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Long-term storage of boar seminal doses contaminated with Proteus vulgaris: a dose-dependent effect on sperm motility and sperm-bacteria interaction

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Abstract:	This study evaluated how *Proteus vulgaris* affects sperm quality and sperm-bacteria interaction in stored semen samples. A strain of *P. vulgaris* resistant to streptomycin, penicillin, lincomycin and spectinomycin was added to boar semen in doses of 10^3, 10^5, 10^6, 10^7 and 10^8 CFU/mL. A sample in which there was no addition of *P. vulgaris* was the negative control. Sperm quality was determined by evaluating sperm motility and morphology using the computer-assisted sperm analysis (CASA) system, and plasma membrane and acrosome integrity using flow cytometry at 0, 1, 2, 4, 6, 8 and 10 days of liquid-storage at 17 ºC. At the same time points, pH, sperm agglutination, sperm-bacteria interaction and bacterial growth were also assessed. There was impaired sperm motility when the infective doses of *P. vulgaris* were equal to or greater than 10^5 CFU/mL with the effect being dose-dependent ($P<0.05$). When infective doses of *P. vulgaris* were of 10^6 to 10^8 CFU/mL there were impairments of plasma membrane and acrosome integrity by Day 1 of storage ($P<0.05$), and alkalinisation of the storing medium by Day 4 ($P<0.05$). Bacterial adhesion increased when infective dose of *P. vulgaris* was greater and as duration of storage increased. *P. vulgaris* had a high affinity for the mid- and principal pieces of sperm cells. It is concluded there were alterations in sperm motility in samples infected with *P. vulgaris* that were associated with bacterial adhesion and medium alkalinisation.
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Cover Letter

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16th December 2019

Prof James E Kinder
Editor
Animal Reproduction Science
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Dear Editors,

We would be very interested in publishing an original manuscript entitled "Long-term storage of boar seminal doses contaminated with *Proteus vulgaris:* a dose-dependent effect on sperm motility and sperm-bacteria interaction" in Animal Reproduction Science. The Manuscript is authored by Delgado-Bermúdez A, Bonet S, Yeste M and Pinart E and it focus on the study of the bacterial contamination by *Proteus vulgaris* on the sperm quality and boar-sperm interaction under long-term liquid storage.

We look forward to hearing from you.

Yours sincerely,

Elisabeth Pinart, PhD

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Kind regards,

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The authors have addressed the reviewers' comments; the revised manuscript has been edited for the author's consideration before the final decision.

Highlights

P. vulgaris affects sperm motility from day 1 of refrigeration at ≥ 10^5 CFU mL^{-1}.

P. vulgaris alters sperm viability from day 1 of refrigeration at ≥ 10^6 CFU mL^{-1}.

P. vulgaris adheres to sperm surface from the first 24h of refrigeration.

P. vulgaris shows high affinity towards mid and principal pieces of boar sperm.

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1 **Title**

2 # Long-term storage of boar seminal doses contaminated with

3 # *Proteus vulgaris:* a dose-dependent effect on sperm motility and

4 # sperm-bacteria interaction

5

6 **Running title**

7 Boar sperm and *Proteus vulgaris*: Is sperm quality affected?

8

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19

20 **ABSTRACT**

21 This study evaluated how *Proteus vulgaris* affects sperm quality and sperm-bacteria
22 interaction in stored semen samples. A strain of *P. vulgaris* resistant to streptomycin,
23 penicillin, lincomycin and spectinomycin was added to boar semen in doses of 10^3, 10^5,
24 10^6, 10^7 and 10^8 CFU/mL. A sample in which there was no addition of *P. vulgaris* was
25 the negative control. Sperm quality was determined by evaluating sperm motility and
26 morphology using the computer-assisted sperm analysis (CASA) system, and plasma
27 membrane and acrosome integrity using flow cytometry at 0, 1, 2, 4, 6, 8 and 10 days of
28 liquid-storage at 17 ºC. At the same time points, pH, sperm agglutination, sperm-
29 bacteria interaction and bacterial growth were also assessed. There was impaired sperm
30 motility when the infective doses of *P. vulgaris* were equal to or greater than 10^5
31 CFU/mL with the effect being dose-dependent (P<0.05). When infective doses of *P.*
32 *vulgaris* were of 10^6 to 10^8 CFU/mL there were impairments of plasma membrane and
33 acrosome integrity by Day 1 of storage (P<0.05), and alkalinisation of the storing
34 medium by Day 4 (P<0.05). Bacterial adhesion increased when infective dose of *P.*
35 *vulgaris* was greater and as duration of storage increased. *P. vulgaris* had a high affinity
36 for the mid- and principal pieces of sperm cells. It is concluded there were alterations in
37 sperm motility in samples infected with *P. vulgaris* that were associated with bacterial
38 adhesion and medium alkalinisation.

39

40 *Keywords*: *Proteus vulgaris*; Boar; semen quality; Sperm-bacteria interaction

41

42

43

44 **1. Introduction**

45 Artificial insemination (AI) is currently associated to the bacterial contamination
46 of semen during collection and dilution (Schulze et al., 2015; Kuster and Althouse,
47 2016; Pezo et al., 2019). Most bacteria present in extended boar semen belong to the
48 Enterobacteriaceae family with the effects of contamination by *Escherichia coli*,
49 *Pseudomonas* spp., *Enterobacter* spp., or *Staphylococcus* spp. having been extensively
50 reported (Bussalleu et al., 2011; Prieto-Martínez et al., 2014; Sepúlveda et al., 2014;
51 Pinart et al., 2017; Bonet et al., 2018). Nevertheless, there are few reports regarding the
52 effects of *Proteus spp.* on sperm quality even though results from some studies indicate
53 *Proteus mirabilis* can survive and proliferate in extended semen (Yániz et al., 2010;
54 Úbeda et al., 2013). *Proteus spp.* is a gram-negative, anaerobic facultative and rod-
55 shaped bacterium that is widely distributed in the natural environment, especially in
56 polluted water or in the soil. This bacterium can reside in the intestines of humans and
57 of wild and domestic animals (Rózalski et al., 1997), and can contaminate semen during
58 collection and processing.

59 In Europe, antibiotic addition to extenders used for semen dilution is regulated
60 by the Council Directive 90/429/EEC. Nevertheless, most bacteria have become
61 resistant to antibiotics, and this allows bacterial proliferation to occur in diluted seminal
62 doses, resulting in a decrease in their sperm quality and fertilisation capacity (Schulze et
63 al., 2015, 2016).

64 In the present study, there was analysis of how *Proteus vulgaris* affects sperm
65 quality and interacts with spermatozoa in liquid-stored boar semen stored at 17ºC. To
66 conduct the study, semen or sperm were analysed for pH, plasma membrane and
67 acrosome integrity, morphology, motility, agglutination and bacterial adhesion of *P.*
68 *vulgaris* to sperm cells in samples contaminated with different infective doses (10^3 to
69 10^8 CFU/mL) of *P. vulgaris*, and stored at 17ºC for 10 days. The aim in conducting this

70 experiment was to mimick how contamination with an antibiotic-resistant *P. vulgaris*
71 strain during semen collection and processing results in decreases in sperm quality and
72 to which part of the sperm cell is there adherence of *P. vulgaris*.
73

74 **2. Materials and methods**

75 *2.1. Semen samples*

76 This study included ten sexually mature Piétrain boars (age: 1.5-2.5 years old)
77 from the same genetic line (Semen Cardona, Cardona, Spain). All males had been
78 previously used in artificial insemination (AI) programs, and produced ejaculates with
79 highly acceptable sperm quality, with there being more than 80% morphologically
80 normal spermatozoa in the ejaculate, 80% total motility, 60% progressive motility, 80%
81 sperm viability and 80% spermatozoa with an intact acrosome (data not shown). Boars
82 were all housed in the same facility with the same husbandry conditions being imposed
83 [i.e., fed the same diet and provided water *ad libitum,* and subjected to a semen
84 collection twice per week using procedures consistent with the guidelines established by
85 the Animal Welfare Directive of the Regional Government of Catalonia, Spain
86 (Generalitat de Catalunya, 2013)].

87 Ejaculates were collected using the gloved-hand technique. The sperm-rich
88 fraction of each ejaculate was filtered through gauze to remove the gel, and diluted in
89 Vitasem® (Magapor, Zaragoza, Spain), a long-term preservation extender, to an average
90 concentration of 35 to 42 $\times$ 10^6 spermatozoa/mL. Samples were cooled to 17 ºC and
91 packaged in 90-mL commercial doses containing 3 $\times$ 10^9 spermatozoa/dose. Two doses
92 per boar were transferred to the Centre for Biotechnology of Animal and Human
93 Reproduction (TechnoSperm), University of Girona (Spain) in a heat-insulating
94 container at 17 ºC.

95

96 *2.2. Infection of seminal doses*

97 For commercial reasons, there could not be determination of the exact
98 composition of Vitasem® extender. According to the information provided by the
99 company, however, the antibiotic composition of this extender is consistent with the
100 guidelines of the European Council Directive 90/429/ECC and the Implementing
101 Regulation 176/2012 by which "the combination of antibiotics in extended sperm
102 should produce an effect on final diluted sperm at least equivalent to the following
103 concentrations: a) not less than 500 μg of streptomycin per ml of definitive solution; b)
104 not less than 500 I.U. of penicillin per ml of definitive solution; c) not less than 150 μg
105 de lincomycin per ml of definitive solution; and d) not less than 300 μg of
106 spectinomycin per ml of definitive solution." These four antibiotics are commonly
107 present in boar semen extenders, alone or in combination.

