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Original Article

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Reliability of the agar based method to assess the production of degradative enzymes in clinical isolates of *Candida albicans*

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Abstract

The aim of this study was to establish a reproducible protocol using the methodology of hyaline zones around the colonies on specific agar plates for phospholipase and proteinase production. This was an *in vitro* double-blind experiment, in which the dependent variables were the enzymatic activity measurements (Pz) for the production of phospholipase (Pz-ph) and the production of secreted aspartyl proteinases (Pz-sap). Three independent variables give rise to different measurement protocols. All measurements were carried out at two different moments by four examiners (E1, E2, E3, and E4). The minimum sample size was 30 *Candida albicans* clinical isolates. Specific agar plates for phospholipase and SAPs production were prepared according the literature. The intra- and inter-examiner reproducibility for each protocol was estimated using the Intraclass Correlation Coefficient (ICC) and its confidence interval (95% CI). Based on the results obtained for both phospholipase and SAPs, there appears to be no consensus on the protocol chosen for each particular examiner. Measuring the colonies in triplicate may be the main factor associated with the increase in measurement accuracy and should therefore take precedence over measuring only one colony. When only one examiner is responsible for taking measurements, a standard protocol should be put in place and the statistical calibration of this researcher should be done prior to data collection. However, if two or more researchers are involved in the assessment of agar plates, our results suggest that the protocols using software to undertake plate reading is preferred.

Key words: Virulence factors, Reproducibility of Results, *Candida albicans*.

Introduction

Among *Candida* species that are frequently isolated from the oral cavity in humans, *Candida albicans* is still the most frequently identified.[1-4] The superficial infection of the oral cavity, associated with the presence of *Candida* spp., is called oral candidiasis and is considered the most common fungal infection in humans.[5] Besides fungal colonization, there are other etiological factors, both local and systemic, which can predispose the development of candidiasis.[1,2,4,6,7]

Moreover, several virulence factors, associated with *Candida* species, are also responsible for promoting the pathogenicity of these microorganisms and, consequently, can interfere with the establishment and persistence of the infection.[4] Some of these factors include the ability to adhere to buccal epithelial cells and abiotic surfaces as well as biofilm production.[8,9] The ability of *Candida* spp. to synthesize extracellular hydrolytic enzymes such as secreted aspartyl proteinases (SAPs) and phospholipases, are also considered fundamental virulence factors for these microorganisms.[4,10-13] The SAPs can degrade a variety of proteins in the host tissues, favoring fungal adhesion and invasion.[14] In addition, this enzyme is essential for destroying the first lines of defense of the oral mucosa.[15] Phospholipase, usually concentrated in the extremities of hyphae,[16] is also actively implicated in the invasion of the host tissues, since it causes the rupture of cell membranes, allowing the fungal cell to penetrate into the cytoplasm.[17]

The measurement of hyaline halos of degradation around yeast colonies on specific agar plates[18,19] has been used in recent studies to analyze enzymatic activity.[12,20] However, data about the consistency of the measurements, including intra- and inter-examiner reliability, are rarely mentioned. Additionally, although some studies have reported that the halos were measured by direct assessment using a caliper[12,21] or on digital images,[22] this information is usually omitted in most studies.[3,10,11,20,23,24] The measurement method may directly interfere with the value obtained,[25] as well as ambient and examiner conditions. Thus, the lack of a standardized methodology makes comparison between results from different studies difficult.

According to the Error Theory, every measurement taken has one component related to the actual value and another to the error, which can be either random or systematic.[26] The latter can seriously affect the statistical analysis and interpretation of the data,[27] so it is essential to assess the magnitude of these errors, by calculating a reliability coefficient.[25] Highly prestigious journals have recently discussed measures to increase the consistency and quality of information in life-sciences papers and the improvement of data reproducibility is one of them.[28,29] Training researchers prior to data collection, the standardization of protocols, and the calibration of equipment are vital steps to improve data reliability. This stage has been neglected in several microbiologic-related papers, which may compromise the quality of the data.

Accurately assessing the virulence factors is fundamental to reduce bias that would certainly influence the result variability and, consequently, the calculation of sample size and the statistical power. Thus, this study assessed the intra- and inter-examiner reproducibility of different readings protocols using the method based on halos of degradation around the colonies on specific agar plates for enzymatic activity *of C. albicans*.

