

1 Bacterial quantification in different plating methodologies

6 Abstract

7 Determining the number of bacterial cells in a product is a fundamental procedure in the
8 inoculant industry, as it ensures its quality. The current legislation in Brazil recommends
9 the spreading technique as the standard procedure to count viable cells per gram or
10 milliliters (mL). However, in order to carry out these analyses, there is a considerable
11 material demand. Hence, the objective of this study was to test an alternative
12 methodology, the microdrop technique, and determine whether it mitigates costs and
13 material demands, with the added benefit of being faster and more accurate for
14 operators. To carry out the experiment, 8 treatments were made in both saline solution
15 (SS) and saline solution (0.9%) + Tween (0.1%), at different concentrations, using a
16 homemade inoculant as inoculum. After plating, bacterial growth was conducted in a
17 growth chamber for 4–6 days. A parameter of 30–300 colonies per plate was used to
18 count colony-forming units (CFU). In addition, contaminant tests were performed for
19 Tryptic Soy Agar (TSA) bacteria and saprophytic fungi (Sabouraud). Dilution samples
20 (10^5) and inoculum from commercial packages were evaluated by Propidiummonoazide
21 quantitative polymerase chain reaction (PMA-qPCR). Despite the microdroplet
22 presenting higher CFU values when compared to the spreading technique, the dilutions
23 did not alter its order of magnitude (10^7 CFU.mL⁻¹). The use of the microdrop method,
24 when combined with Tween (0.1%), increased the unitary CFU value, providing a cost-
25 effective method for counting viable *Azospirillumbrasilense* cell numbers compared to
26 standard culture media.

27 **Keywords:** microbiological parameters; microorganisms; quality control.

30 HIGHLIGHTS

- 31 • Microdrop methodology can be considered similar to standard plating methodology.
- 32 • Tween 80 increases the cell viability.
- 33 • The magnitude of CFU was not affected by dilution proportions.

36 1.INTRODUCTION

37 Since Brazilian legislation obliges inoculant manufacturers to provide
38 information on products' composition on their labels [1], determining bacterial cell
39 numbers is one of the fundamental microbiological procedures in the country, as well
40 as in the food, pharmaceutical, and agricultural industries, among others [2]. This
41 measure guarantees an informed consumer will have the best conditions for
42 choosing, purchasing, and using a product.

43 The legislation further states that commercialized inoculants must contain in
44 their formulation: only the recommended strains; a minimum concentration of viable
45 bacterial cells per g or mL; and no contaminants at 10^5 dilution [1]. Furthermore,
46 colony forming unit (CFU) counting is done through the most probable number
47 (MPN) method, after spreading the material in a solid medium on top of Petri dishes.

48 Polyoxyethylenesorbitanmonooleate (Tween 80) is a non-ionic surfactant widely
49 used as a reductant, dispersant, and stabilizer component in nanoparticles [3]. It can
50 be used as an emulsifier in cosmetics, pharmaceuticals, and food products [4]. It can
51 also impact bacterial growth by directly affecting viability and growth rates, as well
52 as biofilm formation [5].

53 Although the quantification of bacterial inocula has been the subject of many
54 studies, the evaluation of microdrop plating, different dilution proportions using
55 Tween 80 as an adjuvant, and its effect on *Azospirillum* growth still need to be
56 addressed. Thus, this study aimed to analyze different diluent compositions, dilution
57 proportions, and methods for counting bacterial cells, drawing comparisons to the
58 qPCR molecular technique.