108 With regard to bacteria used to infect semen samples, different strains of *Proteus*
109 *vulgaris* were purchased from Spanish Type Culture Collection (CECT, Valencia,
110 Spain; www.cect.org) and evaluated for penicillin, streptomycin, lincomycin and
111 spectinomycin resistance. There was selection of strain CECT167 because it was
112 resistant to the four antibiotics. The strain was cultured in liquid Luria Bertani (LB)
113 broth (Conda/Pronadisa; Madrid, Spain) at 37 ºC for 18 to 24 h in a shaking bath
114 (Memmert Water bath WNB 7-45; Schwabach, Germany). Bacteria concentration of
115 pure *Proteus vulgaris* cultures was assessed using a spectrophotometer (SmartSpec™
116 Plus, Bio-Rad; Irvine, California, USA) at a wavelength of 600 nm (optical density,
117 OD_{600}).

118 For each boar, the two seminal doses were pooled and there was apportioning
119 into six aliquots of 20 mL each. One aliquot was used as a control (non-infected

120 samples), whereas the others were infected with *Proteus vulgaris* (CECT167 strain) at
121 the following initial bacterial concentrations (Day 0): 1×10^3, 1×10^5, 1×10^6, 1×10^7
122 and 1×10^8 Colony Forming Units per mL (CFU/mL). Both non-infected and infected
123 samples were stored in closed tubes at 17 ºC for 10 days and temperature was controlled
124 using a digital system (Magapor®; Zaragoza, Spain).
125
126 *2.3. Semen characteristic analyses*
127 Analyses of semen characteristics of infected and non-infected samples were
128 performed when samples were received in the laboratory (Day 0) and after Days 1, 2, 4,
129 6, 8 and 10 of liquid-storage at 17 ºC. The effects of different infective doses on semen
130 characteristics were determined from the analysis of pH, sperm plasma membrane
131 integrity, acrosome integrity, motility, morphology and agglutination.
132
133 *2.3.1. pH measurement*
134 In non-infected and infected samples, pH was determined at Days 0, 1, 2, 4, 6, 8
135 and 10 of liquid-storage at 17 ºC. Evaluations were performed in triplicate using a
136 digital pH meter Hanna HI-254 pH/ORP Meter (Hanna Instruments, S.L.; Eibar, Spain).
137 For each sample and Day, the mean and its corresponding standard error of the mean
138 (SEM) was calculated.
139
140 *2.3.2. Flow cytometry assays*
141 Flow cytometry was used to evaluate the integrities of plasma membrane
142 (SYBR14/PI) and acrosome (PNA-FITC/PI). In each assay, sperm concentration was
143 first adjusted to 1×10^6 spermatozoa/mL in Beltsville Thawing Solution (BTS) in a
144 final volume of 0.5 mL. Three replicates per infective dose, incubation time and semen

145 characteristics were examined using a Cell Laboratory QuantaSC™ cytometer
146 (Beckman Coulter; Fullerton, California, USA).

147 Information about flow cytometry analyses conducted in the present study is
148 provided based on the recommendations of the International Society for Advancement
149 of Cytometry (ISAC; Lee et al., 2008). Samples were excited with an argon ion laser
150 (488 nm) set at a power of 22 mW. For each particle, characteristics were evaluated and
151 plotted as Electronic Volume (EV, equivalent to Forward Scatter) and Side Scatter (SS).
152 Two optical filters were used with the following optical properties: FL1 (green
153 fluorescence): Dichroic/Splitter, DRLP: 550 nm, BP filter: 525 nm, detection width
154 505-545 nm, and FL3 (red fluorescence): LP filter: 670 nm, detection width: 670 ± 30
155 nm). Sheath fluid flow rate was set at 4.17 μL/min in all analyses and a minimum of
156 10,000 events per replicate were assessed. The threshold of the analyser was adjusted on
157 the EV channel to exclude subcellular debris (particle diameter <7 μm) and cell
158 aggregates (particle diameter >12 μm). The sperm-specific events were positively gated
159 on the basis of EV/SS distributions, and the other events were gated out.

160

161 *2.3.2.1. Plasma membrane integrity*

162 Plasma membrane integrity was determined using the LIVE/DEAD®Sperm
163 Viability Kit (Molecular Probes®, Thermofisher; Waltham, Massachusetts, USA). With
164 this kit there is inclusion of a mixture of two dyes: 1) SYBR14, a membrane permeable
165 fluorochrome that stains sperm heads green (membrane-intact spermatozoa); and 2)
166 propidium iodide (PI), a membrane impermeable fluorochrome that only penetrates
167 disrupted plasma membranes, staining sperm heads red (membrane-damaged
168 spermatozoa). Sperm samples were stained using the protocol described by Garner and
169 Johnson (1995).

170 Spermatozoa with an intact plasma membrane were SYBR14$^+$ and PI$^-$
171 (SYBR14$^+$/PI$^-$), whereas spermatozoa with a damaged plasma membrane could have
172 different staining patterns (SYBR14$^+$/PI$^+$ or SYBR14$^-$/PI$^+$). Non-stained particles
173 (SYBR14$^-$/PI$^-$) were considered to be debris particles.

174

175 *2.3.2.2. Acrosome integrity (PNA-FITC/PI)*

176 Acrosome integrity was evaluated using double staining procedures of sperm
177 cells with the lectin from *Arachis hypogaea* (peanut agglutinin, PNA) conjugated with
178 fluorescein isothiocyanate (FITC) and PI (PNA-FITC/PI test). Sperm samples were
179 stained using the protocol described by Nagy et al. (2003). Because spermatozoa were
180 not previously permeabilized, four different sperm populations were identified (Yeste et
181 al., 2014): 1) spermatozoa with intact plasma and outer acrosome membranes (PNA-
182 FITC$^-$/PI$^-$); 2) spermatozoa with damaged plasma membrane and an outer acrosome
183 membrane that could not be fully intact (PNA-FITC$^+$/PI$^+$); 3) spermatozoa with
184 damaged plasma membrane and without an outer acrosome membrane (PNA-FITC$^-$
185 /PI$^+$); and spermatozoa with a damaged plasma membrane (PNA-FITC$^+$/PI$^-$).
186 Percentages of intact spermatozoa (PNA-FITC$^-$/PI$^-$) were corrected to avoid an
187 overestimation of sperm-events in this quadrant (q_1), as recommended by Petrunkina et
188 al. (2010). Acrosome integrity is expressed as the percentage of viable spermatozoa
189 with an intact acrosome (PNA-FITC$^-$/PI$^-$) for each infective dose and incubation time.

190

191 *2.3.3. Evaluation of sperm motility*

192 Sperm motility was evaluated using the Computer-Assisted Sperm Analysis
193 (CASA) system utilising the protocol described by Pinart et al. (2017). A minimum of
194 three replicates per sample and time point were evaluated, with 1,000 spermatozoa

195 being counted with each analysis. Twenty-five consecutive digitalized frames were
196 acquired in each field, and two motility parameters were assessed: total motility
197 (spermatozoa showing an average path velocity (VAP) ≥ 10 µm/s) and progressive
198 motility (spermatozoa showing percentage of straightness (STR) more ≥ 45%). For each
199 infective dose and day of storage at 17 ºC, sperm motility is expressed as percentages of
200 total and progressively motile spermatozoa.
201
202 *2.3.4. Evaluation of sperm morphology and sperm agglutination*
203 To assess sperm morphology, samples were incubated with 2% formaldehyde in
204 PBS for 5 min at room temperature. Sperm morphology was analysed using the
205 SCA®Production software (Sperm Class Analyser Production, 2010; Microptic SL,
206 Barcelona), and spermatozoa were classified as morphologically normal, with proximal
207 or distal droplets, or aberrant (with head and/or tail anomalies) (Pinart et al., 2017;
208 Bonet et al., 2018). Three replicates of 100 spermatozoa each were evaluated per
209 infective dose and day of storage. Because percentages of spermatozoa with
210 proximal/distal droplets and aberrant spermatozoa were less than 10 % in all trials,
211 sperm morphology is expressed as the percentage of morphologically normal
212 spermatozoa.
213 The same slides were used to evaluate sperm agglutination by determining the
214 presence of white clumps with at least three spermatozoa at 1,000× magnification.
215 Extent of sperm agglutination was determined by counting the number of
216 aggregates/clumps per field and the number of spermatozoa in each clump in 25
217 different fields of view. Agglutination was classified as none, small amount, moderate
218 amount and large amount according to previously established criteria (Pinart et al.,
219 2017).

