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Cell counting and bacterial inoculum standardization by spectrophotometric method for *Bifidobacterium animalis* ssp. *Lactis* INL1

Contagem de células e padronização de inóculos bacterianos pelo método espectrofotométrico para *Bifidobacterium animalis* ssp. *Lactis* INL1

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ABSTRACT
The genus Bifidobacterium is present in the human intestinal microbiota and in abundance in breast milk, conferring probiotic features to this food. However, a large number of variables affect its growth, hindering possible analysis and its use in organic assays. Different direct and indirect methodologies of bacterial cell counting provide results in some units of measurement, such as colony-forming units (CFU) per gram or mL, or even cell number per mL. Standardizing bacterial inoculum used in long-term daily exposure experiments is sometimes difficult due to the numerous variables involved in plaques cultivation and incubation. Considering that, this study sought to standardize adjusted bacterial inoculum from the indirect method of bacterial counting by spectrophotometry, using calibration curve and plating confirmation to ensure the amount of cells to be inoculated. As a result, we found that colony-forming units grew at a 108 rate at 600 nm, within the 0.010–0.006 absorbance range, and that such rate remains regardless of differences within the CFU/mL count. This method can be used to adjust Bifidobacterium animallis ssp Lactis INL1 inoculum, considering its probiotic effect.

Keywords: Spectrophotometry, standardization, colony-forming units.

RESUMO
O gênero Bifidobacterium está presente na microbiota intestinal humana e em abundância no leite materno, conferindo características probióticas a este alimento. Entretanto, um grande número de variáveis afeta seu crescimento, dificultando possíveis análises e seu uso em ensaios orgânicos. Diferentes metodologias diretas e indiretas de contagem de células bacterianas fornecem resultados em algumas unidades de medida, tais como unidades formadoras de colônias (UFC) por grama ou mL, ou mesmo o número de células por mL. A padronização do inóculo bacteriano utilizado em experimentos de exposição diária de longo prazo é às vezes difícil devido às numerosas variáveis envolvidas no cultivo e incubação de placas. Considerando isso, este estudo procurou padronizar o inóculo bacteriano ajustado a partir do método indireto de contagem bacteriana por espectrofotometria, utilizando a curva de calibração e a confirmação de revestimento para garantir a quantidade de células a serem inoculadas. Como resultado, descobrimos que as unidades formadoras de colônias cresceram a uma taxa de 108 a 600 nm, dentro da faixa de absorção de 0,010-0,006, e que tal taxa permanece independentemente das diferenças dentro da contagem UFC/mL. Este método pode ser usado para ajustar o inóculo Bifidobacterium animallis ssp Lactis INL1, considerando seu efeito probiótico.

Palavras-chave: Espectrofotometria, padronização, unidades formadoras de colônias.

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1 INTRODUCTION

Cell count is a method employed in biological assays to verify pertinent microorganisms growth in culture medium. These assays involve monitoring cell concentration, culture medium components, and growth conditions such as temperature and contact with oxygen (Tortora, 2005). Cells quantification is performed by direct counting of cells present within a sample or culture medium, differentiating or not viable and non-viable cells (Trabulsi, 2005). It can also be performed indirectly, whereby cell concentration is determined according to a microorganism metabolites (Guerra, 2016).

Several methods scientifically deemed as conventional or official have been used for a long time with established effectiveness, such as plate count, multiple-tube counting technique, and the Neubauer's chamber counting (Collins, Lyne & Lange, 1989). Advancements in molecular techniques and computing area enabled new microbial detection methods that provide results by short-time analysis. Yet, such methods must have their efficacy proven and equate to conventional methods, aiming to provide reliable and safe data (Guerra, 2016).

The conventional plate count method is the most commonly used for microorganisms counting in microbiology laboratories due to its low-cost and for exempting sophisticated equipment. This method basically consists in plating microorganism aliquots and diluting them in appropriate solid culture medium at optimum growth temperature (Silva, 1999).

Plating can be performed by seeding a defined aliquot (1mL or fractions) on the surface of culture medium, previously distributed in sterile Petri dishes with adequate culture medium containing agar. Diluted sample is seeded with the aid of a Drigalsky's loop, providing total surface spread and complete sample absorption into the agar (Silveira *et al*., 1997).