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60 **2.MATERIALS AND METHODS**

61 **2.1 Procedure**

62 The study was carried out at the Laboratory of Biochemistry and Genetics
63 (LABIOGEN), at the Federal University of Paraná (UFPR) – Setor Palotina, Palotina,
64 Brazil. Three freshly-packed (shelf time of 6 months) refrigerated commercial
65 inoculant bags containing $2 \cdot 10^8$ CFU.mL⁻¹ of *Azospirillumbrasilense* AbV5 and AbV-
66 6 were used in the experiment. For both counting techniques, the evaluated variables
67 were: diluent solution types; dilution proportions; and plating methods, which
68 defined the 8 applied treatments (Table 1). Two diluting solutions were used: saline

69 solution (SS), containing NaCl (0.85%); and SS with an added Tween 80 solution at
70 0.1% (v/v). The evaluated dilution proportions were: 1 mL of product (aqueous
71 liquid inoculant vigorously stirred for 30s) in 9 mL of diluent solution; and 0.1 mL of
72 product in 0.9 mL of diluent solution, resulting in a 10^{-1} concentration for both
73 proportions. The tubes were then shaken for 15–20 min on an orbital table shaker
74 (120 rpm). From this concentration, successive dilutions were performed up to 10^{-7}
75 concentration, by homogenizing the tube for 20s in a vortex in the same final volume
76 (10 mL and 1 mL, respectively, for each of the evaluated dilution proportions).

77 The treatments were repeated 15 times, with 5 samples in each treatment,
78 totalizing 3 technical evaluations. As for plating, two methods were evaluated:
79 spreading and microdrop. For spreading, 0.1 mL of the solution was pipetted onto the
80 central area of the Petri dish containing the culture medium, and the solution was
81 spread over the entire surface of the dish using a Drigalski loop. This experiment was
82 done in tetraplicate. For the microdrop (20 μ L) plating, each plate was divided into
83 four parts corresponding to each dilution (10^{-4} to 10^{-7}) in triplicate. The Congo Red
84 (CR) medium [6] was used in both methods for bacterial growth. Afterward, the
85 plates were placed in an inverted position in a **microbiological incubator**. All **petri**
86 dishes used in both methods were incubated at 28–30 °C for two days. Colony
87 counting was performed 48 hours after the incubation period. The **colony forming**
88 units per milliliter of inoculant (CFU.mL⁻¹) were counted taking into account the
89 dilutions which presented a range of 5–50 colonies per drop, in the case of the drop
90 plate method, and of 30–300 colonies per plate in the spreading method. Gram
91 coloration and microscopic visualization confirmed typical *A. brasilense* morphology
92 in **the CR culture** medium.

93 The formulas for each method differ only in relation to the correction factor,
94 which is 10 for spreading and 50 for microdrop, due to differences in the volume
95 used during pipetting. A control procedure was performed to detect contaminating
96 microorganisms. The procedure consisted of direct seeding from the sample in serial
97 dilution. 0.1 mL were plated on dishes with TSA **agar** **medium** and incubated at 28–
98 30°C for 48–72 hours, to allow detection of bacterial contaminants. The same
99 procedure was performed to verify the presence of saprophytic fungi. Dilutions of the
100 inoculating medium were pipetted onto Sabouraud Agar dishes and incubated for 72
101 hours. Both of the contaminant detection procedures were performed in triplicate.

102 **Table 1.** Methodologies applied to CFU counting

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Treatment	Diluent	Proportion of Diluent	Plating method
1	SS	1 in 9	Spreading
2	SS	0.1 in 0.9	Spreading
3	SS	1 in 9	Microdrop
4	SS	0.1 in 0.9	Microdrop
5	SS + Tween 80 (0.1%)	1 in 9	Spreading
6	SS + Tween 80 (0.1%)	0.1 in 0.9	Spreading
7	SS + Tween 80 (0.1%)	1 in 9	Microdrop
8	SS+ Tween 80 (0.1%)	0.1 in 0.9	Microdrop

SS -Saline solution (0.9%)

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In addition, the validation of this study's results was carried out by PMA-qPCR, using three fresh samples. They were taken on the same day from 10^{-5} dilution from the two-diluent media (SS and SS + Tween [0.1%]). Both were collected with the same proportions as the diluent (1:10 and 0.1:1, respectively). This analysis was carried out at GoGenetic-Curitiba-PR/Brazil, using the protocol adapted for the analysis of *A. brasilense* as a basis [7].