Materials and methods

Study design

This was an *in vitro* double-blind experiment. The dependent variables include enzymatic activity measurements (Pz) for the production of phospholipase (Pz-ph) and the production of SAPs (Pz-sap). In order to analyze Pz-ph, two independent variables were considered: i. type of instrument (digital caliper [DC], and computer program [CP]); ii. number of colony replicates (single colony for each isolate [1C], and triplicate colonies for each isolate [3C]). Only a black background was used behind the plates. For Pz-sap, the same variables were considered but the background color was changed (natural light background [NL], and white background [W]). Thus, the independent variables gave rise to four different protocols for Pz-ph (DC/1C; DC/3C; CP/1C; CP/3C) and eight protocols for PZ-sap (DC/W/1C; DC/W/3C; DC/NL/1C; DC/NL/3C; CP/W/1C; CP/W/3C; CP/NL/1C; CP/NL/3C).

All measurements were performed by four examiners (E1, E2, E3, and E4) at two different moments, with a one-day interval between them. The sample size of 30 *C. albicans* clinical isolates was defined according to the recommendations of Bland[30] ($\alpha = 0.05$; $\beta = 0.20$; $\rho \geq .51$), and a possible loss of unit samples of 10%.

Clinical isolates of *C. albicans*

This study evaluated 30 clinical isolates of *C. albicans* obtained from the yeast culture collection of the Laboratory of Applied Microbiology – UNESP – Univ Estadual Paulista, Araraquara, SP, Brazil. These isolates were previously obtained from dentures surfaces of nondiabetic edentulous patients with oral candidiasis.[13] A reference strain (*C. albicans* ATCC 90028) was also used. All isolates were maintained in yeast-peptone-glucose medium (YEPD: 1% yeast

extract, 2% Bacto peptone, 2% D-glucose, and 2% agar) and frozen at −70°C until use.

Culture conditions and standardization

The microorganisms were subcultured on Sabouraud dextrose agar (SDA; Acumedia Manufacturers Inc., Lansing, MI, USA) plates supplemented with chloramphenicol (0.05 g/l) and incubated at 37°C for 48 h. To prepare the yeast inocula, two loopfuls of the agar cultures were transferred to 5 ml of RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37°C overnight in an orbital shaker (75 rpm). Cells of the resultant cultures were harvested and washed twice with phosphate-buffered saline (PBS, pH 7.2) at 4,000 ×*g* for 5 min. Washed microorganisms were resuspended in PBS and spectrophotometrically standardized at an optical density of 520 nm (Biospectro, Equipar Ltda, Curitiba, PR, Brazil) to a final concentration of 10^8 CFU/ml (colony forming units per ml).[20]

Enzymatic activity

Phospholipase production testing was performed according to the methodology of Price et al.[18] with a few modifications. A base medium was prepared using 10 g of peptone, 30 g of glucose, 57.3 g of sodium chloride, 0.55 g of calcium chloride, and 20 g of agar in 1 l of distilled water.[12] The solution was autoclaved and, after cooling down to 50°C, the base medium was mixed with 80 ml of an egg yolk emulsion with potassium tellurite 0.15% (Laborclin Produtos para Laboratórios Ltda, Pinhais, PR, Brazil). Potassium tellurite was used in the egg yolk emulsion to improve the visualization of the halos of precipitation and the accuracy of measurements. Then, 5-μl aliquots of each cell suspension at 10^8 CFU/ml were inoculated on the egg yolk medium and plates were incubated at 37°C for 7 d.[20]

SAP production testing was performed according to the methodology of Rüchel[19] with a few modifications. The test medium consisted of agar plates containing bovine serum albumin (BSA). The agar medium was prepared using 20 g of dextrose, 1 g of potassium phosphate, 0.5 g of magnesium sulfate, and 15 g of agar in 1 l of distilled water. This solution was autoclaved and cooled down to 50°C. The BSA medium was prepared using 2 g of albumin, 0.2 g of riboflavin, 0.4 g of nicotinic acid, and 0.4 g of thiamine hydrochloride in 1 l of distilled water. This solution was sterilized by filtration with a 0.22 μm membrane filter and mixed with the agar medium. Five-microliter aliquots of each cell suspension at 10^8 CFU/ml were then inoculated on the plates, which were incubated at 37°C for 5 d. The proteolysis activity in the plates was visualized 24–48 h after staining with amido black solution (amido black 0.5%, glacial acetic acid 49.5%, and distilled water 50%).[23]

Pz was measured as the ratio of the diameter of the colony to the total diameter of the colony plus the hyaline zone. Readings using a digital caliper (Mitutoyo Corporation, Tokyo, Japan) were taken directly on the Petri dishes, after placing the plates on predefined background. Digital images were obtained using a digital camera (Canon PC1049, Canon Inc., Japan) placed at 30 cm from plates. The Adobe Photoshop Measurement Tool (Adobe Systems Incorporated, San Jose, CA, USA) was used to perform readings of digital images, after setting the measurement scale (cm). Four blind examiners (two with an advanced level of experience in microbiology and two beginners) performed all readings twice, with a 24-h interval between them.

