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23 **Abstract**

24

25 There are several methods for cultivation of *Paenibacillus larvae*, the causative agent of
26 American foulbrood in honey bees. Protocols for detection of sub-clinical levels of the
27 bacterium from honey and bee samples include heat treatment of samples. The main
28 objective of this study was to investigate if there is variability in temperature resistance
29 among *P.larvae* genotypes, potentially leading to biased diagnose and disease
30 monitoring. The variation in germination and proliferation ability among type collection
31 (N=4) and field isolates (N=4) of *Paenibacillus larvae* representing four different
32 genotypes was investigated. Results demonstrate a significant variability between *P.*
33 *larvae* genotypes in germination rate on solid media as well as in endospore resistance to
34 heat treatment and storage. It is concluded that strains of different genotypes should be
35 included in evaluation of standard laboratory protocols for cultivation of *P. larvae* to
36 avoid bias in disease monitoring and quantification of the pathogen.

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41 **Keywords:** American foulbrood / endospores / genotypes / germination / *Paenibacillus*

42 *larvae*

43 1. Introduction

44 American foulbrood (AFB) is a severe bacterial disease affecting apiculture worldwide.
45 AFB is lethal to infected honey bee larvae and can be devastating to colonies and apiaries
46 if proper control measures are not carried out. The etiological agent, *Paenibacillus larvae*
47 (Genersch et al., 2006) is an endospore-forming, Gram-positive rod. Bacterial sub-typing
48 has demonstrated considerable diversity within the species, and at least four distinct
49 genotypes have been described (Ashiralieva and Genersch, 2006). Traditional diagnosis
50 of AFB is based on observation of clinical symptoms and microbial cultivation of
51 material from infected colonies. Several diagnostic and protocols have been presented, all
52 including heat treatment of samples to avoid contamination of other bacteria and
53 stimulate germination (table 1).

54 The number of bacterial cells in a solution can be measured either by microscopic count
55 (total cell count) or by counting colonies growing on artificial media (viable count). Plate
56 counts can be highly unreliable, and microscopic counts typically reveal far more spores
57 than germinate on any given culture medium. Thus, total spore number can be
58 underestimated by several orders of magnitude (Madigan et al., 2003). Selection of
59 media, choice of temperature treatment as well as awareness of the discrepancy between
60 total cell count and viable cell count are important when performing studies on *P. larvae*.
61 Moreover, the documented genetic variability between isolates raises the question if
62 methods used will yield genotype specific results. Thus, the primary goal of the current
63 study was to investigate if the response to various heat treatments is strain and/or
64 genotype specific. Additionally, we investigated if the germination rate and endospore
65 storage durability is genotype specific.

66 **2. Material and Methods**

67 *2.1 Bacterial strains*

68 Eight strains of *P. larvae* were used in the experiment representing the four different
69 genotypes described using ERIC primers (Genersch et al., 2006). Four of the isolates
70 were commercially available reference or type collection strains: ATCC 9545 (ERIC I),
71 LMG 16252 (ERIC III), LMG 16247, LMG 14427 (ERIC IV). The remaining four strains
72 were isolated from samples of Swedish honey bee brood with symptoms of AFB (field
73 strains), and deposited at the Culture Collection of the University of Gothenburg: CCUG
74 48979, CCUG 48978 (ERIC I), CCUG 48972, CCUG 48973 (ERIC II).

75 Bacterial strains were cultivated on MYPGP-agar plates as described by Nordström and
76 Fries, 1995, and incubated at 35°C and 5% CO₂ for 10-14 days to obtain sporulation.
77 Fresh spore suspensions (stock solutions) were prepared by suspending bacterial colonies
78 in sterile 0.9 % NaCl. Suspensions were checked under microscope to confirm presence
79 of endospores and absence of vegetative cells. The stock spore suspensions were stored at
80 4°C.

81 *2.2 Colony and microscopic count*

82 A ten-fold dilution series from each stock suspension was made by successive dilutions
83 to a final dilution of 10⁻⁶. Ten micro liters of the dilutions were spread over the surface of
84 an agar plate using a sterile spreader. Each dilution was inoculated onto 3 plates to obtain
85 a mean plating value for colony forming units (CFU). Plates representing the dilution
86 with countable (30-100) CFU were used in the final estimation of the number of viable
87 spores in the undiluted stock suspension.