Culture medium may also be added after the 1mL-aliquot or each dilution fractions are transferred to empty and sterile Petri dishes. Then, culture medium containing agar is added and homogenized in circular movements (deep seeding). Plates are incubated and, after growth, these bacteria accumulate in a single locus, forming a colony visible to the naked eye (Franco, 1999).

Surface or depth plating results are expressed in colony-forming units (CFU), which requires counting. Considering difficulties in counting plates with large amounts of colonies, a maximum count limit should be set. To provide statistical significance to the trial, a minimum limit should also be set (Guerra, 2016).

Plate counting methods allow counting viable cells only and isolating colonies, which can be easily identified and studied. However, these are laborious methods for requiring proper

incubation to enable colony growth. Moreover, failures in dilution and/or plating may lead to incorrect counting (Pessôa, 2008; Guerra, 2016).

Another widely used method is the liquid sample turbidity measurement. Such indirect counting technique relies on bacterial cells ability to divert light (light scattering) into a spectrophotometer. The amount of light passing through cell suspension depends on several factors related to cell concentration and suspension culture size, as well as incident light wavelength and intensity (I_0) (Guerra, 2016).

Results observed using these techniques may be expressed in absorbance or transmittance unit, or even as CFU/g or CFU/mL by a calibration curve. Bacterial suspensions optical density is measured using the wavelengths of 540, 600, 620, or 640 nm (Pessôa, 2008).

This method may be performed in two distinct ways: using a test tube containing the culture medium or eliminating it by centrifugation and dilution. The first case requires a tube containing the same culture medium, but uninoculated, to reset the equipment and discount the amount of light absorbed by the medium without microbial growth. The second case requires a considerable amount of sample followed by successive dilutions to contemplate the spectrophotometer reading entire range (absorbance 0.2 - 0.8) (Tortora, 2005; Guerra, 2016).

This indirect method does not enable the distinction between viable and dead cells, and is used to monitor microbial growth based on optical density increase measured at a certain wavelength (Albiero,2013).

Several studies employ the aforementioned methods to determine the number of colonies within a given sample. However, each microorganism has a certain specificity related either to culture medium, required temperature for growth, oxygen requirement or not, cultivation time, among others. (Pessôa, 2008).

Controlling all these parameters is a laborious task, which hinders bacterial suspensions standardization for analysis. Bifidobacteria, for example, only grow in Petri dishes if their ideal multiplication conditions are strictly controlled (Ferreira, 2012). Such issue may compromise assays reproducibility and impair the establishment of an exposure dosage.

Bifidobacteria are Gram-positive, anaerobic, catalase-negative bacteria which optimal growth temperature range varies when isolated from humans (36 to 38ºC) and animals (41 to 43ºC). Temperatures below 20ºC and above 49.5ºC inhibits its growth. The optimum pH for its growth ranges between 6.5 and 7.0, and it shows pH sensitivity below 5.0 (Cronin *et al*., 2011; Ferreira, 2012). Some strategies are employed to improve bifidobacteria survival under oxygen, such as adding ascorbic acid or cysteine to culture medium (Kun *et al*., 2008; Cronin *et al*., 2011).

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The genus Bifidobacterium is present in the human intestinal microbiota and in abundance in breast milk. A newborn in breastfeeding presents 90% of their microbiota composed of bifidobacteria (Turroni *et al*., 2011; Ferreira, 2012).

Standardizing bacterial inoculum used in long-term daily exposure experiments is sometimes difficult due to the numerous variables involved in plaques cultivation and incubation. Considering that, this study sought to standardize adjusted bacterial inoculum from the indirect method of bacterial count by spectrophotometry using calibration curve and plating confirmation to ensure the amount of cells to be further inoculated in oral suspensions in animal models.

