RESEARCH

Performance Equivalence and Validation of a Rapid Microbiological Method for Detection and Quantification of Yeast and Mold in an Antacid Oral Suspension

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ABSTRACT: Alternative and rapid microbiological methods can be effective replacements for more traditional plating approaches for ensuring quality and safety in the pharmaceutical industry. This article compares the efficacy of the Soleris automated method and the traditional plate-count method for the quantitative detection of yeasts and molds at three different microbial bioburden levels. Validation testing was carried out using an antacid oral suspension (aluminum hydroxide 4% + magnesium hydroxide 4% + simethicone 0.4%). Equivalence of data between detection time and colony-forming units was established for both the alternative and the conventional methodologies. Using probability of detection, linear Poisson regression, Fisher's test, and multifactorial analysis of variance (ANOVA), all results from the rapid method were shown to be in statistical agreement with the those of the reference plating procedures. The limits of detection and quantification were statistically similar for both methods (Fisher's exact test, $P > 0.05$), showing that the alternative method is *not inferior* in performance to the reference method. Essential validation parameters such as precision (standard deviation <5, coefficient of variance <35%), accuracy (>70%), linearity (R^2 >0.9025), ruggedness (ANOVA, $P < 0.05$), operative range, and specificity were determined. It was shown that all the test results obtained using the alternative method were in statistical agreement with the those of the standard plate-count method. Thus, this new technology was found to meet all the validation criteria needed to be considered as an alternative method for yeast and mold quantification in the antacid oral suspension tested. However, taking into account that the present validation was carried out utilizing *A. brasiliensis* and *C. albicans* as suitable models for yeasts and molds and with an antacid oral suspension as a pharmaceutical matrix, further investigation will be required to qualify Soleris technology for other environmental isolates and recovery of these isolates from production batches.

KEYWORDS: Alternative microbiological methods (AMM), Rapid microbiological methods (RMM), Validation testing, Yeast and mold quantification, Microbiological quality control.

Introduction

The application of alternative microbiological methods (AMMs) and rapid microbiological methods (RMMs) has been growing over the few last decades, spurred by new technological advances. Health regulatory authorities around the world have been encouraging pharmaceutical manufacturers to use them, as they can offer benefits in execution and automation while improving accuracy, sensitivity, and precision and reducing the microbiological process time compared with that of the traditional ones (1). In addition, these methods exhibit high performance and the ability to analyze a large number of simultaneous samples with automated results, allowing real-time analysis and the possibility of early detection of contamination. As a matter of fact, the use of AMMs is supported by many official documents used for validation guidance. The WHO technical report encourages the use of RMMs as a replacement for conventional methods if the appropriate validation is performed for the pharmacopeial method (2). Also, all pharmacopoeias in their latest versions promote their use as detection methods, offering guides for their implementation in the industry. The European and

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British Pharmacopoeias, for example, recognize new possibilities that allow the implementation of RMMs for taking earlier corrective action as compared with conventional methods, which are generally slow (3–5). Additionally, the Japanese Pharmacopoeia stresses that AMMs can be also applied to viruses and fungi, not only to bacteria. This fact shows the usefulness of AMMs, which help to improve microbial control in critical areas, decreasing the risk of hazardous contamination, among many other advantages (5).

However, despite the broad adoption of AMMs and RMMs in the food industry (6–9), they are almost completely unexplored in the pharmaceutical industry, because they are under strict regulatory limitations that include a stricter microbiological release specification and are expensive and time consuming to validate, these issues being the primary barriers to their adoption (1). Also, due to the strict regulations, the pharmaceutical industry has always tended to be overly cautious in implementing innovative technologies (10). Furthermore, additional internal barriers for the adoption of AMM in the industry include additional validation, dossier submission, cost of equipment, and a slow return on investment. Hence validation is expensive and time consuming, and the inclusion of AMMs as part of an industrial agreement always involves the risk of delaying approval (1). Because of this, the performance of many serious validation studies is essential, and they will be of service for regulatory agencies in helping to make it easier for the pharmaceutical industry to implement such technologies (11). One example of a validation approach used for pharmaceutical preparations was published using the Bioluminex system (an old version of Soleris technology) using nonsterile products such as antacids, suppositories, laxatives, ibuprofen, and aspirin, among others, as pharmaceutical matrices.