Data analysis

A descriptive analysis of the data was carried out and the difference (d) between the Pz values obtained on the first and second reading were calculated, followed by the mean of differences for each examiner ($\bar{d}$) and for each protocol. The intra- and inter-examiner reproducibility of the measurements for each protocol was estimated using the Intraclass Correlation Coefficient (ICC; ρ) and its confidence interval (95% CI), considering the absolute agreement and two-way analysis of variance (ANOVA) model, for single and average measurements (when Pz was calculated after counting one colony and triplicate, respectively).[31] According to the ρ value obtained, the degree of correlation between measurements was classified according to Fermanian.[32]

Results

SAPs

For almost all conditions tested, intra-examiner data showed that the difference calculated for Pz-sap values between the two measurements were numerically smaller when three colonies per sample were analyzed (data not shown). The criteria proposed by Fermanian[32] suggest that ICC values $\geq$ 0.71 can be considered as "Good reproducibility." Figure 1 shows that the majority of protocols with this level of reliability was performed with triplicate readings, especially for E1, E2, and E4. On the other hand, when the measurements were taken using a computer (CP), the 95% CI suggested that counting a single colony or triplicate may not interfere with the intra-examiner reproducibility. However, such findings should be interpreted carefully

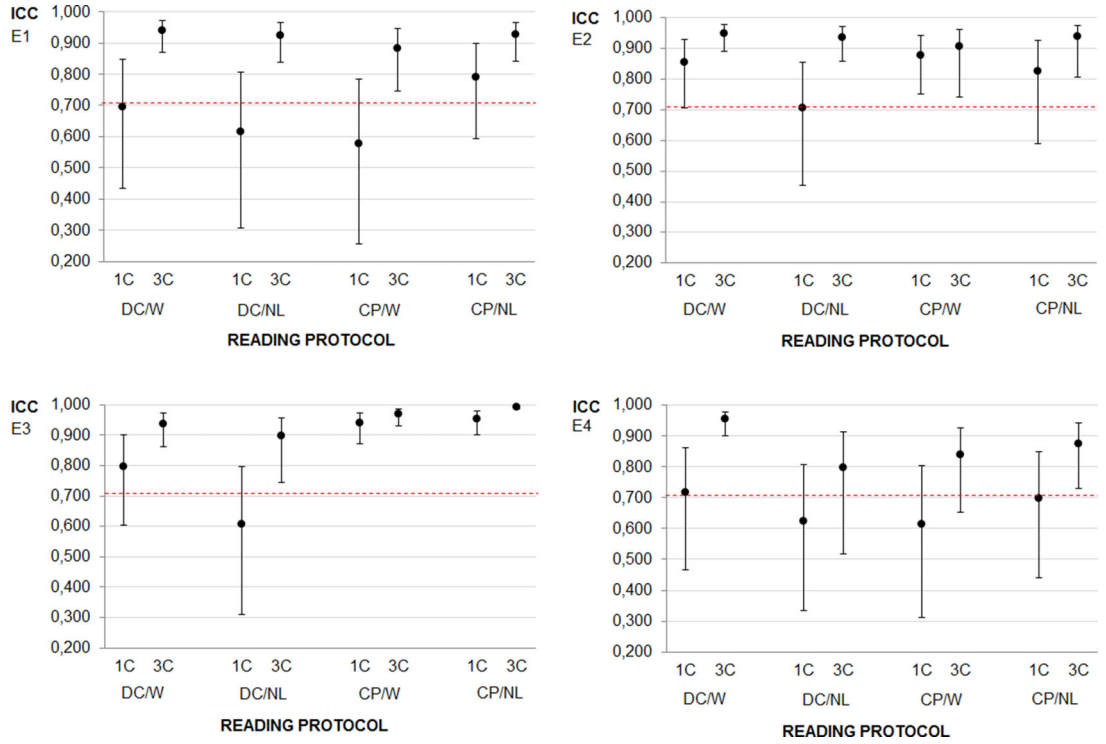

Figure 1. Values obtained for the intraclass correlation coefficient (ICC) and respective confidence interval (95% CI) for the analysis of the intra-examiner reproducibility of Pz-sap values for each measurement protocol and for each examiner (E1, E2, E3, and E4). The dotted line indicates the value of 0.71, which was the criterion adopted as the minimum ICC value to be considered as "good reproducibility" (according to Fermanian 1984).[29]

since the 95% CI for the measurement protocols on a single colony were extremely imprecise, which may have caused the overlapping in the 95% CI limits.