220

221 *2.4. Evaluation of interaction between spermatozoa and bacteria*

222 The extent of interaction between spermatozoa and *Proteus vulgaris* throughout
223 storage at 17 ºC was analysed from the count of the percentage of spermatozoa in
224 contact with bacteria at 1,000× magnification. Samples were prepared as described in
225 the previous section of this manuscript and classified according to the number of
226 bacteria adhered to sperm (Bonet et al., 2018). The number of bacteria adhered to the
227 sperm surface ranged from 1 to 10. Because the percentage of spermatozoa having five
228 or more bacteria adhered was less than 0.5%, these are shown as a single sperm
229 category. There was also determination of the percentages of bacteria adhered to the
230 sperm head and to the sperm tail and the percentages of bacteria adhered to the different
231 head and tail regions of spermatozoa (Bonet et al., 2018).

232

233 *2.5. Assessment of bacterial growth*

234 Bacterial growth was evaluated at each infective dose and time point by plate
235 culturing with Luria Bertani (LB) agar. Dilutions were made with Ringer's solution and
236 plates were incubated at 40 ºC for 72 h, and counted at 24, 48 and 72 h.

237

238 *2.6. Statistical analysis*

239 Statistical analyses were performed using SPSS 25.0 for Windows (IBM®
240 SPSS® Inc.; Armonk, New York, USA). Sperm quality and function variables (% total
241 motile spermatozoa, % progressively motile spermatozoa, % morphologically normal
242 spermatozoa, % membrane-intact spermatozoa, % acrosome-intact spermatozoa, and
243 sperm agglutination), pH, bacterial growth, and variables related to sperm-bacteria

244 interaction were considered as dependent variables. Each seminal dose (n = 10) coming
245 from a separate boar was treated as a biological replicate.
246 Variables were first evaluated for normality (Shapiro-Wilk test) and
247 homoscedasticity (Levene test). A linear mixed model (i.e. with repeated measures) was
248 used to examine differences in responses to infective doses (0, 10^3, 10^5, 10^6, 10^7 and 10^8
249 CFU/mL; inter-subjects factor) and at different storage time points (Days 0, 1, 2, 4, 6, 8
250 and 10; intra-subjects factor). Sidak's *post-hoc* was used for pair-wise comparisons.
251 When needed, data on percentages were recalculated using the arcsine square root (x)
252 transformation to accomplish normality and homoscedasticity. Data were subsequently
253 evaluated using a linear mixed model and *post-hoc* Sidak's test, as previously described.
254 With all statistical analyses, values were considered to be different when there
255 was a $P \leq 0.05$. Results are expressed as means ± standard error of the mean (SEM).
256
257 **3. Results**
258 *3.1. pH*
259 At Day 0, pH was 7.37 ± 0.19. There were similar values throughout the storage
260 period in control samples ($P > 0.05$). In *P. vulgaris* infected samples, pH was relatively
261 more alkaline from Day 4 of liquid-storage at 17 ºC in a dose dependent manner (Table
262 1).
263
264 *3.2. Plasma membrane integrity*
265 The percentages of spermatozoa with an intact plasma membrane ($SYBR14^-/PI^-$)
266 were similar during the first 4 days of liquid-storage at 17 ºC in control samples (P =
267 0.777; Fig. 1). At Day 6, there was a slight decrease (P = 0.032) in plasma membrane
268 integrity with there being similar values for plasma membrane integrity until the end of

269 the experiment (P>0.05). There was a similar pattern for plasma membrane integrity of
270 spermatozoa in samples infected with 10^3 and 10^5 CFU/mL of *P. vulgaris* (P = 0.241).
271 In samples infected with 10^6, 10^7 and 10^8 CFU/mL, the integrity of sperm
272 plasma membrane decreased after Day 1 of storage (P = 0.033). The extent of this
273 reduction in plasma membrane integrity depended on the infective dose.
274 At the end of the experiment, the average decrease in plasma membrane integrity
275 with respect to that on Day 0 was of 15% in non-infected and infected samples when
276 there were infections with 10^3 and 10^5 CFU/mL of *P. vulgaris*, of 22% when there were
277 infections of samples with 10^6 and 10^7 CFU/mL, and of 27% with infections of samples
278 with 10^8 CFU/mL.
279
280 *3.3. Acrosome integrity*
281 In control samples and in samples infected with 10^3 CFU/mL, percentages of
282 spermatozoa with an intact acrosome (PNA-FITC$^-$/PI$^-$) decreased after 4 days of storage
283 (P = 0.026; Fig. 2). In infected samples with 10^5 and 10^6 CFU/mL acrosome integrity
284 was less (P = 0.012) at Day 2, and in samples infected with 10^7 and 10^8 CFU/mL at Day
285 1 of storage (P = 0.003).
286 At the end of the experiment, the average decrease in acrosome integrity in
287 control samples and in samples infected with 10^3 and 10^5 CFU/mL of *P. vulgaris* was
288 17%, in samples infected with 10^6 CFU/mL was 22%, in samples infected with 10^7
289 CFU/mL was 25%, and in samples infected with 10^8 CFU/mL was 30%.
290
291 *3.4. Sperm motility*
292 In control samples, percentages of total (P = 0.008) and progressive (P = 0.002)
293 motile spermatozoa was less after 4 and 6 days of storage at 17 ºC, respectively (Figs. 3

294 and 4). Contamination with *P. vulgaris* resulted in a decrease in total and progressive
295 motility and with the larger infective dose there was a greater alteration in sperm
296 motility. At the end of the experiment, the average decrease in total and progressive
297 sperm motility was 17% and 18% in control samples, 19% and 20% in samples infected
298 10^3 CFU/mL, 23% and 31% in those infected with 10^5 CFU/mL, 24% and 35% in those
299 infected with 10^6 CFU/mL, 27% and 44% in samples infected with 10^7 CFU/mL, and
300 35% and 51% in samples infected with 10^8 CFU/mL.

301

302 *3.5. Sperm morphology and sperm agglutination*

303 The percentage of morphologically normal spermatozoa was 95.4 ± 2.7% at Day
304 0 and was similar during storage at 17 ºC in either non-infected or infected sperm
305 samples (P = 0.895). There were similar amounts of sperm agglutination among control
306 samples and samples infected with *P. vulgaris* (Table 2). There, however, was a small
307 amount agglutination after Day 8 of storage in control samples, from Day 6 in samples
308 infected with 10^3 CFU/mL, from Day 4 in samples infected with 10^5 CFU/mL, after
309 Day 3 in samples infected with 10^6 CFU/mL, and after Day 1 in samples infected with
310 10^7 and 10^8 CFU/mL.

311

312 *3.6. Spermatozoa-bacteria interaction*

313 Percentages of spermatozoa in contact with *P. vulgaris* varied with infective
314 dose of *P. vulgaris* and the day of storage. The larger the infective dose the greater the
315 percentage of spermatozoa bound to bacteria (P = 0.005; Fig. 5). Furthermore, for a
316 specific infective dose, the percentage of spermatozoa to which bacteria were bound
317 increased during the storing period (P<0.003).

318 An effect of infective dose and day of storage was also observed in both the
319 number of bacteria adhered on the sperm surface (Table 3) and the part of the sperm cell
320 to which the bacteria adhered (Table 4, Supplementary Figs. 1 and 2). During the first
321 24 h subsequent to the time of infection, samples infected with 10^3 CFU/mL had more
322 than 90% of spermatozoa with only one bacterium adhered, whereas in those infected
323 with 10^5 to 10^8 CFU/mL this percentage was about 70%. Interestingly, spermatozoa did
324 not have more than three bacteria adhered at any of the infective doses on Day 1. The
325 number of bacteria bound to the sperm surface increased during storage, and with the
326 larger infective dose there were a larger number of bacteria bound to the surface. At the
327 end of the experiment, about 55% of spermatozoa had only one bacterium bound to the
328 surface in samples infected with 10^3 and 10^5 CFU/mL, 50% in those infected with 10^6
329 CFU/mL, and 36% in those infected with 10^7 and 10^8 CFU/mL. At Day 10, the number
330 of bacteria bound to sperm ranged from 1 to 10, regardless of the infective dose.

331 There was binding of *P. vulgaris*, mainly, to the mid-piece, principal piece and
332 acrosomal region of boar spermatozoa (Table 4). Nevertheless, bacteria affinity for
333 these sperm regions was associated with day of storage. In effect, the affinity for mid-
334 (P = 0.010) and principal pieces (P = 0.034) increased throughout storage, whereas the
335 affinity for the acrosomal region decreased ($P<0.05$), regardless of the infective dose.
336 While the percentage of bacteria bound to the acrosomal region was 30.3±1.9% at Day
337 1, there was a decrease to 5.7 ± 1.5% at Day 10 (P = 0.003). With regard to changes in
338 the percentages of bacteria bound to mid- and principal pieces between Days 1 and 10,
339 these numbers increased from 18.2 ± 1.7% to 28.2 ± 2.3% (P = 0.023), and from 41.8 ±
340 2.4% to 50.3 ± 2.4% (P = 0.031), respectively.

341

342 *3.7. Bacterial growth*

343 Throughout liquid-storage at 17 ºC for 10 days, *Proteus vulgaris* (CECT167
344 strain) had similar growth dynamics in all contaminated samples, having a progressive
345 and sustained growth. As duration of storage period increased, differences in CFU/mL
346 among infective doses was correlated with the initial bacterial infection dose, when
347 there was the larger infective dose there was a greater CFU/mL (P = 0.017; Fig. 6).

