play a role in the  
97 transmission of the disease. *Nosema apis* spores expelled with faeces remain viable for over a year.  
98 They also remain contagious in honey (MacInnis et al., 2020) and in bee bodies.

99 The clinical signs of nosemosis are not specific. High infection rates can weaken the colony, leading to  
100 varying levels of depopulation in winter or spring. In the case of *N. apis*, dead bees, bees crawling on  
101 the ground and traces of diarrhoea may be observed on or around the hive. In contrast, the pathogenic  
102 effects of *N. ceranae* on *A. mellifera* colonies are not clearly understood. *N. ceranae* is thought to  
103 contribute to colony weakening, particularly in the presence of other sources of stress (Alaux et al.,  
104 2010, Doublet et al., 2015, Vidau et al., 2011, Zheng et al., 2015). Climate is also thought to have an  
105 effect on the pathogenicity of *N. ceranae*. While it is assumed that in warm areas the chronic stress  
106 caused by *N. ceranae* infections might favour colony death (Higes et al., 2008, Martín-Hernández et  
107 al., 2018), it was shown on the other hand that *N. ceranae* may be more virulent and better adapted

108 than *N. apis* in cold climates (Emsen et al., 2016). It should also be noted that bees can sustain high  
109 infection rates of *N. apis*/*N. ceranae* without apparent symptoms (Meana et al., 2010) (unpublished  
110 data). Given the difficulties of diagnosis, laboratories need to detect and quantify *Nosema* spp. spore  
111 loads in honey bees and to establish a differential diagnosis with other adult honey bee diseases  
112 causing similar disorders (e.g. tracheal acariasis, amoebiasis, chronic paralysis, intoxication, etc.).

113 Nosemosis is not covered by European Union regulations, nor is it included in the list drawn up by the  
114 World Organisation for Animal Health (OIE). However, the disease is regulated at national level in  
115 some countries. The OIE Manual of Diagnostic tests and Vaccines for Terrestrial Animals describes a  
116 diagnosis method for *Nosema disease* based on the detection and the quantification of spores by  
117 microscopy (World Organisation for Animal Health (OIE), 2018). A number of official laboratories  
118 have implemented this method.

119 In response to diagnostic and health issues, and to ensure the quality of the analytical results obtained  
120 within the European Union (EU), the European Union Reference Laboratory (EURL) for Bee Health,  
121 located in the laboratory of the French Agency for Food, Environmental and Occupational Health and  
122 Safety (ANSES) in Sophia-Antipolis (France), organised a InterLaboratory Comparison (ILC) using  
123 microscopy to detect and count *Nosema* spp. spores in crushed bee samples. This was the first test of  
124 this method organised by the EU. All the EU National Reference Laboratories (NRLs) were invited to  
125 participate in the ILC. The overall objective was to assess the ability of laboratories to establish a  
126 correct result using their routine analysis. Four criteria were evaluated: sensitivity, specificity, trueness  
127 and precision of the results. At the same time, a survey was conducted within the network of EU NRLs  
128 in order to collect information on their analytical methods with the perspective of a possible  
129 harmonisation.

130

## 131 **2. Materials and Methods**

### 132 *2.1. Participating laboratories*

133 In June 2017, the EURL for Bee Health organised an ILC. A total of 23 NRLs for Bee Health took  
134 part in this trial, all from EU member states. In order to ensure the confidentiality of results, each  
135 participating laboratory was assigned an individual random code number.

136

## 137 2.2. Reference methods

138 Two reference methods were used to characterise and check the homogeneity and stability of the  
139 samples used in the ILC: i) a microscopy-based method to detect and count *Nosema* spp. spores, and  
140 ii) a PCR-based method to confirm detection of *Nosema* spp. (this method was only used to  
141 characterise the samples, i.e. to verify their negative or positive status). The EURL is accredited by the  
142 French Accreditation Committee (COFRAC) for these two methods in compliance with the  
143 international standard ISO/IEC 17025 on “General requirements for the competence of testing and  
144 calibration laboratories” (NF EN ISO/IEC 17025, 2005).

145 The microscopic method is based on the procedure developed by Cantwell (Cantwell, 1970) and  
146 recommended by the OIE in the Terrestrial Manual (World Organisation for Animal Health (OIE),  
147 2018) intending to detect and evaluate the average infection rate of bees by *Nosema* spp. spores using  
148 microscopy. In brief, the procedure involves crushing bee abdomens (60 bees) with a mortar and a  
149 pestle in ultrapure water at a rate of one millilitre (1 mL) per bee. The suspension is filtered through  
150 two layers of muslin (thin loosely woven cotton fabric) and centrifuged for six minutes at  $800 \times g$  to  
151 eliminate large debris and to purify the spores. The pellets are then resuspended to a homogeneous  
152 suspension in order to restore the initial dilution of 1 mL per bee. Finally, the sample is placed in a  
153 calibrated haemocytometer (Malassez counting chamber) and the microscopic examination is  
154 performed to detect and count *Nosema* spp. spores. The analytical results are both qualitative (negative  
155 versus positive) and quantitative (number *Nosema* spp. spores per mL, i.e. per bee, based on dilution).  
156 It should be noted that measurement uncertainty may be high for the visual counting method. It can  
157 vary depending on the number of *Nosema* spores and particles (e.g. pollen, yeast) present in the bee’s  
158 digestive tract, which may interfere with the detection and identification of *Nosema* spores.

159 The main steps in the molecular method are as follows. First, 80  $\mu$ L of the suspension of crushed bee  
160 abdomens, prepared for the microscopic examination, is used to extract DNA using High Pure PCR  
161 Template Preparation Kit (Roche Diagnostics). The DNA extraction is performed following the  
162 “tissue” protocol without any change. The extracted DNA is resuspended in 200  $\mu$ L of elution buffer  
163 according to the manufacturer’s recommendations and stored at  $-20^{\circ}\text{C} \pm 5^{\circ}\text{C}$  until further analysis  
164 (used as a template in the PCR). The PCR is performed as follows: 25  $\mu$ L of the reaction mixture  
165 containing 1 U Platinum Taq DNA polymerase (Invitrogen), 0.4  $\mu$ M of each primer, 0.4 mM dNTPs  
166 and adjusted with nuclease-free H<sub>2</sub>O to reach a final reaction volume of 20  $\mu$ L plus 5  $\mu$ L of extracted  
167 DNA. The PCR reactions were run in an Eppendorf Mastercycler® Nexus ThermoCycler under the  
168 following cycling conditions: initial denaturation at 94°C for 2 min followed by 35 cycles of 30s at  
169 94°C, 30s at 62°C and 30s at 72°C with a final extension of 7 min at 72°C. The PCR allows the  
170 identification of *Nosema* species (*N. apis* and *N. ceranae*) using the species-specific primers described  
171 by Martin-Hernandez (Martín-Hernández et al., 2007).