The two protocols (DC/W/3C and CP/NL/3C) for which the intra-examiner reproducibility was classified as, at least, good (ICC $\geq$ 0.71) for all examiners were selected for the inter-examiner analysis. Using a white background when taking measurements with a caliper resulted in an ICC = 0.894 and using a natural light background on a computer resulted in an ICC = 0.899, both classified as good reproducibility, with the lower 95% CI limits also included in this classification (0.753–0.953 and 0.754–0.956, respectively). This analysis was repeated without including the E4 data, since the results of this examiner presented the lowest intra-examiner reliability. By removing E4, it was possible to estimate the reproducibility of four protocols, those whose measurements were carried out with triplicate colonies. The results are presented in Table 1. Although the four protocols resulted in ICC values classified as good, only the rotocol CP/NL/3C presented the lower limit of the 95% CI exceeding 0.71.

Phospholipase

The ICC values showed that the protocols tested by all examiners obtained an intra-examiner reproducibility classified as good, with the only exception of DC/1C for E3 measurements, where the lower 95% CI limit was less than 0.71 (Figure 2).

Table 1. Values obtained for the intraclass correlation coefficient (ICC) for the analysis of inter-examiner reproducibility (E1 × E2 × E3) for four different protocols to measure the production of protease (Pz-sap) and their respective confidence interval (95% CI).

Protocol	ICC	95% CI
DC/W/3C	0.870	0.603–0.949
DC/NL/3C	0.786	0.560–0.899
CP/W/3C	0.776	0.217–0.920
CP/NL/3C	0.924	0.825–0.966

Note: DC: digital caliper; CP: computer program; W: white background; NL: natural light background; 3C: Pz values by measuring three colonies of the same sample.

According to the intra-examiner reproducibility results, three protocols were selected to carry out the inter-examiner analysis (Table 2). When the Pz-ph values were obtained by averaging the triplicates, the inter-examiner reproducibility of the measurements taken using both a caliper and a computer were classified as very good. The protocol CP/1C showed an ICC significantly lower than that of protocol CP/3C and its 95% CI limits crossed with those of DC/3C