The Bioluminex system, as an AMM, has been proven to be *not inferior* in performance for the detection of yeast and mold to the standard reference method, showing its applicability as an alternative method to replace the plate-count method (PCM) in the pharmaceutical industry (12).

It is known that the drug product matrix is an important factor for validation results. In the present study, a widely used pharmaceutical product, an antacid oral suspension, was selected. Its active ingredients (aluminum hydroxide and magnesium hydroxide) can relieve an upset stomach through neutralization of digestive acids (13, 14). In this manner, an antacid helps to mitigate a broad spectrum of symptoms, including gastroesophageal reflux disease (GERD) associated with heartburn, biliary reflux, and gastric stress, among many others (15, 16). Thus, the study of this matrix is patently relevant for both pharmaceutical and medical purposes because of the high incidence of patients with gastric problems around the world (17). For example, an estimated GERD prevalence of up to 23.0% has been found in South America and 27.8% in North America (18). Although this is an over-the-counter medicine with a high commercial demand, its availability on the market may be hindered by microbiological quality control. Therefore, it is crucial to expedite these last analytical steps, which involve mold and yeast detection and quantification before the product's release for sale.

According to USP <1111> requirements' acceptance criteria for the microbiological quality of nonsterile dosage for all aqueous preparations for oral use such as antacid oral suspension, there should be a total count for yeasts and molds of <20 CFUs and an aerobic microbial count of <200 CFUs, and the sample should be free of *E. coli*, to fall under the microbiological specification before the product's release for sale (19).

In the pharmaceutical industry, the microbiological process that causes the greatest delay for the finished product's release to the market is the total yeast and mold count, because as is outlined in USP <61>, this microbiological assessment normally takes 5–7 days, whereas the total aerobic microbial count usually takes 3–5 days (20). The validation method proposed in the present article is intended to reduce the microbiological process time for yeast and mold counts from 5–7 days, as the standard reference method usually takes, to 72 h using the AMM. So, the microbiological assessment for the antacid oral suspension is intended to be done in 3 days for total aerobic microbial count and analysis of pathogens such as *E. coli* (using the conventional method), and three days for the total yeast and mold count (using the Soleris system) (6, 21). This fact makes this technology a promising candidate to replace the PCM for yeast and mold analysis, given the huge economic advantage it would generate in the pharmaceutical industry. Antacid oral suspension is the product with the highest number of batches produced in the company due to its high

commercial demand. Therefore, the reduction of microbiological analysis times would help to release the product much faster to the market, reduce warehousing costs, improve efficiency in inventory control, and increase the ability to respond quickly to adverse microbiological results. Because it is an integrated, automated system, it is also less labor intensive, allowing online measurement registration and real-time microbial monitoring and ensuring data integrity and traceability of the analysis (22–23).

As outlined in USP <1223> and demonstrated in other validation investigations (24, 25), Soleris technology could be considered to be an alternative quantitative method for the detection of yeasts and molds through the construction of calibration curves that allow the establishment of numerically equivalent results between enumeration data from the standard reference method and the alternative method (25). Calibration curves would fulfill USP <1223> requirements, such as the determination coefficient ($R^2 \geq 0.9025$) and correlation coefficients ($CC \geq 0.95$). Detection time (DT) data from Soleris equipment software for automatic routine antacid oral suspension assessment of yeasts and molds through a direct comparison with the calibration curve will allow translation of DT into its CFU enumeration equivalent. In this way, the alternative method will have the same regulatory implications as the traditional method for microbiological specifications, because all the Soleris results will be represented in CFUs.

For this purpose, Soleris technology as an automated growth system based on optical variation for rapid detection and quantification of yeasts and molds was validated in the present study. This method is based on the microbial metabolism, using DYM-109C vials. The vial is divided into two zones: the upper part for the sample, and the lower part containing an agar plug with a dye indicator (thymolphthalein). Carbon dioxide generated by the yeasts' and molds' metabolism diffuses from the top to the bottom until it reaches the plug. The zones are divided by a membrane barrier that allows only gas entrance (6, 12, 18). In this way, carbon dioxide and water form carbonic acid, lowering the pH value at the reading zone. This leads to a change of color over time, which is automatically detected and recorded by the automated system (6–9, 18). It is worth noting that both the software and hardware ought to fulfill good manufacturing practice (GMP) requirements, along with the guidelines offered by the Parenteral Drug Association (26, 27).