172

### 173 2.3. Inter-comparison samples

174 The samples from the ANSES collection at Sophia-Antipolis laboratory originated from diagnostic  
175 analyses, field studies, experimental infections and collaborations. The panel included two types of  
176 samples: crushed *A. mellifera* abdomens, prepared according to the reference method described in the  
177 paragraph above, and a filtered suspension of *N. ceranae* spores. The status of each batch of samples  
178 (negative or positive for *Nosema* spp., defined spore load) was based on the results obtained with the  
179 two independent methods described above. The PCR also demonstrated that all positive samples were  
180 infected by *N. ceranae*.

181 Three negative *Nosema* spp. samples (NEG1, NEG2 and NEG3) were prepared using bees from the  
182 ANSES experimental apiary. Three positive *Nosema* spp. samples (POS1, POS2 and POS3) with  
183 different infectious loads (3.47E+06, 4.73E+05 and 1.84E+06 spores per mL, *i.e.* per bee respectively)  
184 were included in the panel (Table 1). POS1 and POS2 were prepared using bees naturally infected



185 with *N. ceranae* from the EURL sample collection, while POS3 was a suspension of *N. ceranae*  
186 spores, prepared from experimentally infected emerging bees and filtered through a 100 µm mesh  
187 sieve.

188 Each crushed sample was distributed in tubes with a volume of 400 µL per tube and the batches of  
189 tubes were stored at  $-20^{\circ}\text{C} \pm 5^{\circ}\text{C}$  until shipment. The panels sent to participants included ten  
190 evaluation samples and a lure sample (Table 1). The lure sample was positive or negative, depending  
191 on the participant, was not evaluated and was added to limit the risk of interlaboratory collusion. Two  
192 positive *Nosema* spp. samples (POS2 and POS3) were sent in triplicate to assess the accuracy and the  
193 trueness of participants' results.

194

#### 195 2.4. Sample homogeneity and stability

196 The EURL conducted homogeneity and stability tests. Homogeneity tests were performed for all  
197 sample batches between February and April 2017, *i.e.* before shipment. The homogeneity of each  
198 batch was tested by means of a duplicate analysis of ten randomly selected samples stored at  $-20^{\circ}\text{C}$ . In  
199 total, 20 results per batch were obtained. The homogeneity criterion of the negative samples was  
200 defined as a number of spores per mL (or bee) not exceeding  $2\text{E}+04$ . This corresponds to the detection  
201 limit of the microscopic counting method using the Malassez chamber. All the negative samples met  
202 this criterion, with no *Nosema* spp. spores detected in any of the selected samples. The homogeneity of  
203 the positive samples was evaluated by calculating the standard deviations (SD) between samples in  
204 compliance with the formula set out in Annex B of international standard NF ISO 13528 (NF ISO  
205 13528, 2015). The analysis was carried out on the number of spores per mL expressed as decimal  
206 logarithm ( $\log_{10}$ ), in order to facilitate data analysis. The homogeneity of the inter-comparison samples  
207 was validated against a target standard deviation value ( $\sigma_{\text{pt}}$ ) of 0.2. This value was based on the results  
208 of an inter-laboratory validation test organised in 2016 within France's official laboratory network. As  
209 specified in Annex B (paragraph B.2.2) of international standard NF ISO 13528, it was necessary for  
210 inter-sample standard deviation (SD) to fall under the critical value of  $0.3 \sigma_{\text{pt}}$ . SD values were  
211 calculated for the three positive samples. They ranged from 0.02 to 0.05  $\log_{10}$ , while the SD/ $\sigma_{\text{pt}}$  ratios

212 were below 0.3 (Table 2). The homogeneity criterion was met and the positive samples were  
213 considered homogeneous.

214 Stability tests were performed on positive batches. Stability was controlled by a duplicate analysis of  
215 three randomly selected samples stored at  $-20^{\circ}\text{C} \pm 5^{\circ}\text{C}$ . In total, six results were obtained for each  
216 sample (Table 3). The tests were carried out one day after the shipment of panels to the participants  
217 (D-0) and at the end of the ILC period (deadline for sending in results) (D-20). The stability of the  
218 positive samples was evaluated in compliance with the criteria set out in Annex B (paragraph B.5.1) of  
219 international standard NF ISO 13528. The results of the stability tests at D-0 and D-20 were compared  
220 to those from the homogeneity tests. The difference between the mean of the homogeneity test results  
221 (mL) and the mean of the stability test results (m2) was less than the critical value of  $0.3 \sigma_{pt}$  (*i.e.*  $|\text{mL} -$   
222  $\text{m2}| \leq 0.3 \sigma_{pt}$ ), thus validating the stability of the positive samples during the trial period (Table 3).

223

#### 224 2.5. Study design

225 The ILC was organised in compliance with the quality requirements described in international  
226 standards ISO/IEC 17043 and ISO/IEC 17025 (NF EN ISO/IEC 17025, 2005, NF EN ISO/IEC 17043,  
227 2015). The samples were packed and shipped between the EURL and NRLs in compliance with  
228 UN3373 regulations (Biological Substance, Category B).