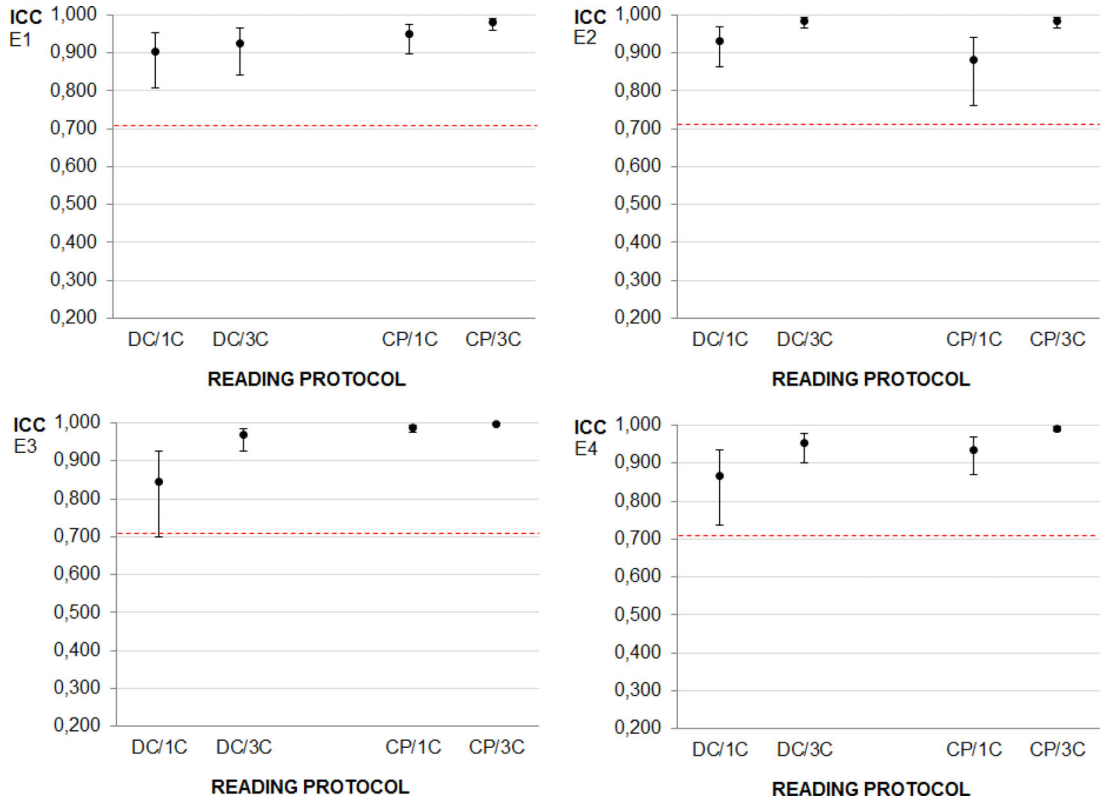

Figure 2. Values obtained for the intraclass correlation coefficient (ICC) and respective confidence interval (95% IC) for the analysis of the intra-examiner reproducibility of Pz-ph values for each measurement protocol and for each examiner (E1, E2, E3, and E4). The dotted line indicates the value of 0.71, which was the criterion adopted as the minimum ICC value to be considered as "good reproducibility" (according to Fermanian 1984).[29]

Table 2. Values obtained for the intraclass correlation coefficient (ICC) for the analysis of the inter-examiner reproducibility (E1 × E2 × E3 × E4) for three different protocols to measure the production of phospholipase (Pz-ph) and its respective confidence interval (95% CI).

Protocol	ICC	95% CI
DC/3C	0.951	0.913–0.975
CP/1C	0.853	0.761–0.920
CP/3C	0.975	0.954–0.987

Note: DC: digital caliper; CP: computer; 1C: Pz-ph values obtained on one colony per sample; 3C: Pz-ph values obtained by counting three colonies from the same sample.

protocol on the significance threshold. Therefore, it can be considered that two Pz-ph protocols showed a greater reliability with regard to measurements taken by different examiners, both by taking measurements in triplicate for each sample.

Discussion

Reliability assessment permits to evaluate the amount of error inherent to a measurement, which will consequently determine the validity of the results. Although the analysis of intra- and inter-examiner reproducibility can provide information about reliability of results, this step has been neglected in several studies of applied microbiology. To our knowledge, no study has compared the consistency of Pz-sap readings according to the measurement protocol. Some authors also have used colonies in triplicate to analyze the production of SAPs in their samples,[12,21,24,33-41] but this decision seems to have been done arbitrarily since no mention of the reason was found. On the other hand, some studies were carried out using a single aliquot for SAP[11] assessment or no information regarding the number of replicates was given in the methodology,[42] which is of particular concern in cases where no statistical calibrations of the researchers were taken before measurements. Overall, readings performed on digital images seemed to increase intra-examiner reproducibility while the type of background resulted in similar consistency. Our literature review identified only one study[22] that mentioned using a computer and three[12,21,43] that mentioned using a caliper, whereas several articles[3,10,11,24,33-39,40-42,44-56] did not mention the method used.

Considering the results obtained in inter-examiner reproducibility, the protocol that presented the highest reliability to measure Pz-sap values was the one that used digital