88 A direct microscopic count of the spore suspensions was made in sub-samples of the
89 stock suspensions using a Helber Bacteria Counting Chamber (Hawksley), according to
90 the manufacturer's recommended protocol. Counting was made using a phase-contrast
91 light microscope (400^x). Three sub-samples of each stock spore suspension were counted
92 and a mean concentration value was calculated. New stock spore suspensions were
93 prepared three times, and each suspension underwent the microscopic/colony count
94 comparison three times, resulting in a total of nine repetitions for each strain.

95 2.3 Heat treatment

96 Aliquots of fresh spore suspensions with known concentrations adjusted to 2.5×10^6
97 spores per milliliter (microscopic count) were heat treated in a block heater (Grant
98 Instruments Ltd) at 80, 85, 90, 95 and 100°C respectively for 10 minutes. One aliquot
99 was left without any treatment and cultivated as untreated control. Ten micro liters from
100 each aliquot were repeatedly spread on 3 agar plates in order to get a mean value of CFU.
101 The experiment was repeated 5 times using all specified *P. larvae* isolates.

102 2.4 Storage

103 Stock spore solutions adjusted to 6×10^5 spores (microscopic count) per milliliter sterile
104 NaCl were stored at 4°C. Colony count was carried out as earlier described and repeated
105 3 times after <1, 6, 12 and 18 months of storage respectively.

106 2.5 Statistical analysis

107 The germination rate of each isolate (nine repetitions per isolate) were compared by using
108 an one-way ANOVA test followed by Fisher's probable least-squares difference (PLSD)
109 test to detect differences between isolates or between genotypes. A two-way ANOVA
110 test was used for analyzing the influence of temperature and genotype on germination

111 rate. The effect from storage on germination rate was tested using a two-way ANOVA
112 with genotype and storage as independent variables

113

114 **3. Results**

115 *3.1 Colony count versus microscopic count*

116 Comparing the average germination rate (viable count versus total microscopic count),
117 revealed a significant variability in spore germination ability between genotypes (Figure
118 1). Spores representing the ERIC IV (LMG 16247, LMG 14427) genotype had a
119 significant ($P < 0.001$, Fisher's PLSD) higher germination rate than all other genotypes
120 (ERIC I, II and III). For example, the two isolates with the ERIC IV genotype had on
121 average a 60-fold increased germination rate compared to spores from the bacterial strain
122 classified as ERIC III (LMG 16252). Strains classified as ERIC I (ATCC 9545, CCUG
123 48979, CCUG 48978) had a significantly higher germination rate compared to the ERIC
124 III genotype ($P = 0.01$, Fisher's PLSD). Comparisons between ERIC I and ERIC II and
125 between ERIC II and ERIC III genotypes were not significantly different ($P = 0.49$ and
126 $P = 0.07$ respectively) regarding germination rate.

127 There were no significant differences in germination rates for isolates within genotype
128 ($P > 0.05$, Fisher's PLSD), except for the genotype ERIC IV, where LMG14427 had a
129 significantly higher germination rate compared to LMG16247 ($P < 0.001$, Fisher's PLSD).

130 *3.2 Temperature sensitivity*

131 The response in germination ability from different heat treatments varied strongly
132 between genotypes (and isolates) and there was a strong interaction between temperature
133 and genotypes (Table 2) suggesting that the effect from temperature is genotype specific.

134 The germination rate of strains belonging to the ERIC I genotype was highly stimulated
135 by increased temperatures (90°C and 95°C, Figure 2a). At 90°C and 95°C there was a
136 significantly higher germination rate of the field isolates representing ERIC I compared
137 to the ERIC II genotype field isolates ($P=0.005$ and $P=0.002$ respectively, t-test; Figure
138 2a). After heating to 100°C, no growth of bacteria classified as the ERIC II genotype
139 could be observed, although 1.4% and 5.5% respectively of the spores from the ERIC I
140 genotype were still able to germinate (Figure 2a). The germination ability of the reference
141 strain representing the ERIC III genotype remained almost unaffected by the heat
142 treatments, while the germination rate of the ERIC IV genotype strains decreased with
143 increasing temperature (Figure 2b).