229 Each participating laboratory was anonymously coded with a 1- or 2-digit random number to ensure  
230 the confidentiality of results. Each of the samples to be blind-tested was coded with the attribution of a  
231 random number between 1 and 11. Participating laboratories received inter-comparison samples with a  
232 laboratory code on each tube. After receiving the package, the laboratories were required to store the  
233 samples at  $-20^{\circ}\text{C} \pm 5^{\circ}\text{C}$  until analysis and to send back their results within 15 days. They were asked  
234 to report the results: i) qualitatively (detected versus not detected, with samples of  $\leq 2 \times 10^4$  spores /  
235 mL being considered as “not detected”), and ii) quantitatively (number of *Nosema* spp. spores per mL,  
236 corresponding to the number of *Nosema* spp. spores per bee). Participating laboratories were required  
237 to conduct calculations in compliance with their own analytical methods and, more particularly,  
238 according to the type of counting chamber used for analysis.

239 Results were evaluated according to four performance criteria:

240 1) Specificity, *i.e.* the ability of the laboratory to give a negative result for a negative sample (NF EN  
241 ISO 22117, 2010). The expected specificity rate was 100% of negative results.

242 2) Sensitivity, *i.e.* the ability of the laboratory to give a positive result for a positive sample (NF EN  
243 ISO 22117, 2010). The expected sensitivity rate was 100% of positive results.

244 3) Trueness, which was evaluated only for positive quantitative results by calculating the z-score in  
245 compliance with international standard NF ISO 13528 (NF ISO 13528, 2015).

246 4) Precision, which was evaluated only for positive quantitative results, by calculating Mandel's k-  
247 value in compliance with international standard NF ISO 5725-2 (NF ISO 5725-2, 1994).

248

#### 249 *2.6. Technical survey of the analytical methods employed by the ILC participants*

250 This study was the first step in a process to evaluate the level of harmonisation across the European  
251 NRL network for the diagnosis of *Nosema* spp. by microscopy. Participating laboratories were asked  
252 to use their own routine methods to analyse the ILC panel of samples. Concurrently with the test, and  
253 in order to gather information on these methods, the EURL asked participants to complete an online  
254 survey (using Sphinx iQ2 software, version 7.4.0.0, Le Sphinx Développement), detailing each stage  
255 of their routine procedure, from grinding the sample to interpreting the results.

256

#### 257 *2.7. Statistical analysis of results*

258 In the first instance, a qualitative analysis was conducted in order to evaluate the sensitivity and  
259 specificity of results. Conformity was assessed using the reference values obtained during the  
260 homogeneity study. Sensitivity and specificity rates were calculated using the formula below, set out  
261 in standard NF ISO 22117 (NF EN ISO 22117, 2010):

262 - Sensitivity rate:  $r_{SE} = n_+ / E_{(n+tot)} \times 100\%$  (where  $n_+$  is the number of positive results found and  
263  $E_{(n+tot)}$  is the total number of expected positive samples).

264 - Specificity rate:  $r_{SP} = n. / E_{(n-tot)} \times 100\%$  (where n. is the number of negative results found and  
265  $E_{(n-tot)}$  is the total number of expected negative samples).

266 The second step was to analyse the quantitative results from the positive samples included in triplicate  
267 in the panel (POS2 and POS3). The value assigned to each positive sample was established as the  
268 consensus value for the results of participants, in compliance with the procedure described in  
269 Appendix C of international standard NF ISO 13528 and corresponding to the robust average of  
270 participants. The individual results of each participant were then compared to this value, taking  
271 account of standard uncertainty. SD for the ILC assessment ( $\sigma_{pt}$ ) was calculated using the results  
272 obtained by participants. The trueness of the results was evaluated by means of the z-score, which  
273 expressed the ratio between the observed deviation from the mean value and the standard deviation of  
274 the ILC ( $\sigma_{pt}$ ). It was calculated in compliance with standard NF ISO 13528 (NF ISO 13528, 2015).  
275 The level of precision was evaluated through the graphical representation of Mandel's k-values, in  
276 compliance with international standard NF ISO 5725-2 (NF ISO 5725-2, 1994). Mandel's k-values are  
277 intra-laboratory statistics calculated for each sample and each participant. They correspond to the ratio  
278 between the standard deviation of the participant's results and the average standard deviation of the  
279 sample.

280

### 281 **3. Results**

#### 282 *3.1. Analysis of qualitative results*

283 Participants identified all the positive samples. Sensitivity was therefore satisfactory for all the  
284 participants and complied with the expected rate of 100%. However, the analysis revealed a non-  
285 compliance in specificity for the laboratory with code No. 21, which gave a positive result for sample  
286 NEG3. Specificity was therefore satisfactory for 95.6% of participants (22 of 23).

287 Across the network of the 23 participating EU NRLs, the global sensitivity rate for results was 100%  
288 while the global specificity rate was 98.6% (Table 4).

289

290           3.2. *Analysis of quantitative results*

291 All the results of the participants were included in the analysis (Supplemental information, Table S1).  
292 The performance of participants was evaluated by sending three replicates of two different loaded  
293 samples (POS2 and POS3), as described in the section “Materials and methods”. Figures 1A and 1B  
294 show the results of the 23 participants for sample POS2 and sample POS3 respectively. The values  
295 assigned to samples POS2 and POS3 are 5.57 and 6.19 log<sub>10</sub> respectively (with a standard deviation of  
296 0.18 and 0.16 log<sub>10</sub>). The mean and standard deviation were estimated through a robust analysis of all  
297 participant data, in compliance with algorithm A described in Appendix C of international standard  
298 NF ISO 13528. As the uncertainties relating to the values assigned to the two positive samples could  
299 be considered as negligible, they were not included in the interpretation of the results, making it  
300 possible to use a z-score for the evaluation.

301

302           3.3. *Assessment of the trueness of results*

303 The z-score values are shown in two histograms (Fig. 2). Figures 2A and 2B show the z-score of each  
304 participant for the three repetitions of results with POS2 and POS3, respectively.

305 As a reminder, if the z-score is 0, the measured value (x) corresponds to the assigned value (x\*). The  
306 interpretation of z-scores is set out in international standard NF EN ISO / IEC 17043 as: (i) if  $|z| \leq$   
307 2.0 then the value of z is considered to be acceptable, (ii) if  $2.0 < |z| < 3.0$  the value of z is  
308 considered to give a warning signal, and (iii) if  $|z| \geq 3.0$  the value of z is considered to be  
309 unacceptable and generates an action signal.