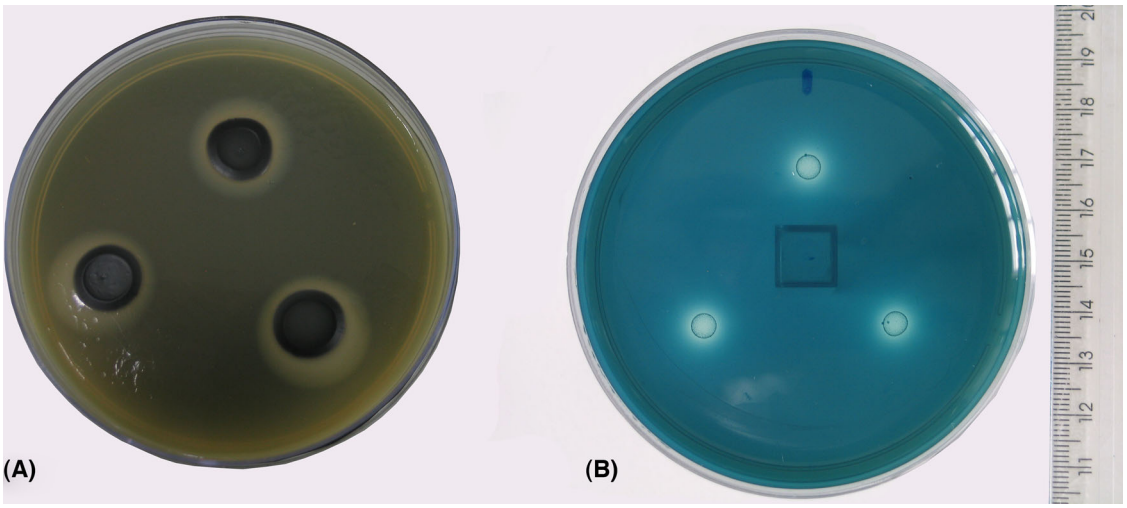

Figure 3. Images of the samples assessed for the production of phospholipase (A) and SAPs (B).

photographs and took measurements on a computer, on a natural light background, and with measurements being carried out on triplicate colonies. This result reinforces the importance of measuring the enzymatic activity of SAPs in triplicate. It also reveals the importance of assessing the statistical calibration between the different researchers when taking the same measurement, since only one of the eight protocols assessed showed data with sufficient reliability when four different examiners were involved in performing the analyses.

Results from Pz-ph evaluation suggested that carrying out measurements in triplicate for phospholipase is not essential to obtain adequate intra-examiner reproducibility.

Thus, it seems that accurate measurements were more easily obtained in the analysis of phospholipase, which may be explained by the characteristics of the colony and halos formed. The methodology used promoted the formation of large colonies and halos with well-defined borders, which did not occur in the SAPs analysis (Figure 3). Moreover, the use of egg yolk with potassium tellurite allowed the formation of dark colonies contrasting with the yellow color of the surrounding halos, which possibly improved the visualization of the halos of degradation and the accuracy of measurements. In the literature, few studies stated to have measured only one colony.[11,57] On the other hand, there were more studies carried out in triplicate,[4,12,20,21,33-38,40,55,58,59] but there was no information about the calibration of the researchers, which makes it difficult to discuss the accuracy of the measurements taken in each case.

Data also suggested that the use of digital images did not increase intra-examiner reliability in an evident manner, but the estimated ICC values showed that the protocol that maximized the intra-examiner reproducibility for all examiners was the CP/3C. Although the measurements taken in triplicate were not primordial with regard to the intra-examiner reliability, when measurements were taken simultaneously by different researchers, the assessment of more colonies helped increase the accuracy. Thus, taking measurements using a computer or a digital caliper, in triplicate, seem to be the best protocols, which allow accurate data from different examiners.

Many investigations used the agar-based methodology to assess the enzymatic profile of yeasts, but the examiner calibration before the readings are rarely mentioned. So, it was very difficult to make comparisons of our results and even to discuss our findings based on the literature. In general, it was accepted that differences between repeated measurements of the same sample may result from the instrument/method itself and the circumstances in which the measurements are performed, such as luminosity and the calibration of the examiner/equipment.[25] Based on the results obtained for both phospholipase and SAPs, there appears to be no consensus on the protocol chosen for each particular examiner. Measuring the colonies in triplicate may be the main factor associated with the increase in measurement accuracy and should therefore take precedence over measuring only one colony. When only one examiner was responsible for taking measurements, a standard protocol should be put in place and the statistical calibration of this researcher should be done prior to data collection. However, if two or more researchers are involved in the assessment of agar plates, our results suggest that the protocols using digital images to undertake plate reading are preferred.

It is important to point out that the methodology for SAPs involves staining with amido black to help visualize the halo, but despite this, the halo limits were still not easily

identifiable, which probably resulted in differences between repeated readings of the same sample. This measurement issue for SAPs may explain the fact that some authors opted to use the agar plate method only to determine phospholipase and use alternative methods such as colorimetry and fluorimetry to further investigate SAPs activity.[23,59,60-62] It is essential to highlight the importance of standardization and verification of examiner calibration prior to taking the measurements to ensure that the results are reliable. Although there are more sensitive and modern tests to investigate enzyme activity,[52,55,56,63] the halo method is scientifically accepted,[18,19] frequently used,[23,24,33-36,42,44-47,58,59,60,61] and its advantages lie in its low cost and ease of use, so it is worthwhile to conduct studies that help obtain reliable measurements from it.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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