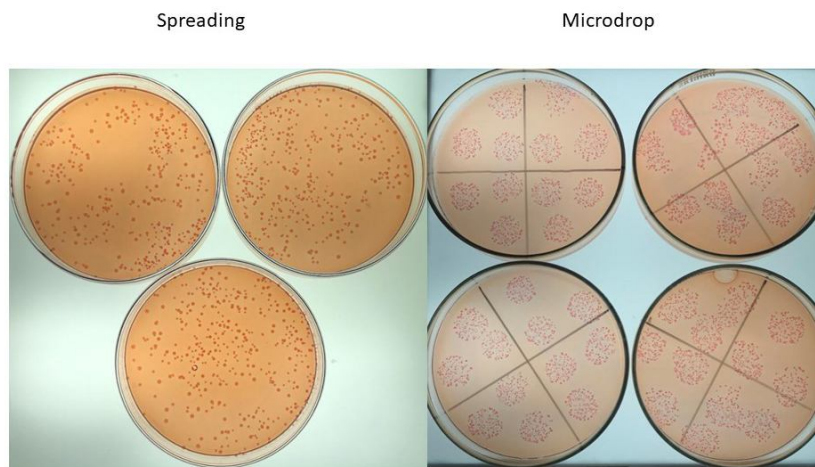
2.2 Data Analysis

The data obtained (CFU.mL⁻¹) were converted into log₁₀ and subsequently underwent Analysis of Variance for Two Factors-Two-Way (ANOVA), considering treatments and experiments as fixed factors. Two-Way-ANOVA was performed for each of the considered methods (spreading and microdrop). A Tukey test ($p < 0.05$) was performed to determine the averages of the experiments performed for each evaluated treatment. The data were arranged in Boxplot charts. Statistical Analyses were conducted in the R Software Version 4.0.2 [8].

5.RESULTS

After plating, all treatments showed bacterial growth (Fig. 1). The use of Congo Red dye in the medium helped in distinguishing *Azospirillum*, forming red spots and easing the counting process. As expected, the plated dilutions (10^{-4} to 10^{-6}) in all applied methods did not differ in order of magnitude (10^7). To make comparisons easier, we opted to show only 10^{-5} dilution results, which are comparable to PMA-

131 qPCR dilution results – therefore, the data from the other experiments were not
132 discussed here. The CFU results from all treatments are listed in Table 2. Spreading
133 showed a high mean range for CFU (22.08 to 92.33) in the evaluated treatments,
134 while microdrop showed a CFU mean ranging between 18.04 to 28.70 in both diluent
135 proportions. Analyzing CFU in each treatment for both plating techniques, spreading
136 showed a higher standard deviation compared to microdrop (SS 0.1-0.9) and
137 promoted higher CFU growth in SS+Tween(1-9) (Fig. 2A). Comparing all treatments
138 in the same plating methodology (Fig. 2B), microdrop promoted a more consistent
139 CFU, with lower variation than spreading. Tween 80, when added to the dilution
140 medium, incremented the CFU number in both plating techniques. On the other hand,
141 spreading showed a higher variation between treatments, with the SS + Tween in the
142 1:10 proportion treatment showing a significant increase in CFU number (Fig. 2B).
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145 **Fig 1.** Visual aspect of the plating methodologies. A. Spreading -treatment SS (1-9).
146 B. Microdrop SS-TW (0.1-0.9). SS-Saline solution (0.9%); TW-Tween 0.1%.

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148 However, when data were adjusted to mL^{-1} , all treatments showed the same
149 order of magnitude ($\text{CFU} \cdot \text{mL}^{-1}$), reaching similar values (10^7), although there was a
150 numerical difference among the spreading (SS 1-9) and microdrop (SS+TW 1-9)
151 treatments. Tween, as a diluent in both plating and dilution methods, was responsible
152 for a 4-fold and 1.6-fold (regarding 1-9 dilution) increment in average $\text{CFU} \cdot \text{mL}^{-1}$
153 values compared to the saline solution (Table 2), as well as a 1.3-fold increase in 0.1-
154 0.9 dilution for the microdrop technique. The exception was spreading (0.1-0.9
155 dilution), which showed a 0.34-fold decrease in CFU count.