310 Five participants delivered unacceptable results, with a  $|z| \geq 3.0$ : the participants with code No. 20  
311 and No. 27 for sample POS2, the participants with code No. 5 and No. 21 for sample POS3, and the  
312 participant with code No. 40 for both samples. These results were assessed as non-compliant.

313 At ILC level, trueness was satisfactory (*i.e.*  $|z| < 3.0$ ) for 92.8% of the results provided by  
314 participants.

315

316           3.4. *Assessment of the precision of results*

317 For 23 participants and three repeats, the critical Mandel's k-value at the 1% significance level was  
318 2.08. Given that less than 25% of k-values were below the critical value, the data of all participants  
319 could be used for analysis. The Mandel's k-values (Fig. 3) show that 87% of participants (20  
320 laboratories) achieved good repeatability with both samples. Three participants encountered problems  
321 with this criterion: the participant with code No. 27 for sample POS2, and the participants with code  
322 No. 5 and No. 21 for sample POS3.

323

### 324 3.5. Technical survey of analytical methods employed

325 The online survey was sent out in June 2017. Of the 23 ILC participants, seven laboratories (30%)  
326 were accredited for the diagnosis of nosemosis by microscopy. Most methods (75%) were  
327 recommended by or adapted from the OIE Manual. The other methods were internal or based on  
328 methods published in literature. One laboratory (No. 21) was using a method for the first time with the  
329 ILC.

330 A wide diversity of counting chambers was used for counting *Nosema* spp. spores (Fig. 4). The Bürker  
331 and Neubauer chambers were the most frequent choices (25% and 30% respectively). It should be  
332 noted that two participants did not perform routine counting, preferring to make a semi-quantitative  
333 evaluation of the *Nosema* spp. spore load (giving rise to analytical results such as “sporadic  
334 occurrence”, “weak infestation”, “moderate infestation”, “strong infestation”, depending on the  
335 number of spores observed per microscopic field).

336 About 60% of the laboratories used a single microscopic preparation (*i.e.* one microscopic slide  
337 prepared from the abdomen suspension), while around 40% used several slides (*i.e.* several  
338 microscopic slides prepared from the suspension) in order to calculate the mean of the different counts  
339 as their final result. Moreover, some laboratories carried out several counts on the same microscopic  
340 slide. One laboratory used two preparations, each slide being counted twice by different analysts.

341 To finish, it should be also noted that some laboratories adjusted the spore-counting protocol  
342 depending on the sample (*e.g.* spore load). In the event of a high number of spores, for instance, 60%  
343 of laboratories said that they diluted the suspension.

344

#### 345 **4. Discussion**

346 The ILC was carried out with 23 NRLs from the EU. Its purpose was to evaluate their level of  
347 competence in using microscopy to detect and quantify *Nosema* spp. spores in crushed honey bee  
348 abdomens. Of the 23 participants, 18 laboratories (78%) obtained compliant results for all evaluation  
349 criteria (specificity, sensitivity, trueness and precision) (Table 5). Nine results, attributed to five  
350 participants, failed to meet the defined criteria: i) one for a lack of specificity, ii) five for a lack of  
351 trueness, iii) three for a lack of precision. A lack of trueness was observed for laboratory No. 40,  
352 which globally over-estimated the number of spores in the two positive samples included in triplicate  
353 as part of the panel (POS2 and POS3). This laboratory was accredited for the method used; its  
354 procedure was based on a single count with a Malassez chamber. The results of participant No. 20  
355 showed a lack of trueness for sample POS2, tending – in contrast – towards under-estimation. The  
356 method employed by this laboratory for the ILC relied on a single microscopic preparation counted  
357 once with a Neubauer chamber. The results of participant No. 21 showed a lack of trueness, precision  
358 and specificity. It should be noted that this laboratory did not apply a routine method, which could  
359 explain these non-compliances. Likewise, participant No. 27 encountered problems of trueness and  
360 precision. This participant did not make routine use of a counting chamber, but gave semi-quantitative  
361 results expressed by crosses in the current analysis (the number of crosses depending on the number of  
362 *Nosema* spp. spores observed in the microscopic field). A lack of trueness and precision was also  
363 observed for laboratory No. 5, although this participant was using an accredited method, based on the  
364 preparation of two microscopic slides counted twice by two different analysts using a Bürker-Türk  
365 chamber.

366 Following the ILC, the laboratories undertook an investigation in collaboration with the EURL to  
367 identify and resolve the causes of the non-conformities observed. In one case, the anomaly of trueness  
368 was directly linked to an error in the formula used for converting the number of spores counted with  
369 the haemocytometer in “spores per mL”. The problems of trueness and precision were also explained  
370 by a possible incomplete defrosting of the tubes and/or a lack of vortexing before analysis, resulting in

371 insufficient homogenisation of the samples. Moreover, one laboratory indicated that diluting the most  
372 heavily loaded samples would have improved the precision of its results and should have been carried  
373 out. However, it should be noted that some participants obtained satisfactory results in the ILC without  
374 diluting the samples. Although no reason was specifically determined, a confusion between *Nosema*  
375 spp. spores and refractive artefacts could explain the anomaly in specificity, given that the laboratory  
376 had little experience in using this method. In 2018, to check the efficacy of the corrective actions  
377 carried out, the EURL sent a second panel of samples to the two laboratories interested in taking part  
378 in a new assay. The results were satisfactory, proving that the causes of the non-conformities had been  
379 resolved.

380 The ILC on *Nosema* spp diagnosis by microscopy was the first assay involving the official laboratories  
381 of EU member states. Concerning the network of the 23 participating NRLs, test results showed global  
382 specificity of 98.6%, and global sensitivity of 100%. From a quantitative standpoint, trueness and  
383 precision were satisfactory for 92.8% and 93.5% of results respectively. These data demonstrated the  
384 performance of the network to provide the reliable analytical results that are essential to ensuring the  
385 quality of surveillance and study data. Although the analytical methods used by the NRLs were based  
386 on the same principle set out in the OIE Terrestrial Manual, they implemented different technical  
387 procedures for preparing microscopic slides and counting. The data collected did not reveal any  
388 apparent link between the diversity of procedures and the conformity of results.