144 3.3 Storage resistance

145 The durability of endospores showed great variation between genotypes and there was a
146 strong interaction between genotype and storage time (Table 3), suggesting that the
147 storage effect on germination ability is genotype specific. The *P. larvae* type reference
148 isolates ATCC 9545 (ERIC I), LMG 16252 (ERIC III) and LMG 16247 (ERIC IV)
149 showed no decrease in germination ability after 18 months of storage (figure 3b), whereas
150 field strains produced significantly less colonies after 18 months of storage (Figure 3a,
151 $P=0.93$ and $P<0.001$ respectively, t-test). When comparing field strains, the ERIC II
152 isolates appeared to lose some germination ability already after 6 months, whereas the
153 germination rate of the ERIC I genotype remained unaffected after 12 months of storage
154 (Figure 3a).

155

156 **4. Discussion**

157 Our results clearly demonstrate a significant variability between *P. larvae* genotypes in
158 the germination rate (Figure 1) on solid media (MYPGP, 5% CO₂), in heat treatment
159 resistance of the spores (Figure 2a, b), and in durability (Figure 3a, b). Thus, standard
160 laboratory protocols for estimating *P. larvae* infection levels and spore densities will
161 clearly yield different results depending on the investigated genotype of *P. larvae*.

162 *4.1 Germination rate*

163 The significance of variation in germination and proliferation ability among *P. larvae*
164 genotypes becomes particularly serious when the objective is to assess the bacterial load
165 in a sample. Quantifying cells from strains where only 1 % of the countable spores
166 produce colonies on solid media probably leads to a gross underestimation of the
167 potentially infectious microbial load. Hence, evaluation of new media effectiveness or
168 new diagnostic methods should include more than one genotype of *P. larvae* for more
169 complete and accurate estimates of spore loads or infection levels. It must also be
170 recognized that in their natural environments (i.e. in the mid-gut of the honey bee larvae)
171 germination rates of *P. larvae* endospores is highly likely to reach levels not observed in
172 the laboratory on any solid media. The two isolates representing the ERIC IV-genotype
173 (LMG 162427, LMG 14427) are superior among the investigated bacterial strains in their
174 ability to utilize the presented media for germination and proliferation (Figure 1). It could
175 be argued that these reference strains are domesticated and better adapted to normal
176 laboratory conditions compared to strains isolated directly from diseased larvae (Palková,

177 2004). However, the LMG 16252 (ERIC III-genotype) commercially available reference
178 strain is seemingly not as well adapted to *in vitro* growth (Figure 2b).

179 4.2 Heat treatment

180 Heat treatment of endospores is a standard procedure in many protocols for cultivation of
181 *P. larvae*. The result from this study clearly demonstrates that some isolates are more
182 sensitive to heat than others (Figure 2a, b). Thus, it is strongly advisable to include *P.*
183 *larvae* strains of more than one genotype in the evaluation of standard laboratory
184 protocols. Also field isolates in this study responded very different to heat treatment. The
185 germination rate of the endospores of the ERIC I genotype was strongly stimulated by
186 heating to 90° and 95°C, while the field isolates of ERIC II type responded negatively to
187 all temperature treatments (Figure 2a). This will undoubtedly lead to a higher probability
188 of finding *P. larvae* isolates of the ERIC I genotype compared to isolates of the ERIC II
189 genotype when protocols including heating to 90°C and above are used. For field
190 monitoring of disease prevalence or spore contamination levels, this is a most serious
191 bias. The presented results suggest that the apparent dominance of the ERIC I genotype
192 in field isolates of *P. larvae* (unpublished data) may partly be due to the lower probability
193 of detecting the more heat sensitive ERIC II genotype. The fact that germination of
194 endospores of the ERIC I genotype are stimulated by heat treatments, whereas spores of
195 ERIC II genotype lose germination ability with increasing temperature, further decreases
196 the probability of detecting isolates representing the latter genotype. This is a problem,
197 since some kind of heat treatment is necessary to avoid contamination of other micro-
198 organisms when monitoring for *P. larvae*, in particular when adult bee samples are used.
199 Thus, evaluation of standard laboratory protocols must be a balance between detection of

200 endospores representing all genotypes and avoiding overgrowth of contaminating micro-
201 organisms.