2 MATERIAL AND METHODS

2.1 SAMPLE PREPARATION

Bifidobacterium animalis subsp. *Lactis* INL1 (Zacarías *et* al., 2011) was prepared from frozen isolated strain from matrix supplied by the Institute of Industrial Lactologia (INLAIN) – Universidad Nacional del Litoral (UNL), Santa Fé, Argentina. Culture and activation were performed as follows:

After unfreezing, the *B. lactis* INL1 sample was reactivated in anaerobiosis in 10mL MRS broth at 37 °C for 24 hours in the proportion of 1:100 added to 1% cysteine at 10%. Then, samples were recultivated under the same conditions. This procedure was repeated twice to that cells overnight multiplication potential was greater.

Cell viability was tested by incubating two inoculums (of one stria) of the obtained culture – one in aerobiosis (in which no growth was observed in the culture medium) and the other in anaerobiosis jar, in MRS agar at 37ºC for 48 h.

Cell growth capacity was determined by the traditional plate count method. Cells obtained after overnight were diluted in series, plated on surface in MRS agar, and incubated in anaerobiosis jar with anaerobic gas generator at 37ºC for 72 h. Then, plates with growth ranging from 3 to 400 colonies were used to count colony-forming units (Silveira *et al*., 1997; Guerra, 2016).

2.2 INOCULUM STANDARDIZATION:

From the obtained overnight culture suspension, serial dilutions from $^{-}1$ to $^{-}7$ were performed in PBS buffer. The final three ($^{-}5$, $^{-}6$, and $^{-}7$) were submitted to absorbance evaluation at 600nm of each dilution, in triplicate. PBS was used for controls.

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For CFU counting, 0.1mL of the same dilutions per surface were plated and incubated in anaerobiosis jar with anaerobic gas generator at 37ºC for 72 h, in plates with MRS medium (Man Rogosa and Sharp). Counting were adjusted by dilution factor.

2.3 COUNTING CHART CREATION:

Obtained results were estimated in a graph whereby y-axis refers to dilution-related counting in CFU/mL and x-axis to dilution-related absorbance values. Linear regression was used to evaluate the experimental data best representative equation. "R" and "R2" values were analyzed.

2.4 EXPERIMENTAL DESIGN AND STATISTICAL TREATMENT:

Results were tabulated and variance analysis (ANOVA) and Student's T-test ($p<0.05$) were used to statistically verify results accuracy among trials.

3 RESULTS AND DISCUSSIONS

After performing tests described in the methodology to standardize inoculum and cell counting, we developed the following table using means obtained by plate counting and turbidity measurement of the respective sample.

Table 1: Absorbances and colony-forming units mean values.

Dilutions	CFU/mL (10^8)	ABS
-5	3.5	0.010
-6	5.3	0.007
-7	7	0.006

The following graph helps to better visualize and compare colony-forming units and dilutions absorbance (600nm):