389 The trial used crushed abdomen samples. It would not have been feasible to use whole bees as *Nosema*  
390 spp. infection in honeybees is heterogeneous between individuals and not experimentally controllable.  
391 However, using whole bees as inter-comparison samples would have made it possible to evaluate the  
392 analytical methods in their entirety, *i.e.* including the first stages of the method: sampling for analysis,  
393 bee preparation, buffer type, grinding process and filtration procedures. The data collected by the  
394 questionnaire revealed a wide diversity in practices that could also influence results. For instance,  
395 some laboratories do not use only bee abdomens but whole bees or digestive tracts only in their  
396 routine analyses (16% and 8% of the participants respectively). Moreover, around 40% of the  
397 laboratories do not filter the suspension before microscopic examination. The presence of a significant  
398 quantity of particles and artefacts (such as pollen or yeast present in the digestive tract) could have an



399 impact on the visual detection and counting of spores. A harmonised approach is certainly necessary  
400 for these early stages of the method.

401 Finally, the survey found that most NRLs did not have a diagnosis threshold for the clinical disease of  
402 noseiosis (*i.e.* a *Nosema* spp. spore load suggestive of an overt infection). Even when this threshold  
403 did exist, it varied from one million to several million or nine million spores per bee for *N. apis* (20%  
404 of laboratories having a threshold for this species). In the case of *N. ceranae*, two laboratories had a  
405 diagnosis threshold of one million of spores per bee. A recent study conducted in North America  
406 (Canada, Ontario) found that high levels of *N. ceranae* infections were significantly associated with  
407 reduced bee populations and food stores in colonies, and indeed suggested a intervention threshold of  
408 one million of spores per bee (Emsen et al., 2020). Several laboratories also said that the clinical signs  
409 observed in apiaries were considered in the interpretation of results. Harmonising the way in which  
410 results are interpreted, taking account not only of the spore load, but also the clinical signs and  
411 associated field information (e.g. presence of other sources of stress), would consolidate diagnostic  
412 modalities for noseiosis in the long term.

413 To conclude, the results of the ILC on *Nosema* spp. spore detection and counting were satisfactory  
414 overall. However, it should be mentioned that the panel, consisting of ten samples (three negatives and  
415 three positives, including two in triplicate) was relatively small. Further investigations with larger  
416 sample sizes should be conducted to consolidate the results and the conclusions of this study. In  
417 addition, the small number of anomalies identified must be relativised in view of the significant  
418 measurement uncertainties of the visual counting method and the absence of a scientifically  
419 established threshold for the diagnosis of *N. apis* and *N. ceranae*.

420

#### 421 **Declaration of Competing interest**

422 The authors confirm that there are no known conflicts of interest associated with this publication.

423

#### 424 **Funding**

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426 Safety, Brussels.

427 Table 1. Composition of the test panel of samples sent to participating laboratories.

Samples	Microscopic examination			Nosema species	Criterion evaluated
	Detection	Counting** (spores/ml)	SD*** (spores/ml)		
<b>POS1</b>	Positive	3.47E+06	2.83E+05	<i>N. ceranae</i>	Sensitivity
<b>POS2*</b>	Positive	4.73E+05	8.39E+04	<i>N. ceranae</i>	Sensitivity
<b>POS3*</b>	Positive	1.84E+06	2.01E+05	<i>N. ceranae</i>	Sensitivity
<b>NEG1</b>	Negative	-	-	-	Specificity
<b>NEG2</b>	Negative	-	-	-	Specificity
<b>NEG3</b>	Negative	-	-	-	Specificity
<b>Lure</b>	Positive	7.04E+05	1.96E+05	<i>N. ceranae</i>	
	or negative	-	-	-	not evaluated

428 \* Samples tested in triplicate

429 \*\* Mean of the homogeneity study results

430 \*\*\* Standard deviation evaluated within the homogeneity study

431

432 Table 2. Homogeneity of positive samples

433

Sample code	m1	SD	SD/ $\sigma_{pt}$	Criteria $SD/\sigma_{pt} \leq 0.3$
<b>POS1</b>	6.54	0.02	0.11	OK
<b>POS2</b>	5.67	0.05	0.22	OK
<b>POS3</b>	6.26	0.03	0.19	OK

434

435 m1:  $\log_{10}$  of the mean of the results obtained by the homogeneity study

436 SD: inter-sample standard deviation

437  $SD/\sigma_{pt} \leq 0.3$ : homogeneity criterion according to Annex B of international standard NF ISO 13528

438

439

440 Table 3. Stability of positive samples

441

	Sample code	m1	m2	m1-m2	(m1-m2)/ $\sigma_{pt}$	Criteria (m1-m2)/ $\sigma_{pt} \leq 0.3$
	<b>POS1</b>	6.54	6.50	0.04	0.17	OK
<b>D0</b>	<b>POS2</b>	5.67	5.63	0.04	0.17	OK
	<b>POS3</b>	6.26	6.22	0.04	0.23	OK
	<b>POS1</b>	6.54	6.50	0.04	0.18	OK
<b>D20</b>	<b>POS2</b>	5.67	5.63	0.04	0.18	OK
	<b>POS3</b>	6.26	6.21	0.05	0.28	OK

442

443 m1:  $\log_{10}$  of the mean of the results obtained by the homogeneity study

444 m2:  $\log_{10}$  of the mean of the results obtained by the stability study

445 (m1-m2)/ $\sigma_{pt} \leq 0.3$ : stability criterion according to Annex B of international standard NF ISO 13528