In this study, we report the validation of this automated system for the quantification of yeasts (*Candida albicans*) and molds (*Aspergillus brasiliensis*) at three different microbial burden levels, which enables the establishment of the microbiological specification for all aqueous preparations for oral use, with high confidence. An antacid oral suspension containing 4% aluminum hydroxide, 4% magnesium hydroxide, and 0.4% simethicone was chosen. The main purpose of this validation study was to prove that the AMM's entire performance is *not inferior* to that of the conventional PCM. In order to accomplish the validation of the alternative method, two steps were followed. First, an equivalence of results was established between the CFUs counted in a standard PCM and the DT measured by the automated system (indicating a positive test result or presence of microorganisms). The second step was to prove that the validation criteria such as linearity, operative range, equivalence of results, accuracy, limit of detection (LOD) and limit quantification (LOQ), precision, ruggedness, and specificity were established in accordance with the United States Pharmacopeia (24, 28). This study aims to assist health regulatory authorities by providing documented proof that will assist in the approval of these new technologies in the pharmaceutical industry.

Materials and Methods

AQ: 1

Reagents

Gastro Full oral suspension (4% aluminum hydroxide + 4% magnesium hydroxide + 0.4% simethicone) was chosen for use in this study. Two antacid matrices were tested, each with the same formulation but elaborated with raw materials provided by two different suppliers for the USP grade aluminum hydroxide and USP grade magnesium hydroxide. These two suppliers were Khemik S.A.S. (antacid suspension 1) and Corinter S.A.S. (antacid suspension 2). The strains *A. brasiliensis* (Cat. No. ATCC 16,404), *C. albicans* (Cat. No. ATCC 10,231), *Pseudomonas aeruginosa* (Cat. No. ATCC 9027), *Escherichia coli* (Cat. No. ATCC 8739), *Staphylococcus aureus* (Cat. No. ATCC 6538), *Salmonella typhimurium* (Cat. No. ATCC 14,028), and *Bacillus subtilis* (Cat. No. ATCC 6633) were studied. Other reagents, including tryptic soy broth (TSB) (Scharlab, code 02-200), sabouraud dextrose agar (SDA) (Neogen, cat. No. NCM0008), tryptic soy agar (TSA) (Scharlab, code 02-200), and Tween 20 (Scharlab, code 73,625), were used as provided.

Rapid Microbiological Method

Soleris 128 instruments and supplies were acquired from Catver S.A.S., Neogen Corporation representatives in Colombia. The system includes four incubator drawers (128 vial places), with a precise temperature control for each drawer (15°C–60°C, ± 0.5°C), and dedicated software and computer. The design qualification (DQ), installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ) of the system were satisfactory fulfilled by both user and supplier, according to PDA guidelines (27). DYM-109C vials, approved by AOAC Research Institute (License #051,301), were used to quantify the representative yeast and mold (*C. albicans* and *A. brasiliensis*).

Inoculum Standardization

C. albicans and *A. brasiliensis* were reactivated from frozen stocks in SDA and incubated for 48 h at 20°C–25°C. After the growth time was completed, isolated colonies were taken and resuspended in 0.9% saline solution until they reached a McFarland standard of 2 (equivalent to 1.0×10^8 CFU/mL). Then a serial dilution was performed for both strains to assure work was performed with the dilutions when they reached a count of 10–100 CFU/mL.

Suitability of the Method

Antacid oral suspension utilizes methylparaben and propylparaben as active preservatives. Parabens are very effective in preventing the growth of yeasts, molds, and to a lesser extent bacteria (29). However, a check of the historical microbiological analyses for the antacid oral suspension finished product showed that there is no historical contamination susceptibility for either pathogenic bacteria or total aerobic microbials (including yeasts and molds).