156 The presence of contaminants in TSA and Sabouraud medium was also analyzed
157 (Table 3). In some treatments, contamination was detected across different
158 experiments, pointing to pre-existing contamination of the commercial inoculant
159 used.

160 Regarding the CFU.mL⁻¹ quantification results through the PMA-qPCR
161 technique (Table 4), the experiments revealed that the commercial inoculant aliquot
162 showed a population of 10⁹ CFU, 10 times higher than expected and announced on
163 the product label (2x10⁸ CFU. mL⁻¹). The sample quantification showed a variation
164 among dilutions from 2.36x10⁴ to 5.30x10⁵ CFU.mL⁻¹ over the course of repetitions.
165 We also identified a variation of 1.19 to 5.04x10⁹ CFU. mL⁻¹ among repetitions
166 evaluated in the commercial inoculant samples.

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168 **4.DISCUSSION**

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170 The formulation of inoculants must provide a suitable microenvironment,
171 combined with physical protection for a prolonged period, to prevent a rapid decline
172 of the introduced *Azospirillum* until its use in planting [9]. Culture-dependent
173 methods have several advantages as practical techniques to quantify bio-inoculants,
174 since they only detect viable cells and, therefore, can identify inoculants that are
175 competitive and persist over time [10]. On the other hand, the plate-counting
176 approach strongly underestimates the active microbial biomass. Therefore, the
177 population sizes of bioinocula could be severely underestimated by cultivation
178 methods [11].

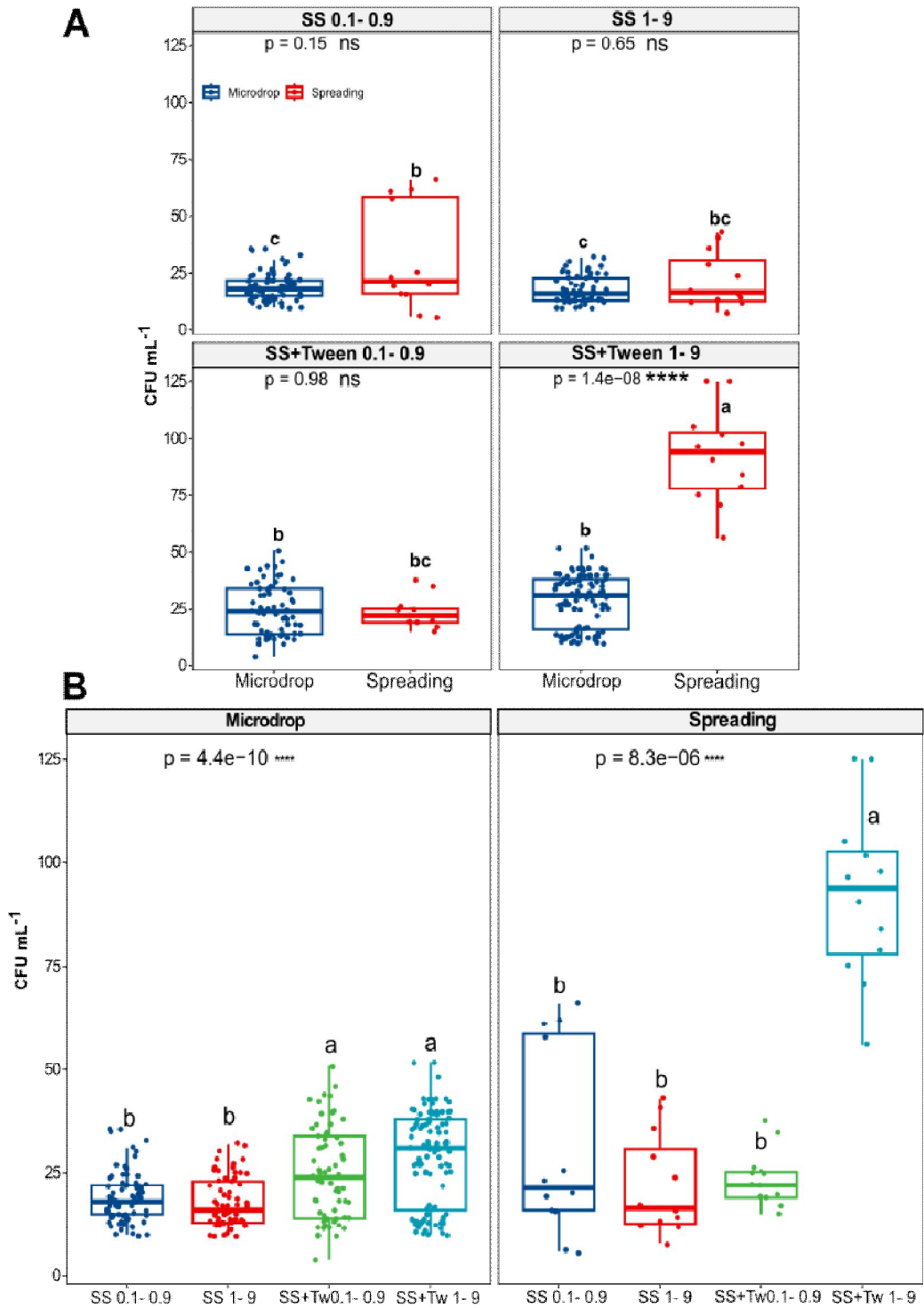
179 The evaluation of counting methods has been the research subject of many
180 authors [12–14]. All of them found that microdrop showed a higher CFU mean than
181 spreading in all treatments: 1.5-fold (SS+Tw1-9); 5.2-fold (SS+Tween 0.1-0.9); 4.1-
182 fold (SS 1-9); and 3-fold (SS 0.1-0.9).