446

447 Table 4. Sensitivity and specificity rates attained by each participating laboratory.

Laboratory Code	Sensitivity <sup>a</sup> (%)	95% confidence interval (%)	Specificity <sup>b</sup> (%)	95% confidence interval (%)
lab1	100	64.6 to 100	100	43.9 to 100
lab2	100	64.6 to 100	100	43.9 to 100
lab3	100	64.6 to 100	100	43.9 to 100
lab4	100	64.6 to 100	100	43.9 to 100
lab5	100	64.6 to 100	100	43.9 to 100
lab6	100	64.6 to 100	100	43.9 to 100
lab7	100	64.6 to 100	100	43.9 to 100
lab8	100	64.6 to 100	100	43.9 to 100
lab15	100	64.6 to 100	100	43.9 to 100
lab16	100	64.6 to 100	100	43.9 to 100
lab17	100	64.6 to 100	100	43.9 to 100
lab18	100	64.6 to 100	100	43.9 to 100
lab20	100	64.6 to 100	100	43.9 to 100
lab21	100	64.6 to 100	66.7	20.8 to 93.8
lab22	100	64.6 to 100	100	43.9 to 100
lab23	100	64.6 to 100	100	43.9 to 100
lab24	100	64.6 to 100	100	43.9 to 100
lab25	100	64.6 to 100	100	43.9 to 100
lab26	100	64.6 to 100	100	43.9 to 100
lab27	100	64.6 to 100	100	43.9 to 100
lab38	100	64.6 to 100	100	43.9 to 100
lab40	100	64.6 to 100	100	43.9 to 100
lab41	100	64.6 to 100	100	43.9 to 100
<b>Overall</b>	<b>100 (161/161)</b>	<b>97.7 to 100</b>	<b>98.6 (68/69)</b>	<b>92.2 to 99.7</b>

448

449 <sup>a</sup> Calculation of the sensitivity rate was based on 7 samples (see Table 1).

450 <sup>b</sup> Calculation of the specificity rate was based on 3 samples (see Table 1).

451 Table 5. Participant fulfilment of proficiency test criteria.

Criteria	Participant code																							
	1	2	3	4	5	6	7	8	15	16	17	18	20	21	22	23	24	25	26	27	38	40	41	
<b>Specificity</b>	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
<b>Sensitivity</b>	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
<b>Trueness</b>	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes	No	Yes	No	Yes	
<b>Precision</b>	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	

452

453

454 Supplemental information

455 Table S1. Raw data, mean and standard deviation (expressed in log<sub>10</sub>) obtained by each participating  
456 laboratory.

Laboratory Code	Sample code									
	POS1 <sup>a</sup>	POS2 <sup>b</sup>			POS2 Mean (SD)		POS3 <sup>b</sup>			POS3 Mean (SD)
lab1	6.25	5.46	5.35	5.37	5.40 (0.06)	6.06	5.98	6.01	6.02	(0.04)
lab2	6.46	5.64	5.38	5.60	5.54 (0.14)	6.17	6.26	6.24	6.22	(0.04)
lab3	6.28	5.35	5.11	5.20	5.22 (0.12)	5.92	6.08	5.89	5.96	(0.11)
lab4	6.44	5.54	5.48	5.60	5.54 (0.06)	6.16	6.27	6.30	6.24	(0.07)
lab5	6.52	5.82	5.92	5.93	5.89 (0.06)	6.28	6.71	6.68	6.56	(0.24)
lab6	6.40	5.62	5.61	5.67	5.63 (0.03)	6.30	6.23	6.27	6.26	(0.04)
lab7	6.66	5.64	5.63	5.70	5.66 (0.04)	6.40	6.20	6.38	6.33	(0.11)
lab8	5.89	5.61	5.63	5.64	5.63 (0.02)	6.22	6.21	6.16	6.20	(0.04)
lab15	6.42	5.67	5.62	5.68	5.65 (0.03)	6.49	6.26	6.30	6.35	(0.12)
lab16	6.42	5.51	5.61	5.52	5.55 (0.05)	6.13	6.13	6.10	6.12	(0.02)
lab17	6.40	5.61	5.62	5.59	5.61 (0.01)	6.23	6.28	6.25	6.25	(0.03)
lab18	6.36	5.48	5.85	5.54	5.62 (0.20)	6.19	6.26	6.24	6.23	(0.03)
lab20	5.98	5.35	5.18	5.00	5.18 (0.18)	5.75	5.92	5.72	5.80	(0.11)
lab21	5.96	5.23	5.64	5.65	5.50 (0.24)	6.07	5.48	5.78	5.78	(0.29)
lab22	6.29	5.56	5.58	5.56	5.56 (0.01)	6.18	6.20	6.22	6.20	(0.02)
lab23	6.35	5.66	5.76	5.78	5.73 (0.06)	6.20	6.29	6.23	6.24	(0.05)
lab24	6.46	5.74	5.85	5.81	5.80 (0.05)	6.24	6.26	6.28	6.26	(0.02)
lab25	6.08	5.35	5.38	5.34	5.36 (0.02)	5.87	6.17	6.16	6.07	(0.17)
lab26	6.40	5.81	5.74	5.51	5.69 (0.16)	6.16	6.27	6.31	6.25	(0.08)
lab27	6.29	5.00	5.66	5.38	5.35 (0.33)	6.13	6.03	5.88	6.01	(0.12)
lab38	6.40	5.51	5.44	5.46	5.47 (0.04)	6.17	6.10	6.22	6.16	(0.06)
lab40	6.86	6.15	6.15	5.78	6.02 (0.21)	6.81	6.79	6.95	6.85	(0.09)
lab41	6.40	5.51	5.60	5.56	5.56 (0.05)	6.25	6.25	6.23	6.24	(0.01)

457

458 <sup>a</sup> log<sub>10</sub> of the POS1 sample: one result per participant.

