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Bacterial Quantification in Different Plating Methodologies

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Determining the number of bacterial cells in a product is a fundamental procedure in the inoculant industry, as it ensures its quality. The current legislation in Brazil recommends the spreading technique as the standard procedure to count viable cells per gram or milliliters (mL). However, in order to carry out these analyses, there is a considerable material demand. Hence, the objective of this study was to test an alternative methodology, the microdrop technique, and determine whether it mitigates costs and material demands, with the added benefit of being faster and more accurate

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for operators. To carry out the experiment, 8 treatments were made in both saline solution (SS) and saline solution (0.9%) + Tween 80 (0.1%), at different concentrations, using a homemade inoculant as inoculum. After plating, bacterial growth was conducted in a growth chamber for 4–6 days. A parameter of 30–300 colonies per plate was used to count colony-forming units (CFU). In addition, contaminant tests were performed for Tryptic Soy Agar (TSA) bacteria and saprophytic fungi (Sabouraud). Dilution samples (10^5) and inoculum from commercial packages were evaluated by Propidium monoazide quantitative polymerase chain reaction (PMA-qPCR). Despite the microdroplet presenting higher CFU values when compared to the spreading technique, the dilutions did not alter its order of magnitude (10^7 CFU.mL^{-1}). The use of the microdrop method, when combined with Tween 80 (0.1%), increased the unitary CFU value, providing a cost-effective method for counting viable *Azospirillum brasilense* cell numbers compared to standard culture media.

Keywords: Microbiological parameters; microorganisms; quality control.

1. INTRODUCTION

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

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

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

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

2. MATERIALS AND METHODS

2.1 Procedure

The study was carried out at the Laboratory of Biochemistry and Genetics (LABIOGEN), at the Federal University of Paraná (UFPR) – Setor Palotina, Palotina, Brazil. Three freshly-packed (shelf time of 6 months) refrigerated commercial inoculant bags containing 2.10^8 CFU.mL^{-1} of *Azospirillum brasilense* AbV5 and AbV-6 were used in the experiment. For both counting techniques, the evaluated variables were: diluent solution types; dilution proportions; and plating methods, which defined the 8 applied treatments (Table 1). Two diluting solutions were used: saline solution (SS), containing NaCl (0.85%); and SS with an added Tween 80 solution at 0.1% (v/v). The evaluated dilution proportions were: 1 mL of product (aqueous liquid inoculant vigorously stirred for 30s) in 9 mL of diluent solution; and 0.1 mL of product in 0.9 mL of diluent solution, resulting in a 10^{-1} concentration for both proportions. The tubes were then shaken for 15–20 min on an orbital table shaker (120 rpm). From this concentration, successive dilutions were performed up to 10^{-7} concentration, by homogenizing the tube for 20s in a vortex in the same final volume (10 mL and 1 mL, respectively, for each of the evaluated dilution proportions).

The treatments were repeated 15 times, with 5 samples in each treatment, totalizing 3 technical evaluations. As for plating, two methods were evaluated: spreading and microdrop. For spreading, 0.1 mL of the solution was pipetted onto the central area of the Petri dish containing the culture medium, and the solution was spread over the entire surface of the dish using a Drigalski loop. This experiment was done in

Table 1. Methodologies applied to CFU counting

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%)

tetraplicate. For the microdrop (20 µL) plating, each plate was divided into four parts corresponding to each dilution (10^{-4} to 10^{-7}) in triplicate. The Congo Red (CR) medium [6] was used in both methods for bacterial growth. Afterward, the plates were placed in an inverted position in a microbiological incubator. All petri dishes used in both methods were incubated at 28–30 ^{o}C for two days. Colony counting was performed 48 hours after the incubation period. The colony forming units per milliliter of inoculant (CFU.ml^{-1}) were counted taking into account the dilutions which presented a range of 5–50 colonies per drop, in the case of the drop plate method, and of 30–300 colonies per plate in the spreading method. Gram coloration and microscopic visualization confirmed typical *A. brasilense* morphology in the CR culture medium.

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

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].