202 4.3 Storage resistance

203 Durability of the endospores demonstrated great variability between genotypes. There
204 was a striking difference in spore survival between field and type collection isolates
205 (figure 3a, b). The germination ability of the endospores of investigated reference strains
206 remained unaffected after 18 month storage (Figure 3b). It is likely that the generally
207 higher germination rate of the reference strain LMG 16247 as well as improved storage
208 durability is a result of bacterial adaptation to laboratory conditions. There is also a trend
209 that after 12 month of storage, field isolates of the ERIC I type remain unaffected,
210 whereas the germination ability of the ERIC II type isolates has decreased (Figure 3a). It
211 is well known that *P. larvae* endospores may remain viable for decades (Shimanuki,
212 1997). However, even if some spores may remain viable over long periods, our data
213 clearly demonstrate an influence from storage time on spore viability, and that the storage
214 effect on germination ability differs between genotypes of the bacterium. The durability
215 data in this study is small and the time of storage relatively short, but the indicated
216 differences in spore viability after storage are clearly worth to be studied further.

217 It could be argued that spore survival and germination success are factors strongly
218 influencing differences in virulence among strains and genotypes of *P. larvae*. In this
219 perspective, some of the field strains (ERIC II genotype) may be less virulent at colony
220 level since the viability of the endospores seems to decrease more rapidly than the
221 viability of the spores of the ERIC I type. Moreover, the lower resistance to heat may also

222 hamper the spread of this bacterial genotype in comparison to other isolates of the
223 pathogen.

224 **5. Conclusion**

225 We can conclude that development and evaluation of standard laboratory protocols for *P.*
226 *larvae* should always include strains of different genotypes, since the variation in
227 germination ability and the response to heat treatment is so variable. This conclusion is
228 emphasized by discrepancies in germination ability after heat treatment, not only between
229 field isolates and laboratory cultures, but also between field isolates of different
230 genotypes.

231

232 **Acknowledgement**

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234

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280 honey. Acta Biotechnol. 16, 57-64.

281

282

1 **Table 1.** Temperature and duration of heat treatment in various investigations of the
 2 presence of *Paenibacillus larvae* spores in samples of honey and adult bees

3

Publication	Heat treatment of bee samples	Heat treatment of honey samples
Pernal and Melathopoulos, 2006	85° C for 15 min	85° C for 15 min
Hansen, 1984	-	88-92° C for 5 min
Hornitzky and Karlovskis, 1989	80° C for 15 min	-
Dingman and Stahly, 1983	-	65° C for 15 min
Schuch et al., 2001	-	80° C for 15 min
Nordström et al., 2002	85° C for 10 min	88° C for 10 min
Hornitzky and Clark, 1991	-	80° C for 15 min
Alippi et al., 2004	-	85-90° C for 10 min
Lindström and Fries, 2005	91° C for 10 min	-
Genersch and Otten, 2003	-	90° C for 6 min
Steinkraus and Morse, 1996	-	80° C for 10 min
Goodwin et al., 1996	93° C for 20 min	-
Nordström and Fries, 1995	-	80-82° C for 10 min
Shimanuki and Knox, 1988	-	80° C for 10 min

4

5 **Table 2.** ANOVA table for germination rate with heat treatment (untreated, 80, 85, 90,
6 95 and 100°C) and genotype (ERIC I, II, III and IV) as independent variables

7

Variable	Degrees of freedom	Mean square	F-value	P-value
Temperature	5	0.108	18.71	<0.001
Genotype	3	0.560	96.71	<0.001
Temperature*Genotype	15	0.066	11.42	<0.001
Residual	227	0.006		

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10 **Table 3.** ANOVA table for germination rate with storage time (<1 month, 6, 12 and 18
11 months) and genotype (ERIC I, II, III and IV) as independent variables

12

Variable	Degrees of freedom	Mean square	F-value	P-value
Storage time	3	0.014	2.99	0.038
Genotype	3	0.649	133.64	<0.001
Temperature*Genotype	9	0.021	4.27	<0.001
Residual	69	0.005		

13

14 **List of captions**

15

16

17 **Figure 1.** Average germination rate (% , viable count versus total microscopic count) of
18 all investigated and untreated strains (N=9 for each strain). Error bars represent standard
19 error.