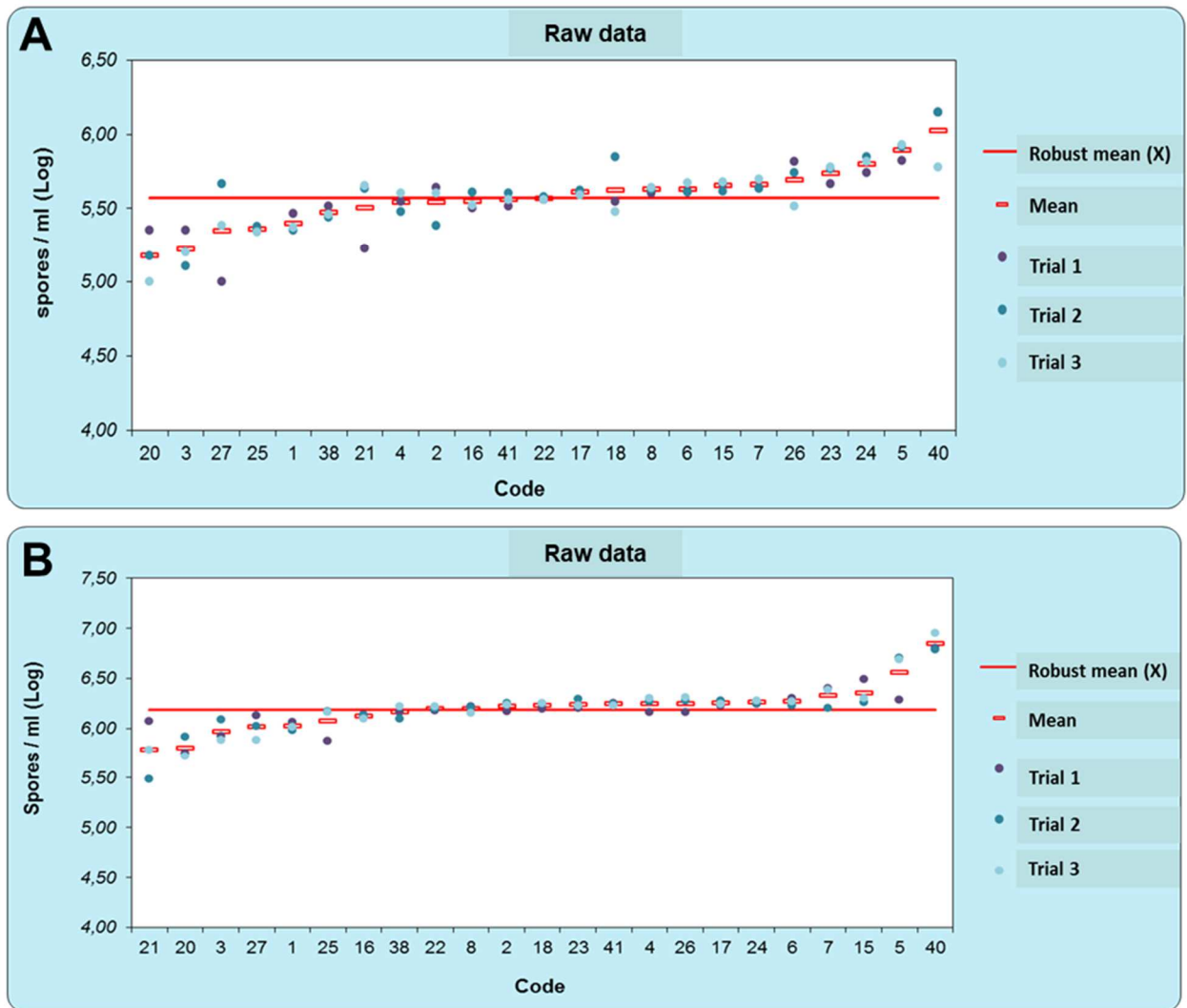
459 <sup>b</sup> log<sub>10</sub> of the POS2 and POS3: three results per participant.

460

461



462 Figure 1. Experimental results of participants when quantifying *Nosema* spp. spores in the ILC  
 463 samples. Each participant tested three replicates of the POS2 sample (Fig 1A) and three replicates of  
 464 the POS3 sample (Fig 1B). The bullet points indicate the number of *Nosema* spp. spores per bee  
 465 (expressed in  $\log_{10}/\text{bee}$ ) quantified by microscopy. The empty red box is the mean value found by each  
 466 participant. The red lines indicate the robust mean (X).

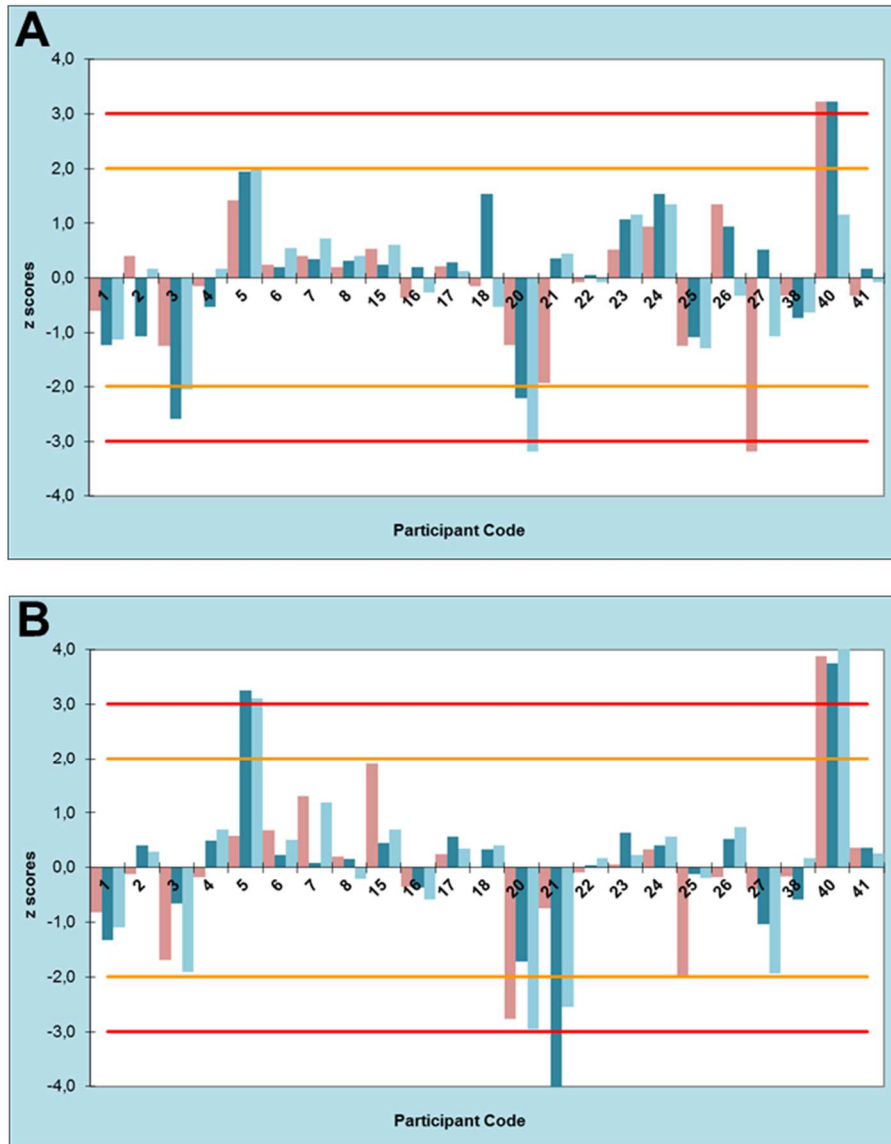


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469

470 Figure 2. Z-scores calculated for each participant quantifying *Nosema* spores in the inter-laboratory  
471 comparison samples. The boxes indicate the individual z-score for the three replicates of the POS2  
472 sample (Fig 2A) and POS3 sample (Fig 2B) tested by each participant. The yellow and red lines  
473 indicate the limits of  $\pm 2$  and  $\pm 3$  respectively.