183 Comparing diluters (SS and SS plus Tween), there was a decrease of 3–4 fold in
184 CFU.mL⁻¹ and a high variation in spreading methodology when using only SS (1:10
185 and 1:100, respectively) (Fig. 2B). Reduction in variation was also observed by
186 Thomas *et al.* [15], who noted that the continuous use of the spreader on the agar
187 surface after the exhaustion of free moisture caused irrecoverable damage to
188 vegetative bacterial cells.

189 In another study, Thomas *et al.* [16] emphasized that this significant reduction in
190 CFU can occur during spreading as a result of physical impact injuries to bacterial
191 cells, depending on the spreader - either by Drigalski loop or squeegee -, due to
192 rupture of vegetative cells from contact with a hard surface. However, in the current
193 study, spreading in a proportion of 1:10 plus Tween resulted in the highest CFU
194 value (4 times higher) compared to using a saline solution as a diluent, showing a
195 0.34-fold reduction in 1:100 proportion (Fig. 2). This result can highlight the
196 pipetting and dilution series errors during the plating process, among others. Senger
197 *et al.* [13] pointed out the differences in *Bacillus* CFU counting methods, differing
198 basically on the growth medium used. The authors showed that standard serial
199 dilution and spread Red Congo plating reached the highest CFU number.

200 The presence of Tween 80 (0.1%) was beneficial to cell growth, especially in the
201 microdrop technique, which showed the highest CFU count (1.6-fold in 1:10 and 1.3-
202 fold in 1:100 compared to the saline solution) (Table 2). The use of Tween reduced
203 the possibility of overlap between the drops, which facilitated the microdrop
204 counting in our experiments. Reitermayer *et al.* [17] reported the effect of Tween 80
205 on cell viability, metabolic activity, protein release, and propidium iodide uptake in
206 *Lactobacillus plantarum*. Pillay *et al.* [18] showed a high cost-benefit use of Tween
207 80 to increase the isolation efficiency of Gram-positive and negative organisms.