20

21 **Figure 2a, b.** Average germination rate (%) of field isolates (a) and reference isolates (b)
22 after heat treatment (N=5 for each isolate). Error bars represent standard error.

23

24 **Figure 3a, b.** Average germination rate (%) of field isolates (a) and reference isolates (b)
25 after 6, 12 and 18 months of storage (N=3 for each strain). The ERIC IV genotype is only
26 represented by strain LMG 16247 in this experiment. Error bars represent standard error.

27

28

29

30

Figure 1

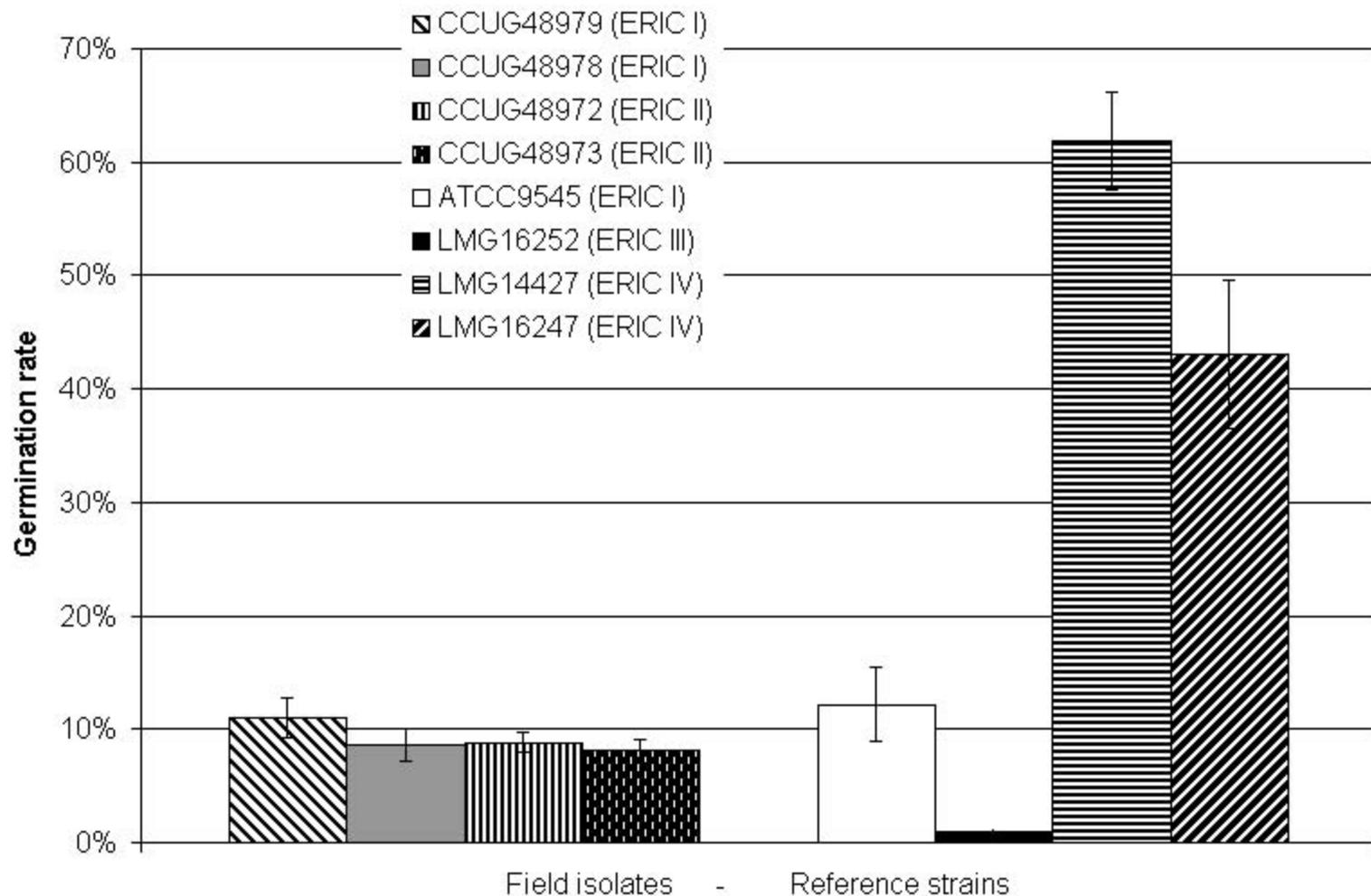