3. 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-qPCR dilution results – therefore, the data from the other experiments were not discussed here. The CFU results from all treatments are listed in Table 2. Spreading showed a high mean range for CFU (22.08 to 92.33) in the evaluated treatments, while microdrop showed a CFU mean ranging between 18.04 to 28.70 in both diluent proportions. Analyzing CFU in each treatment for both plating techniques, spreading showed a higher standard deviation compared to microdrop (SS 0.1-0.9) and promoted higher CFU growth in SS+Tween (1-9) (Fig. 2A). Comparing all treatments in the same plating

methodology (Fig. 2B), microdrop promoted a more consistent CFU, with lower variation than spreading. Tween 80, when added to the dilution medium, incremented the CFU number in both plating techniques. On the other hand, spreading showed a higher variation between treatments, with the SS + Tween in the 1:10 proportion treatment showing a significant increase in CFU number (Fig. 2B).

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

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

Regarding the $CFU.mL^{-1}$ quantification results through the PMA-qPCR technique (Table 4), the experiments revealed that the commercial inoculant aliquot showed a population of 10^9 CFU, 10 times higher than expected and announced on the product label ($2x10^8$ CFU. mL^{-1}). The sample quantification showed a variation among dilutions from $2.36x10^4$ to $5.30x10^5$ $CFU.mL^{-1}$ over the course of repetitions. We also identified a variation of 1.19 to $5.04x10^9$ CFU. mL^{-1} among repetitions evaluated in the commercial inoculant samples.

4. DISCUSSION

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

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

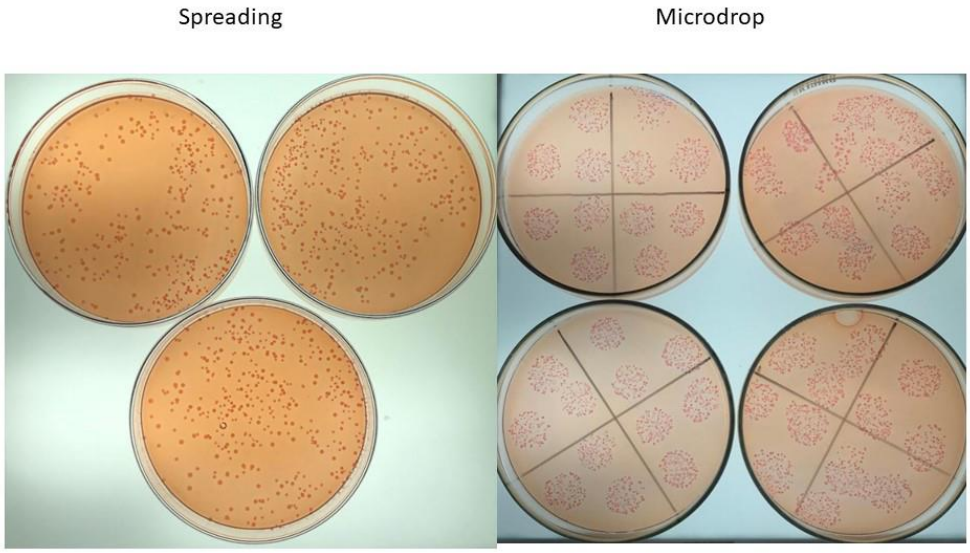

Fig. 1. Visual aspect of the plating methodologies. A. Spreading -treatment SS (1-9). B. Microdrop SS-TW (0.1-0.9). SS-Saline solution (0.9%); TW-Tween 0.1%

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

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

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

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

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

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

In order to check a gold technique of viable bacteria number counting, the PMA-qPCR method was applied. In this technique, samples are pre-treated with intercalating nucleic acid dyes - propidium monoazide (PMA) -, due to its ability to penetrate dead or membrane-compromised cells which inhibit the qPCR, avoiding false positive results [25]. Regarding the dilution sample quantification by the PMA-qPCR technique (Table 4), the qPCR showed an increase in *Azospirillum* content in the

commercial inoculant used in this study (10^9), pointing to bacterial growth after packing and during storage, since the commercial bag showed an initial population of 10^8 cells.