348

349 **4. Discussion**

350 Microorganisms are usually present in commercial seminal doses, the sources of
351 contamination being of animal and/or non-animal origin (Maroto-Martin et al., 2010;
352 Schulze et al., 2015). Contaminations of animal origin result from systemic and/or
353 urogenital tract infections or in transfer from the penile surface of the boar into the
354 semen, whereas contaminations of non-animal origin can occur during semen collection
355 and processing (Maes et al., 2008). Many of these non-animal sources can subsequently
356 be a seeding point when successive semen samples are collected and exposed to that
357 source of contamination (Schulze et al., 2015). Most bacteria that are in commercial
358 seminal doses are not considered primary pathogens in swine, however, these bacteria
359 induce alterations in sperm quality, which compromise the fertilizing capacity of sperm
360 in these seminal doses (Bussalleu et al., 2011; Prieto-Martínez et al., 2014; Pinart et al.,
361 2017; Bonet et al., 2018). Much effort, therefore, is necessary to prevent the
362 contamination of seminal doses during handling.

363 Commercial extenders can contain a single antibiotic or an antibiotic cocktail to
364 inhibit bacterial growth, so the number of bacteria that have become resistant to
365 antibiotics has increased (Schulze et al., 2016). Antibiotic resistance has provided an
366 impetus for formulating antibiotic-free extenders that contain antimicrobial agents,
367 mainly antimicrobial peptides (Puig-Timonet et al., 2018); for developing new

368 strategies for hypothermic storage of the semen at 5 °C (Waberski et al., 2019); or to
369 reduce the extent of bacterial infection using single layer centrifugation (Morrell, 2019).
370 The knowledge of chemical and physical interaction between spermatozoa and bacteria
371 is essential to identify and develop alternative procedures of sanitary control of sperm
372 samples, which could favour the reduction or even result in elimination of the use of
373 antibiotics. To develop a general model about the effects of *Proteus vulgaris* on sperm
374 quality and longevity, and on sperm-bacteria interactions in refrigerated semen, in the
375 present approach there was use of a commercial strain resistant to penicillin,
376 streptomycin, lincomycin and spectinomycin, the antibiotics most commonly used in
377 semen extenders.

378 Results of previous studies indicate that members of *Proteus* spp. (either *Proteus*
379 *vulgaris* or *Proteus mirabilis*) can be found in boar semen, with an incidence around 2%
380 (Maroto-Martín et al., 2010; Úbeda et al., 2013). Nevertheless, there are few reports
381 about the detrimental effects on sperm quality, mainly because this bacterium
382 contaminates boar ejaculates along with other types of bacteria. Gao et al. (2018)
383 reported that *Proteus mirabilis* releases outer membrane vesicles during sperm storage,
384 which alter mitochondrial membrane potential and induce apoptotic-like changes at a
385 concentration greater than 10 μg/mL. These vesicles can also adhere to the plasma
386 membrane, thus affecting the capacity of spermatozoa to attach to the oocyte surface
387 (Gao et al., 2018).

388 As expected, with use of long-term extenders, there was preservation of semen
389 pH, plasma membrane integrity and sperm motility of control samples during liquid-
390 storage at 17 °C in the present study. Contamination with *P. vulgaris* resulted in a slight
391 alkalinisation of the medium after Day 4 of storage when there were infections with 10^6
392 CFU/mL or larger infective doses of *Proteus vulgaris*. In a recent study, *E. coli*

393 contamination was found to induce a slight alkalinisation of the medium at infective
394 doses equal or less than 10^6 CFU/mL (Pinart et al., 2017). In contrast, semen
395 contamination with *Enterobacter cloacae* results in a moderate amount of acidification
396 (Prieto-Martínez et al., 2014), whereas contamination with *Pseudomonas aeruginosa*
397 did not result in a change of the pH (Sepúlveda et al., 2014). Even though medium
398 acidification as a result of bacterial contamination is associated with reduced sperm
399 survival (So et al., 2011; Prieto-Martínez et al., 2014), there have been few reports on
400 the effects of alkalinisation on sperm function and survival. There have been some
401 suggestions that alkalinisation and acidification are indicative of the release of residual
402 metabolism products from bacteria into the medium (Pérez-Llano et al., 2001);
403 however, studies focusing on the changes of substrate composition of the medium
404 during liquid-storage and/or bacterial contamination of seminal doses are lacking. While
405 results from the current study indicate that slight medium alkalinisation in *Proteus*
406 *vulgaris* contaminated samples does not affect plasma membrane and acrosome
407 integrity, these findings do support that this type of contamination results in impairment
408 of progressive sperm motility.

409 In the present study, there were effects of *P. vulgaris* contamination on sperm
410 quality in long-term stored samples with there being effects of infective dose. The least
411 bacterial infective dose of 10^3 CFU/mL had little effect on sperm quality and the
412 infective dose of 10^5 CFU/mL only affected progressive sperm motility, however
413 infections doses of 10^6 CFU/mL or greater led to alterations in plasma membrane and
414 acrosome integrity and, especially, sperm motility in a dose-dependent manner.
415 Consistent with the present results, Gao et al. (2018) reported that the effects of *Proteus*
416 *mirabilis* in stored semen samples are dependent on the extent to which outer acrosome
417 vesicles are released by this bacterium into the medium and that, at infective doses of 5

418 µg/mL or less, these releases of outer acrosome vesicles do not affect the sperm quality.
419 Similarly, *Clostridium perfringens* (Sepúlveda et al., 2013; Pinart et al., 2017; Bonet et
420 al., 2018) and *Pseudomonas aeruginosa* (Sepúlveda et al., 2014) have little effect on
421 sperm quality at infective doses equal to or less than 10^6 CFU/ml. In contrast, other
422 bacterial contaminants, such as *Escherichia coli* or *Enterobacter cloacae*, have
423 deleterious effects on sperm quality at all infective doses evaluated, with the extent of
424 these sperm quality alterations being dose-dependent (Berktas et al. 2008; Bussalleu et
425 al., 2011; Prieto-Martínez et al. 2014; Pinart et al., 2017; Bonet et al., 2018).

426 An interesting finding in the present study is that alterations in sperm motility
427 induced by *P. vulgaris* at the infective doses of 10^5 CFU/mL or larger did not correlate
428 with disruptions in plasma membrane integrity or sperm agglutination. Consistent with
429 results from the present study, *P. mirabilis* induces alterations in sperm motility of
430 stored boar sperm samples by altering the mitochondrial membrane potential due to
431 increased reactive oxygen species (ROS) concentrations in the semen samples (Gao et
432 al., 2018). Sperm motility has also been described as the most reliable variable to
433 evaluate effects of bacterial contamination of boar seminal doses by *Escherichia coli*,
434 *Pseudomonas aeruginosa*, *Clostridium perfringens* or *Enterobacter cloacae* (Berktas et
435 al. 2008; Bussalleu et al., 2011; Prieto-Martínez et al. 2014; Sepúlveda et al., 2014;
436 Pinart et al., 2017; Bonet et al., 2018).

437 Findings in the present study, when there were light microscopy assessments,
438 indicate there was a lack of morphological sperm alterations in response to
439 contamination of semen samples with *P. vulgaris*. Even though there was a lack of
440 morphological alterations, it cannot be discounted that there may have been
441 ultrastructural sperm abnormalities, as previously reported in bacteria contaminated

442 semen samples from humans (Diemer et al., 2000) and boars (Bonet et al., 2018; Gao et
443 al., 2018).

444 As described for both *E. coli* and *C. perfringens* (Diemer et al., 2000, 2003;
445 Prabha et al., 2009; Kala et al., 2011; Pinart et al., 2017; Bonet et al., 2018), *P. vulgaris*
446 adheres to the plasma membrane of boar spermatozoa immediately after contamination
447 of samples. Nevertheless, based on the percentage of spermatozoa bound to bacteria and
448 the number of bacteria bound after 24 h of contamination, *P. vulgaris* had a lesser
449 adherence rate to the sperm surface than the previous described bacterial species (Bonet
450 et al., 2018). Binding of *P. vulgaris* to the boar sperm surface, however, increased
451 during semen storage at 17 ºC in a dose-dependent manner. There are few previous
452 reports about the effects of bacterial adhesion on sperm quality of liquid-stored boar
453 semen. Consistent with results in a previous study in which samples infected with *E.*
454 *coli*, where the adhesion of this bacterium to sperm cells was related with the
455 progressive decrease in plasma membrane integrity and sperm motility throughout
456 storage (Bonet et al., 2018), results from the current study validate there is a marked
457 relationship between bacterial adhesion of *P. vulgaris* and impaired sperm motility.