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210 **Fig. 2.** Comparison between plating methodologies. A. Results of methodologies in
 211 each dilution treatment. B. Results of methodologies in all treatments applied.

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213 Besides the high correlation between parametric and nonparametric methods for
214 counting methods [19], both have pros and cons that must be considered. As an
215 advantage, the microdrop technique can save time and material, with bacterial
216 solution spots showing greater uniformity and less dispersion. However, as
217 disadvantages, not only is microdrop a labor-intensive technique, but it carries the
218 risk of microdrop overlapping, making counting difficult by underestimating the
219 number of bacterial cells. Di Salvo *et al.* [20] cited that microdrop allows the
220 inclusion of more replicates using fewer petri dishes and culture medium amounts,
221 making it a more cost-effective method than the spread plate technique.

222 Herigstad *et al.* [21] defended drop plate as a more accurate technique than
223 spreading. However, this method is not recommended to enumerate bacterial taxa
224 that show a swarming type of motility. On the other hand, spreading bacteria
225 cultivated on solid media followed by counting the colony-forming units has been
226 the gold standard method for the enumeration of live bacteria [22] and shows the true
227 density of microorganisms by plating a larger volume [21]. Also, spreading
228 simplifies counting, since the colonies are spread over a higher counting area, which
229 is why results showed a higher standard deviation in bacterial counting. According to
230 the literature, only plates containing between 25 and 300 colonies are selected for
231 counting [1, 23]. Despite the drawbacks, dependent plating methods exhibited better
232 colony morphology, thus facilitating the identification of bacterial genera, making
233 them standard counting methodologies [24]. Culture-dependent methods may end up,
234 in turn, underestimating bacterial colony counts, since in situations of agglomeration,
235 colonies may overlap each other and be considered as a single CFU [2].

236 The positive bacteria and fungi genera observed in some dilutions and
237 experiments raise the hypothesis that the commercial inoculant bags used may have
238 been previously contaminated during transport or storage since they had been
239 recently produced. These contaminants, mainly bacteria, made many plaques and
240 their counts (CFU) non-viable and, according to Brazilian law (which allows no
241 contaminants at 10^5 dilution in commercial inoculants), the inoculum was discarded.

242 In order to check a gold technique of viable bacteria number counting, the PMA-
243 qPCR method was applied. In this technique, samples are pre-treated with
244 intercalating nucleic acid dyes - propidiummonoazide (PMA) -, due to its ability to
245 penetrate dead or membrane-compromised cells which inhibit the qPCR, avoiding
246 false positive results [25]. Regarding the dilution sample quantification by the PMA-

247 qPCR technique (Table 4), the qPCR showed an increase in *Azospirillum* content in
248 the commercial inoculant used in this study (10^9), pointing to bacterial growth after
249 packing and during storage, since the commercial bag showed an initial population of
250 10^8 cells.

251 The data also showed a variation among dilutions from 10^4 to 10^5 CFU.mL⁻¹ in the
252 course of experiments, which can possibly be explained by bacteria death in diluent
253 media before the samples were analyzed, which only happened 24 hours later. Shi *et al.*
254 [26] compared the results of the PMA-qPCR and plate count from lactic acid bacteria
255 obtained from milk and showed that the results of the plate count were lower than those
256 of the PMAq-PCR method, probably because some of the cells in the samples were in a
257 viable but non-culturable (VBNC) state. Also, da Cunha *et al.* [7] using bacterial plate
258 counting and PMA-qPCR to monitor *A. brasilense* FP2 viable cell counting, observed
259 similar results in both methods, although PMA-qPCR could estimate culturable and
260 non-culturable cells, while plate counting could only count viable ones. On the other
261 hand, Soto-Muñoz *et al.* [27] showed agreeing results between both methods when
262 bacteria were freeze-dried or fluidized bed-dried. After spray-drying, however, cell
263 numbers obtained by PMA-qPCR (which assesses intact cells) were two orders of
264 magnitude higher than those obtained by plate-counting. Lee *et al.* [24] compared six
265 different counting methods for soil bacteria and DNA quantification methodology, and
266 showed a 100-fold increase in cells (g.dry wet soil⁻¹) compared to the result obtained
267 from the spreading method, which suggests the need for careful interpretation of
268 bacterial numbers. Moreover, another drawback of the routine use of this technique is its
269 high cost.