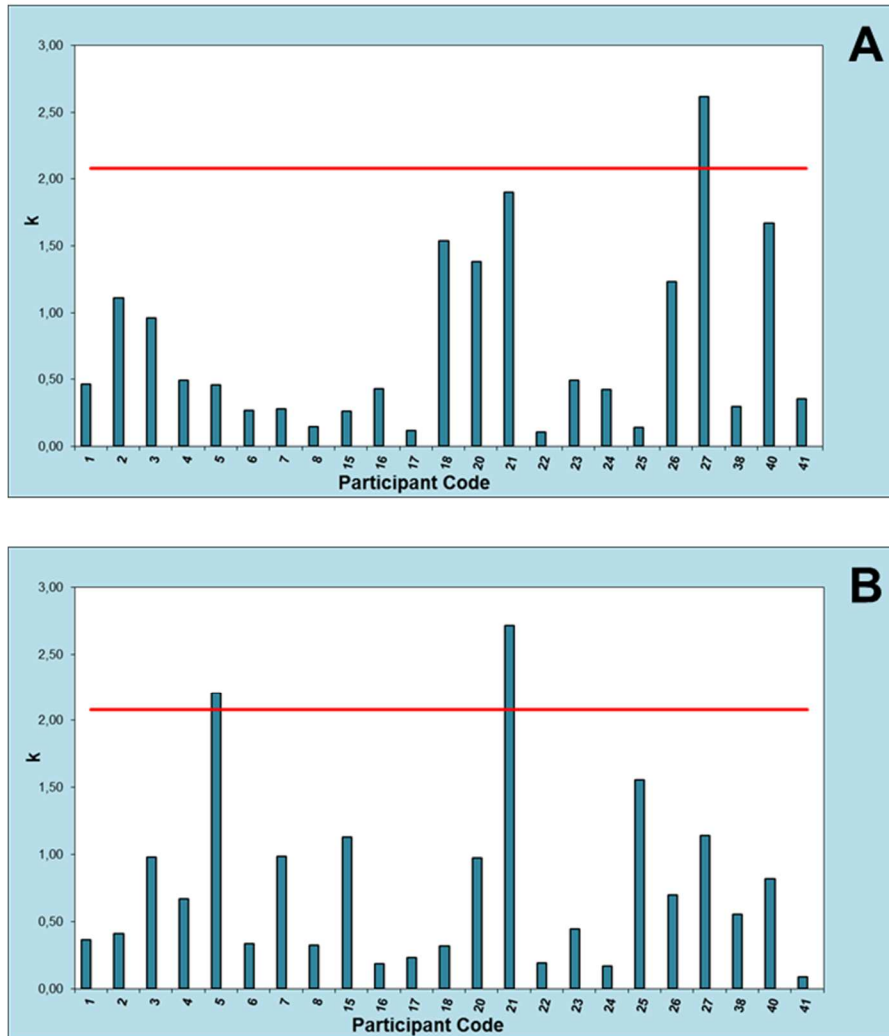


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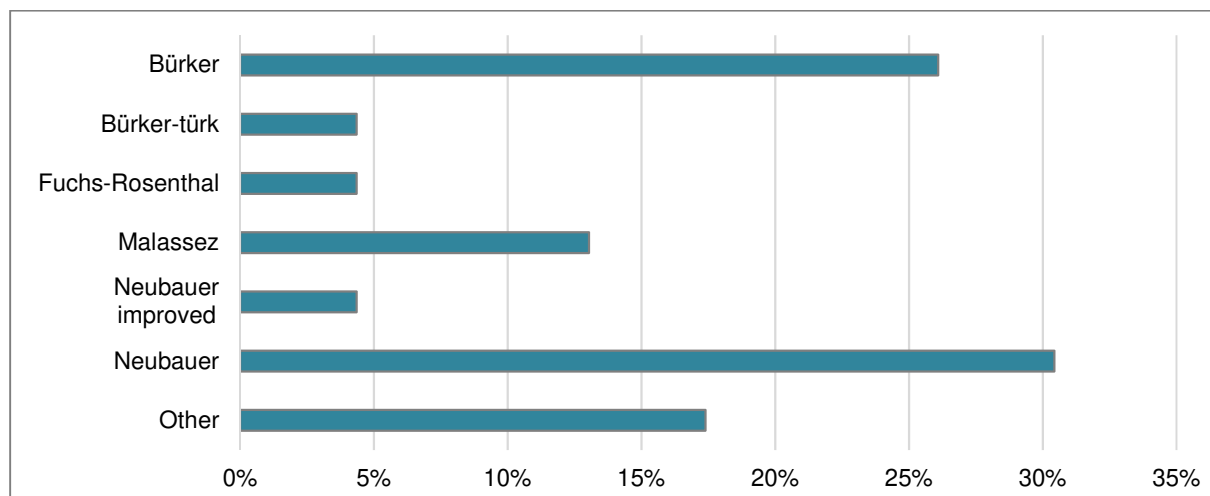
476

477 Figure 3. Mandel's k-value calculated for each participant quantifying *Nosema* spores in the inter-  
478 laboratory comparison samples. The boxes indicate the individual z-score for the three replicates of  
479 the POS2 sample (Fig 3A) and POS3 sample (Fig 3B) tested by each participant. The 1% significance  
480 level is indicated by the red line ( $k = 2.08$ ).



481  
482  
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484

485 Figure 4. Type of counting chambers used by the 23 participants.



486  
487

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