458 An unexpected finding in the present study was that not only did the infective
459 dose of *P. vulgaris* and duration of storage affect the percentage of spermatozoa in
460 contact with bacteria and the number of bacteria adhered to sperm cells, it was
461 ascertained that this affinity was for a specific part of the sperm. When there were
462 relatively smaller infective doses and shorter semen storage periods in the present study,
463 *P. vulgaris* had a greater affinity for the acrosomal region, mid- and principal pieces;
464 however, the affinity for the acrosomal region was less when there was the larger
465 infective doses and when semen storage was for a longer period. This change in
466 bacterial affinity may reflect changes on in the molecular composition of the sperm

467 plasma membrane during liquid-storage (reviewed from Pinart & Puigmulé, 2013). In
468 contrast with the findings in the present study, in a previous study there was an effect of
469 infective dose and duration of semen storage on bacterial adhesion but not on bacterial
470 affinity when semen samples were infected with *E. coli* and *C. perfringens* (Bonet et al.,
471 2018).
472

473 **5. Conclusions**

474 The results of the present study indicate, for the first time, that boar semen
475 contamination with *Proteus vulgaris* leads to severe impairment of progressive sperm
476 motility when infective doses are greater than 10^5 CFU/mL, due to bacterial adhesion to
477 the sperm flagellum and there are moderate extents of semen alkalinisation. The
478 relatively lesser plasma membrane and acrosome integrity when there were infective
479 doses of 10^6 CFU/mL or larger does not, however, appear to be related to bacterial
480 adhesion or alkalinisation of the semen.
481

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487

488 **Conflict of interest**

489 No author discloses any conflict of interest.
490

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615 **Figure legends**

616 **Fig. 1.** Percentage of spermatozoa with an intact plasma membrane (mean ± SEM) in
617 sperm samples from *Sus domesticus* where there were different infective doses of
618 *Proteus vulgaris* and storage at 17 ºC for 10 days; CFU/mL values indicate the
619 approximate initial bacterial count; Different superscripts (a,b) indicate differences
620 between values at the same time-point ($P < 0.05$; $n = 10$)
621
622 **Fig. 2.** Percentage of spermatozoa with an intact acrosome (mean ± SEM) in sperm
623 samples from *Sus domesticus* where there were different infective doses of *Proteus*
624 *vulgaris* and storage at 17 ºC for 10 days; CFU/mL values indicate approximate initial
625 bacterial count; Different superscripts (a-c) indicate differences between values at the
626 same time-point ($P < 0.05$; $n = 10$)
627
628 **Fig. 3.** Percentage of total motile spermatozoa (mean ± SEM) in sperm samples from
629 *Sus domesticus* where there were different infective doses of *Proteus vulgaris* and
630 storage at 17 ºC for 10 days; CFU/mL values indicate the approximate initial bacterial
631 count; Different superscripts (a-c) indicate differences in values at the same time-point
632 ($P < 0.05$; $n = 10$)
633
634 **Fig. 4.** Percentage of progressive motile spermatozoa (mean ± SEM) in sperm samples
635 from *Sus domesticus* where there were different infective doses of *Proteus vulgaris* and
636 storage at 17 ºC for 10 days; CFU/mL values indicate the approximate initial bacterial
637 count; Different superscripts (a-d) indicate differences in values at the same time-point
638 ($P < 0.05$; $n = 10$)
639

640 **Fig. 5.** Percentage of spermatozoa with bacteria adhered (mean ± SEM) in sperm
641 samples from *Sus domesticus* where there were different infective doses of *Proteus*
642 *vulgaris* and storage at 17 ºC for 10 days; CFU/mL values indicate the approximate
643 initial bacterial count; Different superscripts (a-d) indicate differences in values at the
644 same time-point ($P < 0.05$; $n = 10$)

645

646 **Fig. 6.** Bacterial growth (CFU/mL; mean ± SEM) in sperm samples from *Sus*
647 *domesticus* where there were different infective doses of *Proteus vulgaris* and storage at
648 17 ºC for 10 days; CFU/mL values indicate approximate initial bacterial count;
649 Different superscripts (a-f) indicate differences in values at the same time-point ($P <$
650 0.05; $n = 10$)

651

652 **Supplementary Fig. 1.** Percentage of bacteria adhered to the sperm head (mean ±
653 SEM) in sperm samples from *Sus domesticus* where there were different infective doses
654 of *Proteus vulgaris* and storage at 17 ºC for 10 days; CFU/mL values indicate the
655 approximate the initial bacterial count; Different superscripts (a-c) indicate differences
656 in values at the same time-point ($P < 0.05$; $n = 10$)

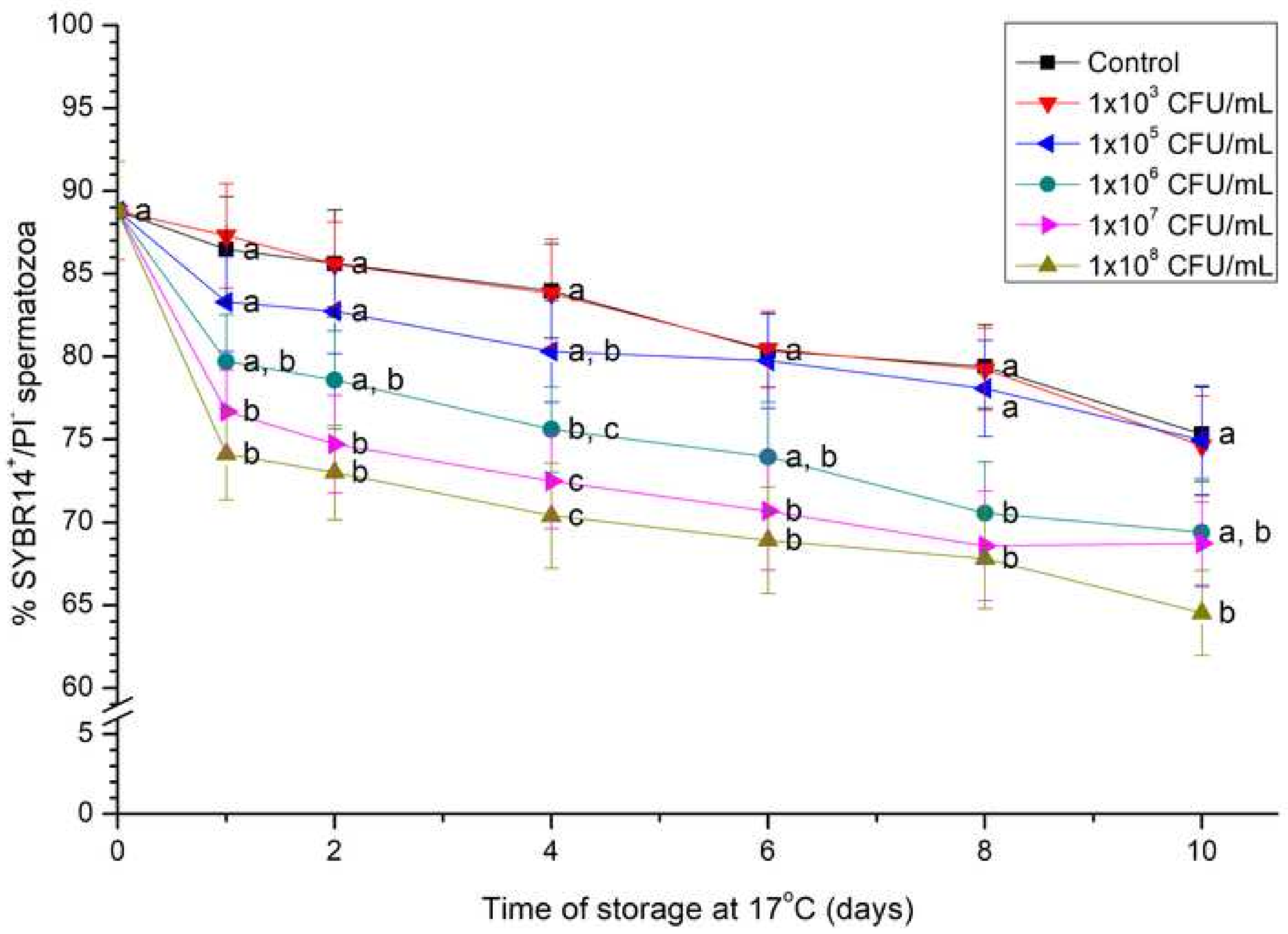
657

658 **Supplementary Fig. 2.** Percentage of bacteria adhered to the sperm tail (mean ± SEM)
659 in sperm samples from *Sus domesticus* where there were different infective doses of
660 *Proteus vulgaris* and storage at 17 ºC for 10 days; CFU/mL values indicate the
661 approximately initial bacterial count; Different superscripts (a-c) indicate differences in
662 values at the same time-point ($P < 0.05$; $n = 10$)