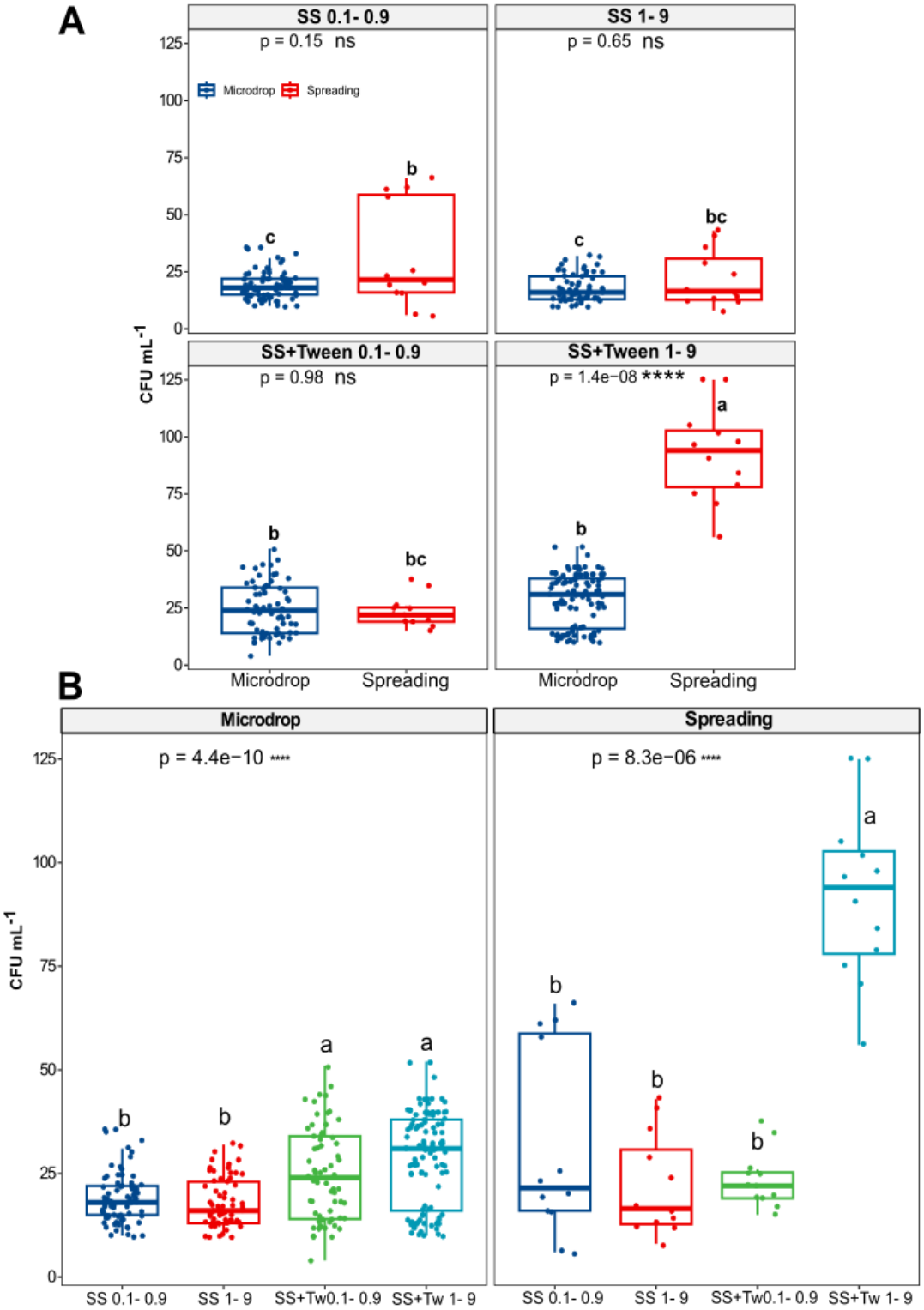

Fig. 2. Comparison between plating methodologies. A. Results of methodologies in each dilution treatment. B. Results of methodologies in all treatments applied

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

	Mean CFU (10^5)		CFU (10^5) corrected		CFU.mL^{-1}	
Treatment	**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}: Nº of counted colonies x 10 x dilution factor (10^5) ** CFU.mL^{-1}: Nº of counted colonies x 50 x dilution factor (10^5)*

Table 3. Results from contaminants checking in TSA e Sabouraud at 10^5 dilution

	SS+Tween (1 – 9)			SS+Tween (0.1 - 0.9)			SS (1 - 9)			SS (0.1 - 0.9)		
Repetitions	**1st**	**2nd**	**3rd**	**1st**	**2nd**	**3rd**	**1st**	**2nd**	**3rd**	**1st**	**2nd**	**3rd**
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 10^5)

	Detection (Ct)- Quantification (CFU.mL^{-1})				
Repetitions	**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

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

5. CONCLUSIONS

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

HIGHLIGHTS

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

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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