Figure 2a

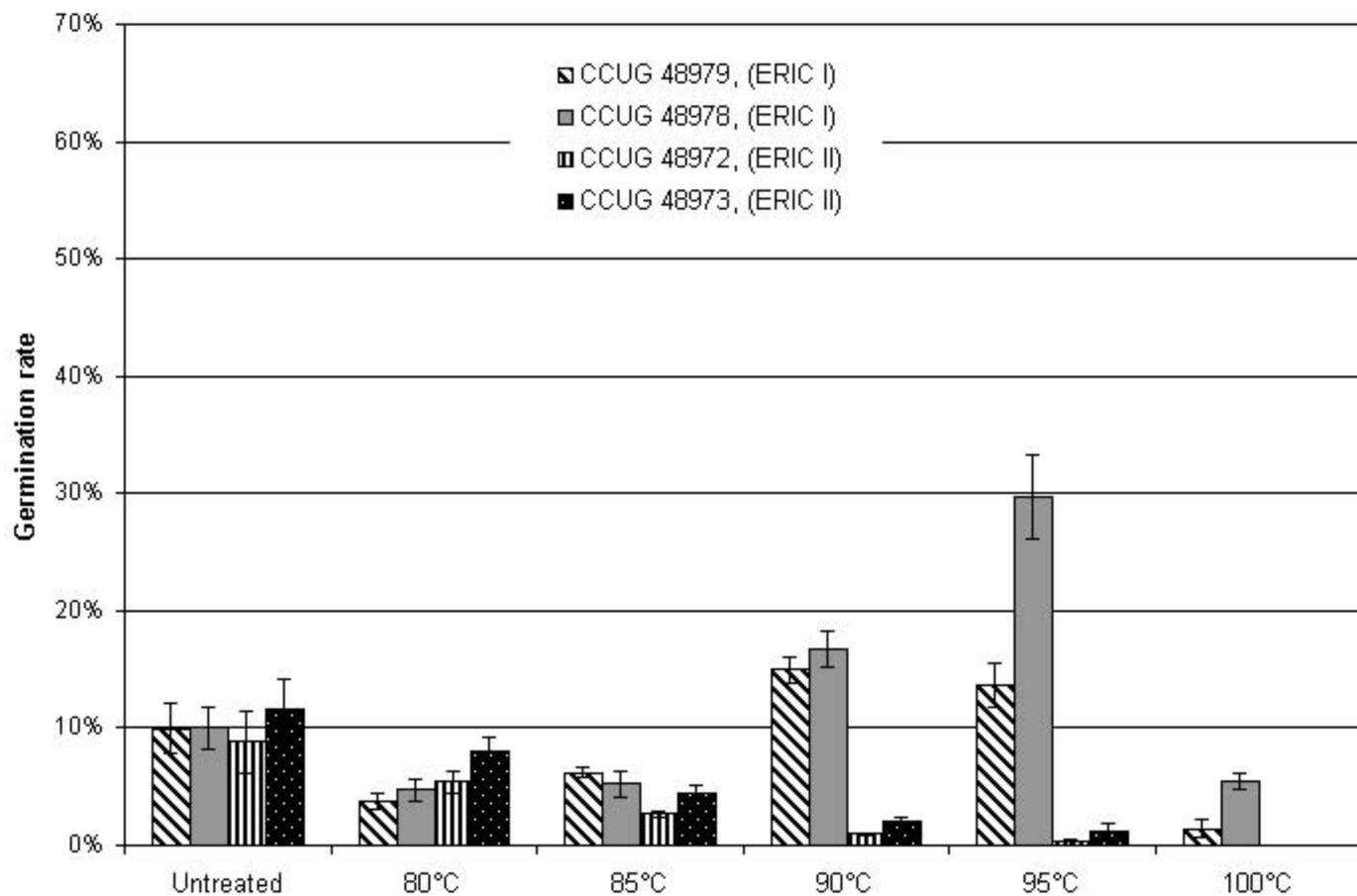


Figure 2b

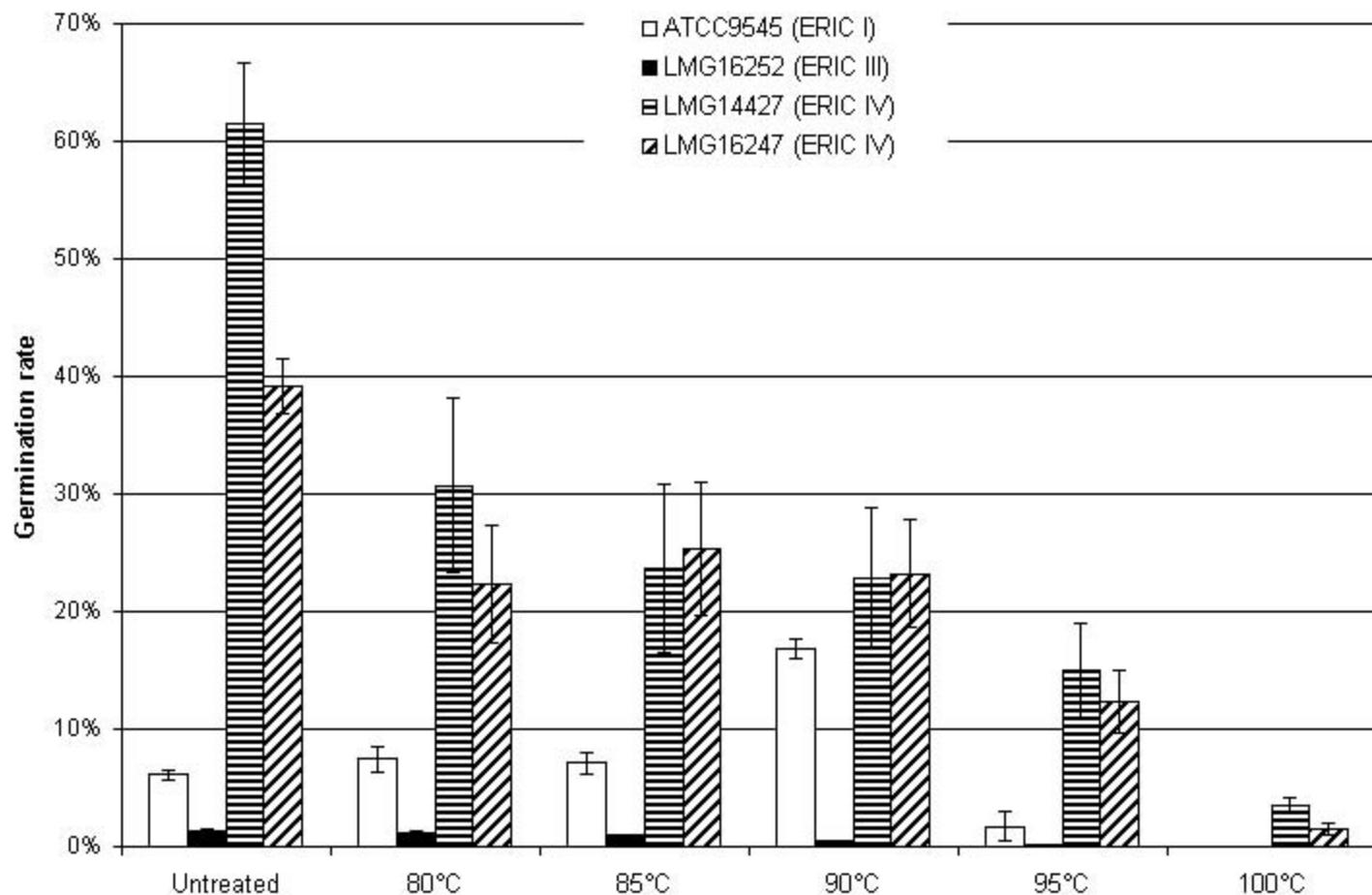


Figure 3a

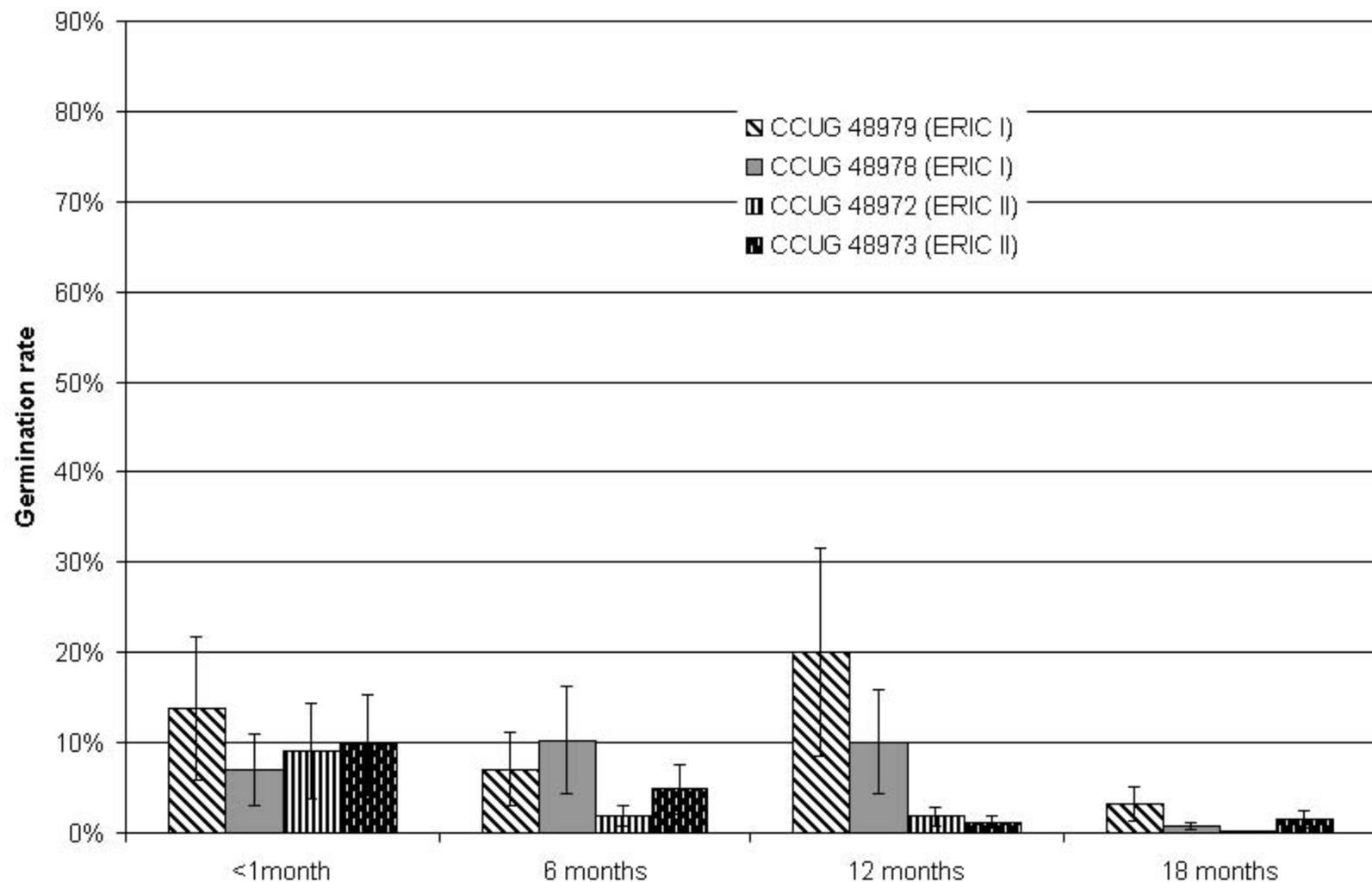


Figure 3b