663

Figure

Click here to access/download;Figure;Fig. 1. Plasma membrane integrity.tif



Figure
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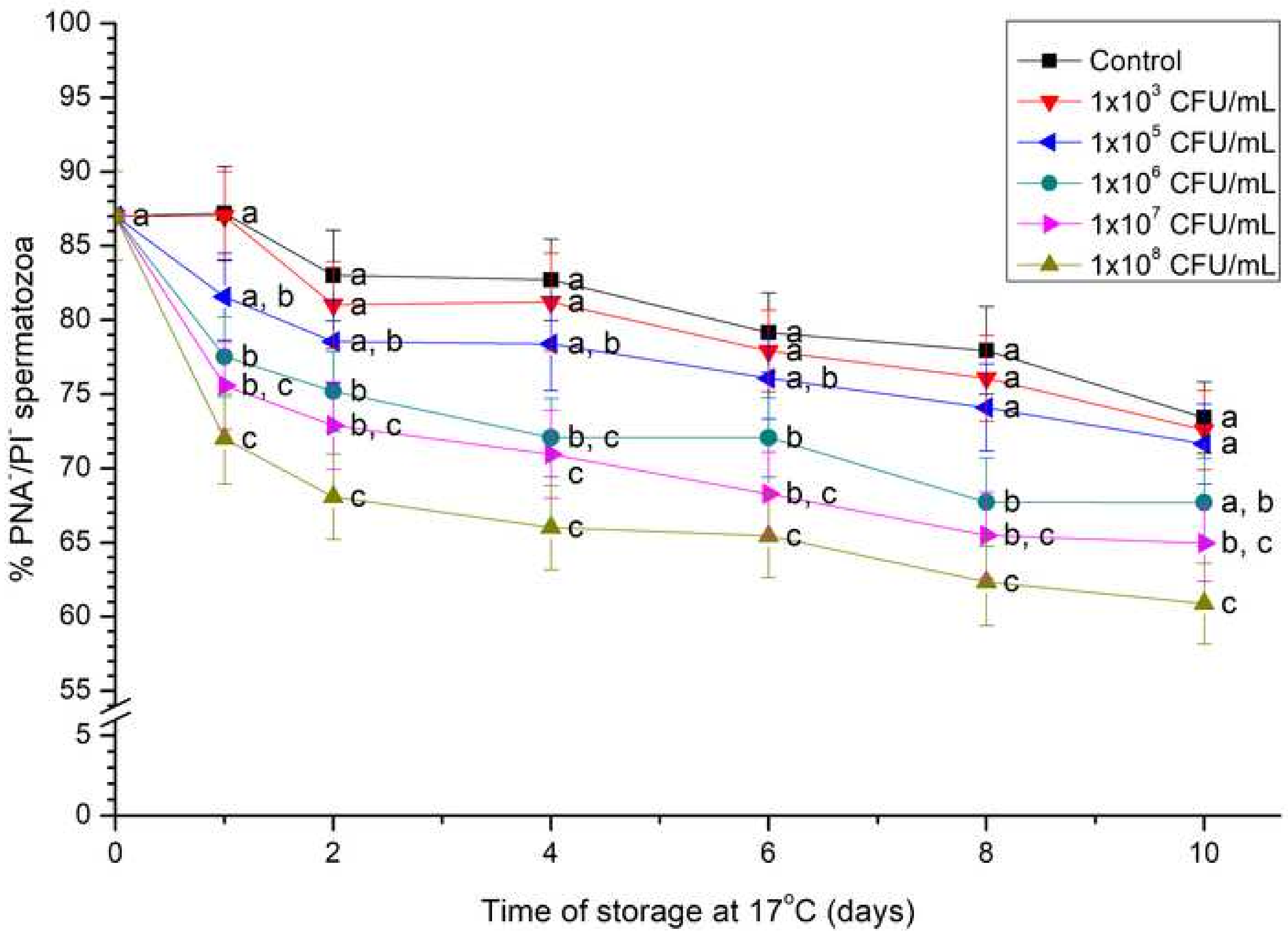
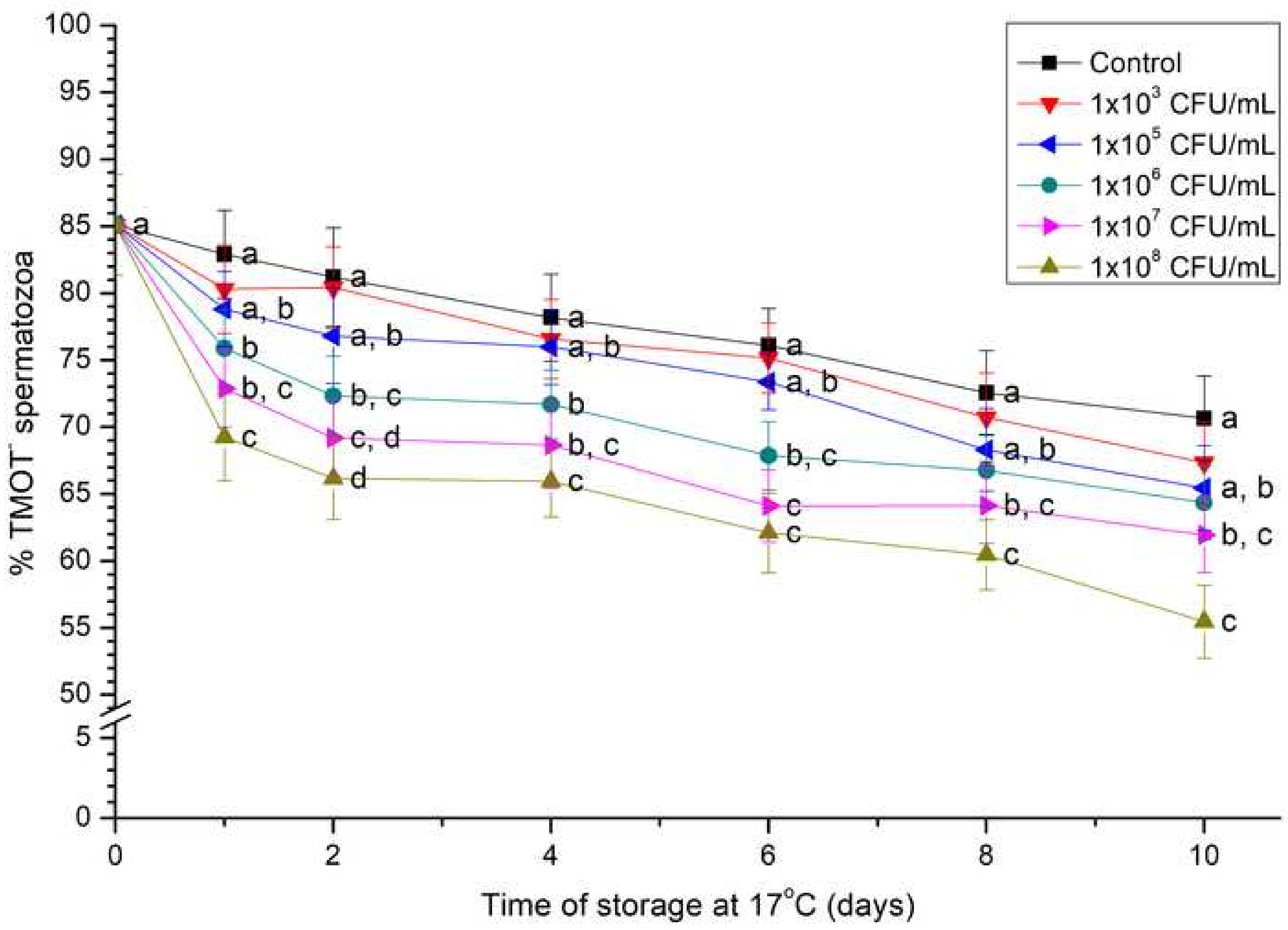