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272 **Conclusions**

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274 In conclusion, this study showed that microdrop plating and the incorporation of
275 Tween 80 0.1% provided a cost-effective method for generating higher viable cell
276 yields of *A.brasilense* compared to other standard culture media (saline solution and
277 spreading). Dilution proportions of 1:10 or 1:100 did not change the order of
278 magnitude of bacterial population count in samples. PMA-qPCR results confirmed

279 the bacterial concentration in diluted samples, despite variations (10-fold) found in
280 samples, as well as in the commercial inoculant package.

281

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296 name, version, model, and source of the generative AI technology and as well as all input
297 prompts provided to the generative AI technology

298 Details of the AI usage are given below:

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Table 2. Results from the colony forming counting of *Azospirillum* sp. obtained from plating methodologies

Treatment	Mean CFU (10 ⁵)		CFU (10 ⁵) corrected		CFU.mL ⁻¹	
	Spreading	Microdrop	Spreading	Microdrop	Spreading*	Microdrop**
SS+TW1-9	92.33±20.81	28.7±11.27	923.3	1435	9.23x 10 ⁷	14.35x10 ⁷
SS+TW 0.1-0.9	23.58±6.90	24.73±11.16	235.8	1237	2.35x10 ⁷	12.37x10 ⁷
SS 1-9	22.08±12.25	18.04±5.94	220.8	902	2.20x10 ⁷	9.02x10 ⁷
SS 0.1-0.9	31.58±23.09	19.08±6.09	315.8	954	3.15x10 ⁷	9.54x10 ⁷

SS – Saline solution (NaCl 0.85%). TW -Tween 80 * CFU.mL⁻¹: N^o of counted colonies x 10 x dilution factor (10⁵) ** CFU.mL⁻¹: N^o of counted colonies x 50 x dilution factor (10⁵)

Table 3. Results from contaminants checking in TSA e Sabouraud at 10⁵ dilution

Repetitions	SS+Tween (1 – 9)			SS+Tween (0.1 - 0.9)			SS (1 - 9)			SS (0.1 - 0.9)		
	1 st	2 nd	3 rd	1 st	2 nd	3 rd	1 st	2 nd	3 rd	1 st	2 nd	3 rd
Sabouraud	NC	NC	NC	NC	NC	C	NC	NC	NC	C	NC	NC
TSA	NC	NC	NC	C	NC	C	NC	C	NC	NC	NC	NC

C- presence of contaminants; NC- **absence** of contaminants.

Table 4. Results from *Azospirillum* sp CFU counting using qPCR, (dilution 10⁵)

Repetitions	Detection (Ct)- Quantification (CFU.mL ⁻¹)				
	Commercial Inoculant	SS + Tween (1 – 9)	SS + Tween (0.1 – 0.9)	SS (1 – 9)	SS (0.1 – 0.9)
1	1.90±0.39x10 ⁹	5.30±1.13x10 ⁵	3.47±0.47x10 ⁵	2.09±0.87x10 ⁵	7.70±1.51x10 ⁴
2	5.04±0.42x10 ⁹	1.53±0.28x10 ⁵	9.51±0.77x10 ⁴	1.33±0.50x10 ⁵	8.08±1.54x10 ⁴
3	1.19 ±0.08x10 ⁹	2.82±0.41x10 ⁵	4.41 ± 0.31x10 ⁵	6.04± 0.77x10 ⁴	2.36± 0.12x10 ⁴

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