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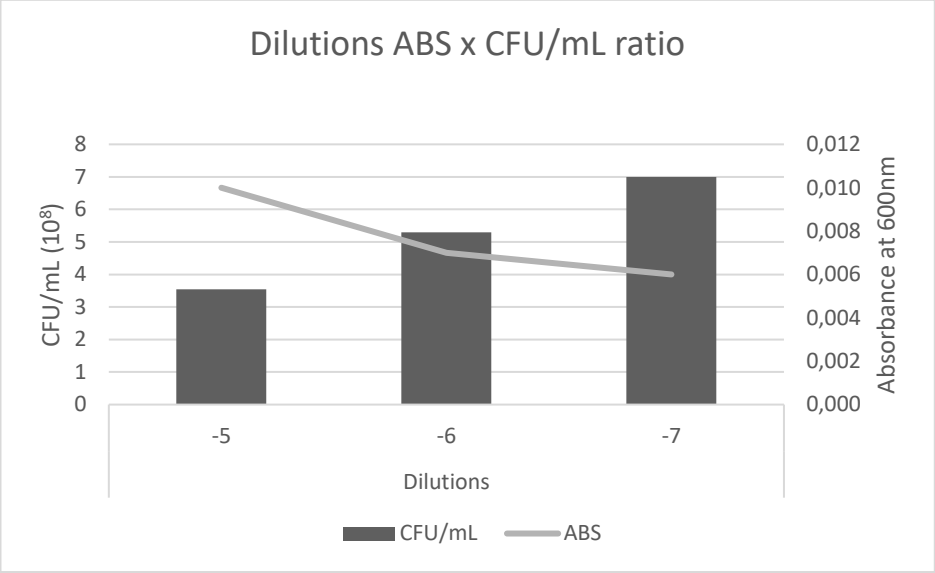
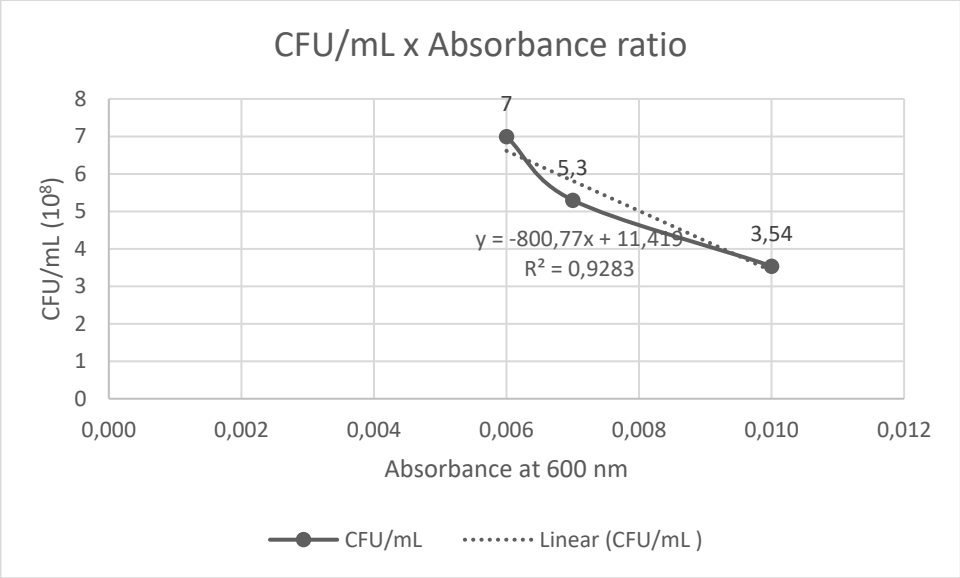
Graph 1: Comparison between plate counting and absorbance regarding dilution.



The following graph was developed to understand the correlation between absorbance and colony growth:

Graph 2: Correlation between absorbance and colony-forming units.



By applying linear regression analysis, we verified variables to have a 92% impact on the set. However, the analysis of variance (ANOVA) found a significant difference between obtained means (p= 0.17), which differed from that found by the Student's T test (p=0.03).

Considering that, dilutions, absorbance, and colony-forming units growth capacity were correlated. At 600 nm, within the 0.010–0.006 absorbance range, colony-forming units grew at a 10^8 capacity.

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To be of physiological importance to the consumer, probiotics added in foods or other products of human consumption must attain high populations. Vinderola *et.al* (2011) discusses the maintenance of the viability of microorganisms of the *genera Lactobacillus* and *Bifidobacterium*, stating that probiotics should be added to foods at 10^7–10^8 CFU/g or mL concentrations, without participating in fermentation and barely modifying their concentration during product preparation.

The Brazilian legislation indicates that probiotic food must contain a minimum viable quantity within the 10^8 to 10^9 CFU/mL range in the daily recommendation of ready-to-eat products; provided that the company proves its effectiveness, lower values may be accepted (BRASIL, 2008). As for criteria established by FAO/WHO (2001), for a product to be considered probiotic, it must contain at least 10^6 to 10^7 CFU/mL of the microorganism during its shelf life. Thus, our results are in line with the recommendations.

Inoculating the correct amount of microorganisms is important to ensure a probiotic effect for the consumer.

4 CONCLUSION

The divergences found in our study may be justified by the issue in the delicate cultivation of microorganisms of the analyzed genus, stressing that spectrophotometry is an indirect counting method that does not differentiate viable cells from those that are not. Yet, we found a correlation between dilutions, absorbance, and colony-forming units growth capacity, so that growth capacity remains at 10^8 even if the CFU/mL count is not optimal. Considering that, the method can be used to adjust *Bifidobacterium animallis* ssp *Lactis INL1* inoculum, aiming at its probiotic effect.

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