Figure
Click here to access/download;Figure;Fig. 3. Total motility.tif



Figure

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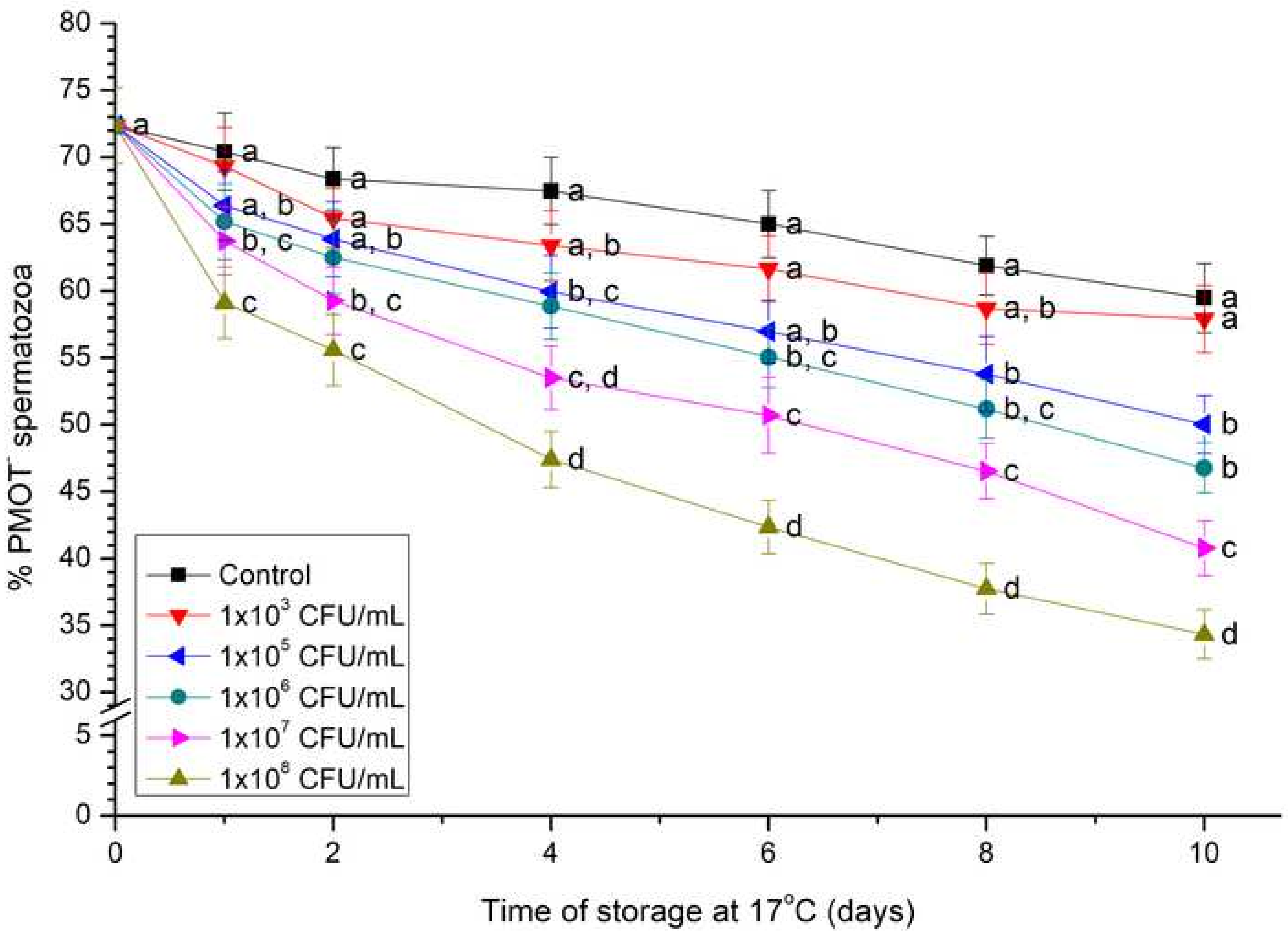
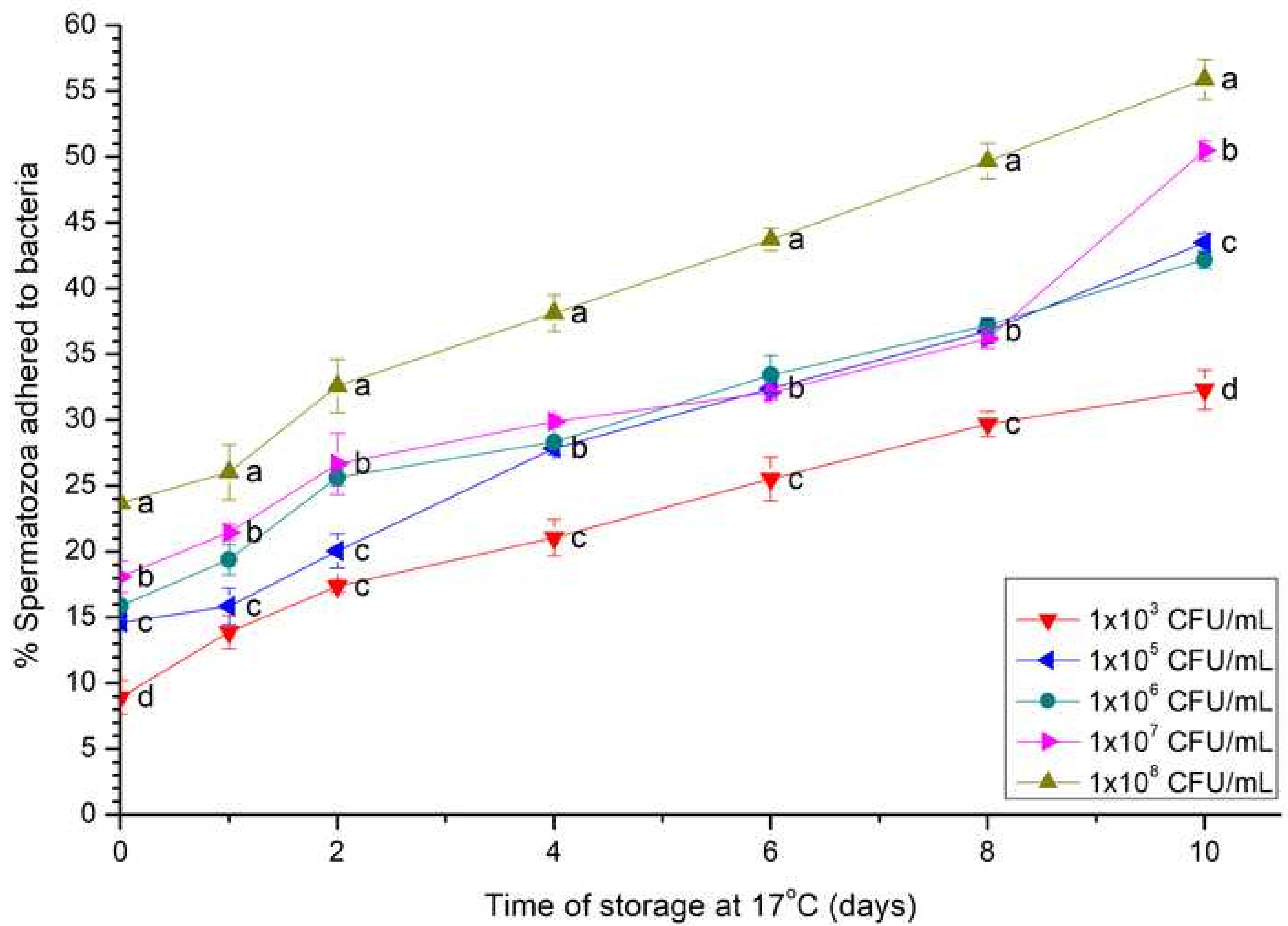
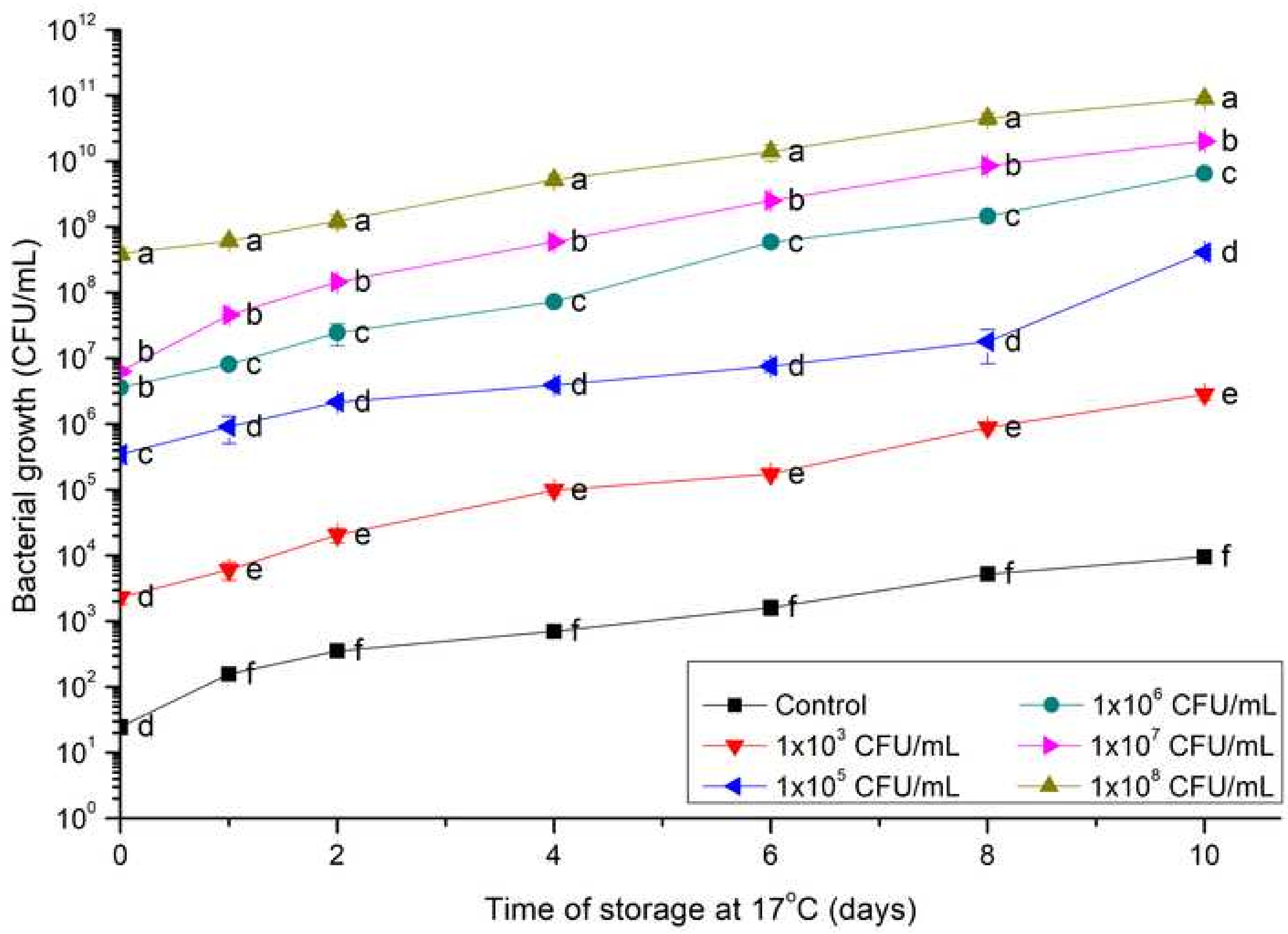


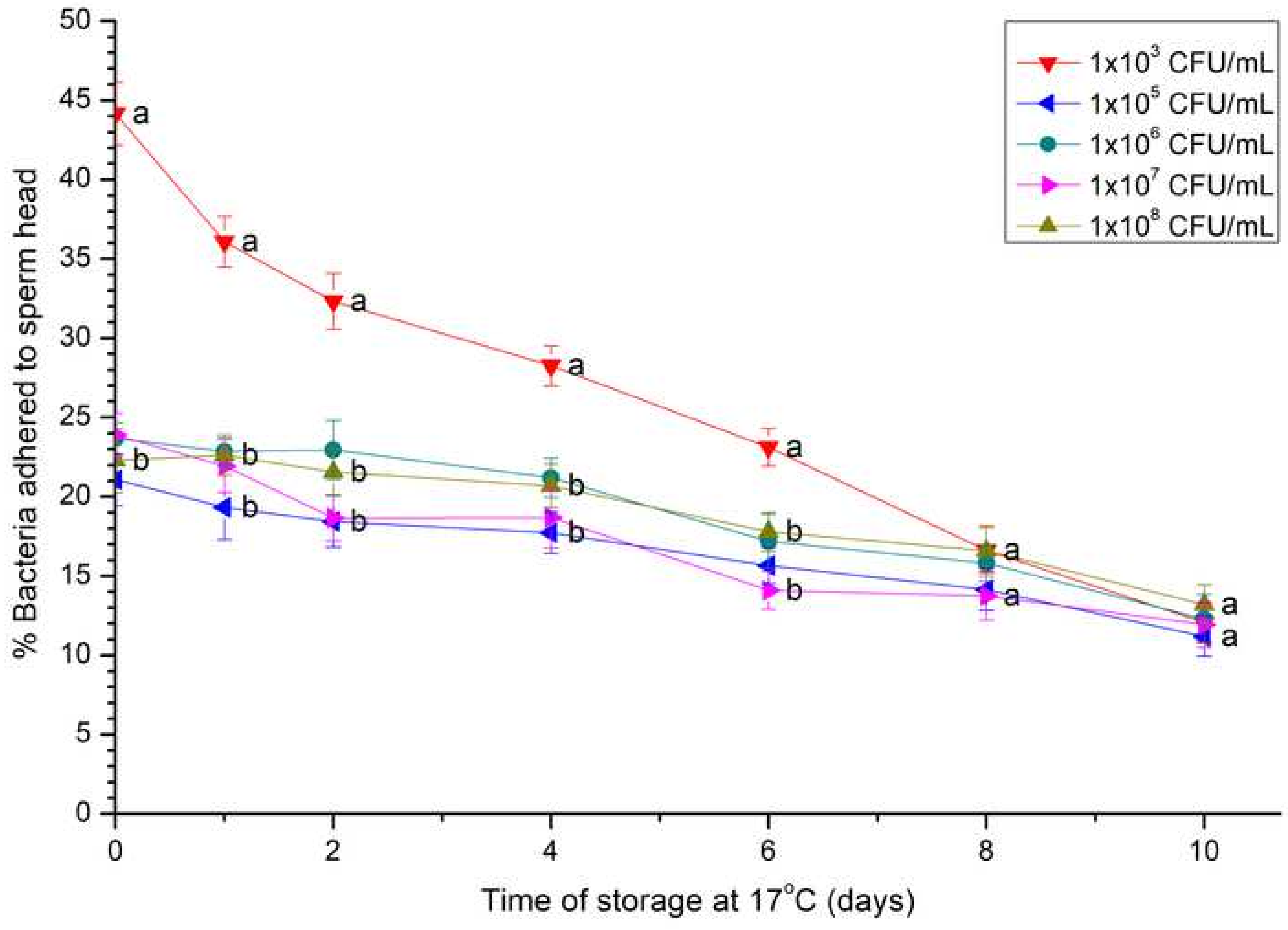
Click here to access/download;Figure;Fig. 5. Spermatozoa adhered to bacteria.tif



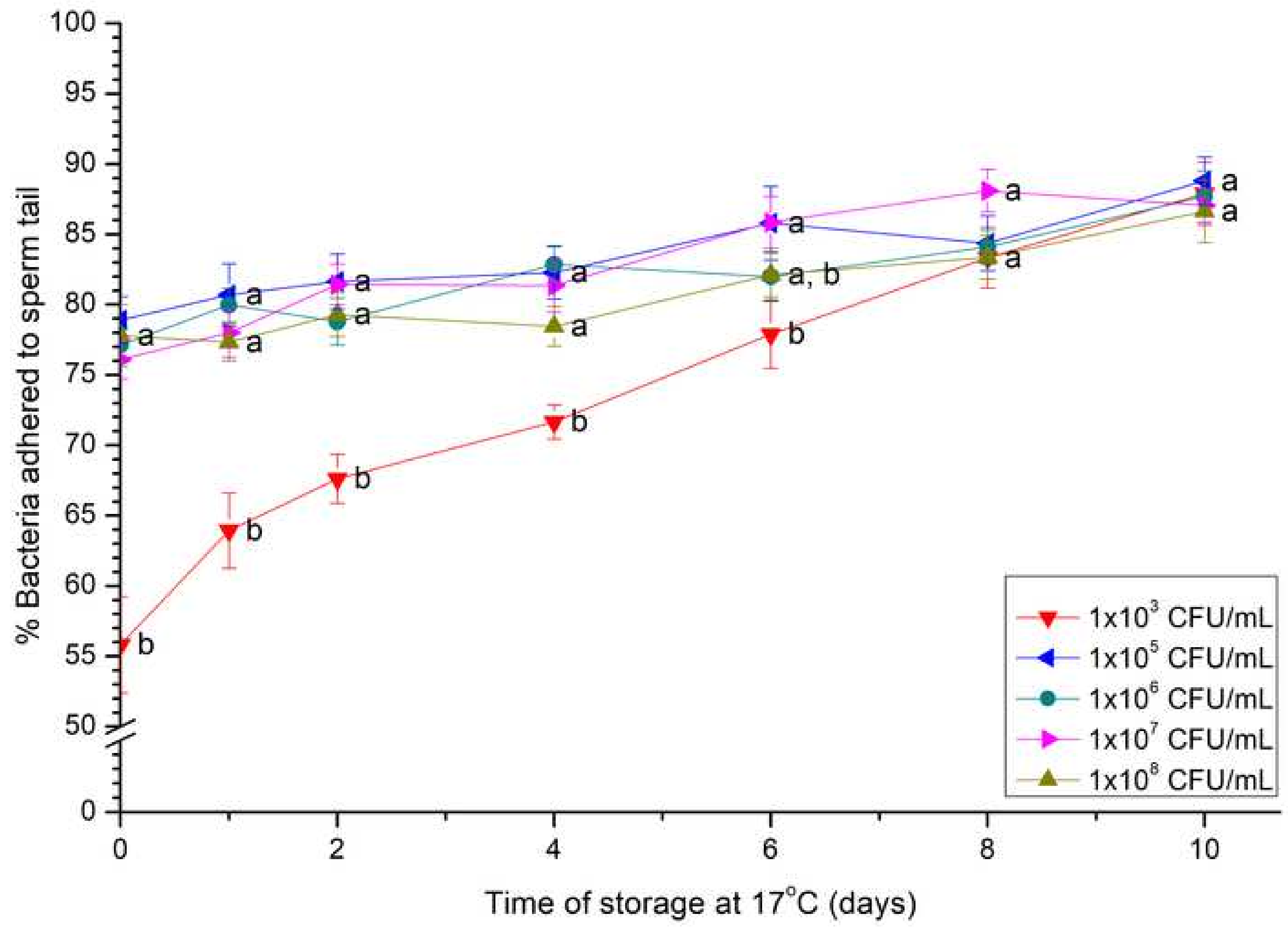
Click here to access/download;Figure;Fig. 6. Bacterial growth.tif



Click here to access/download;Figure;Suppl. Fig 1. Bacteria adhered to head.tif



 Click here to access/download;Figure;Suppl. Fig 2. Bacteria adhered to tail.tif



Table

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Conflict of interest

Girona, 16th of December of 2019

I hereby state that

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The authors declare that there is **no conflict of interest** that could be perceived as prejudicing the impartiality of the research reported.

Professor Elisabeth Pinart
University of Girona

AUTHORSHIP STATEMENT

Manuscript title: Long-term storage of boar seminal doses contaminated with Proteus vulgaris: a dose-dependent effect on sperm motility and sperm-bacteria interaction

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in the *Hong Kong Journal of Occupational Therapy*.

Authorship contributions

Please indicate the specific contributions made by each author (list the authors' initials followed by their surnames, e.g., Y.L. Cheung). The name of each author must appear at least once in each of the three categories below.

Category 1

Conception and design of study: E. Pinart, M. Yeste, ______, ______;

acquisition of data: A. Delgado-Bermúdez, E. Pinart, M. Yeste;

analysis and/or interpretation of data: E. Pinart, A. Delgado-Bermúdez, M. Yeste, S. Bonet

Category 2

Drafting the manuscript: E. Pinart, A. Delgado-Bermúdez, ______;

revising the manuscript critically for important intellectual content: E. Pinart, M. Yeste,

S. Bonet, ______.

Category 3

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