In order to avoid any interference and prevent false negatives, the antacid suspension's methylparabens and propylparabens were neutralized. Polysorbate (Tween 20), a highly ethoxylated compound, was used to neutralize the activity of parabens, leading to the yeast's and mold's recovery by the AMM and the PCM. First, 1 mL of inoculum dilution (10–100 CFU/mL) was added to a Schott bottle and mixed with 90 mL of broth in the presence of the selected neutralizing agent (40 g/L of Tween 20 in TSB). Then, 10 mL of the antacid suspension was added and vigorously shaken to ensure sample homogenization. Different controls were performed as follows: control for the antacid product preservative (1 mL inoculum +10 mL antacid product + TSB, without Tween 20), control for the neutralizing agent toxicity (1 mL inoculum + TSB + Tween 20, without antacid product), and a negative control (TSB +Tween 20, without inoculum or antacid product).

Each test was done in triplicate, and the recovery of the microorganisms was counted and averaged by the PCM, following USP guidelines (16). Three preservative inactivation times (20, 30, and 40 min) were tested for both the antacid suspensions (three different lot numbers for each one). It was found that the shortest period (20 min) was enough for the recovery of both microorganisms (separate tests). From this procedure, recovery measurements for each strain were simultaneously performed via the AMM and the PCM to set up equivalence between the methods and generate a calibration curve for the validation (21).

Calibration Curve

Once the efficacy and nontoxicity of the neutralizing agent was proven, the suitability of the method (previously described) was repeated to produce a stock solution. From it, a serial dilution was performed, as recommended by Neogen's representation (30). From each dilution (D1–D8), 1 mL was placed directly into each DYM-109C vial, and they were incubated for 72 h at 23.5°C in the equipment. The vials were inoculated in duplicate. Simultaneously, agar plates were also inoculated in duplicate with 1 mL of each dilution by the spread plate technique. The plates were incubated at 23.5°C for 7 days. These series of experiments were performed at least ten times for each microorganism. An uninoculated DYM-109C vial was used as a negative control, and an inoculated vial with 1 mL of the dilution (count of 10–100 CFU/mL) was used as a positive control. Every DT recorded by the system within the incubation period (3 days), confirmed also by color change, was an indication of the presence of microorganisms. Data generated by both the alternative and conventional method were plotted in Minitab to generate calibration curves by plotting DTs (measured by the AAM) relative to the corresponding log CFU values (obtained by the PCM). In this way, more than a hundred data points were plotted for both microorganisms, *C. albicans* (129 data) and *A. brasiliensis* (136 data).

COLOR

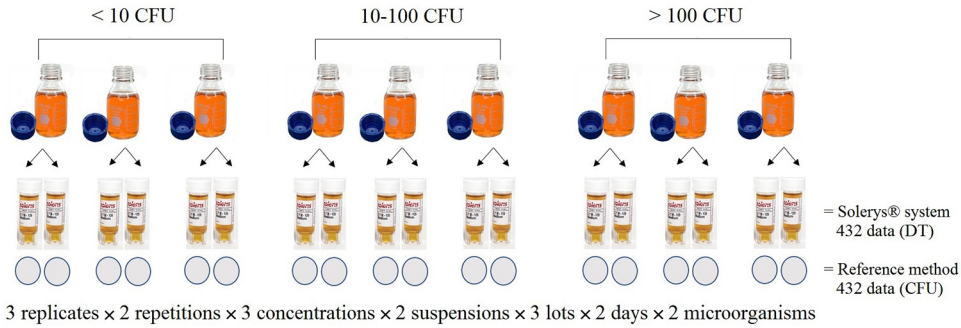

Figure 1

Experimental design used for the validation testing of the AMM vs PCM. AMM, alternative microbiological method; DT, detection time; PCM, plate count method.

Validation Testing

Validation of the alternative method was performed by reproducing the suitability of the method once again but using three different microbial bioburden levels (<10 CFUs, 10–100 CFUs, and >100 CFUs). For each case, 1 mL was mixed with 100 mL of TSB (without either Tween 20 or an antacid suspension) and used as a positive control. An uninoculated DYM-109C vial was used as a negative control. For each concentration, three replicates were made, each of them with two repetitions (see Figure 1). This procedure was performed for both antacid suspensions (three different lot numbers for each one), on two different days, and for both microorganisms. Thus, a total of 432 DT values were automatically collected by the system (AMM), and simultaneously 432 CFU values were also measured via the standard reference method (PCM). These data were fed into the statistical study to analyze validation parameters in accordance with USP requirements (30). These criteria include: linearity, equivalence of results, accuracy, LOD and LOQ, precision, ruggedness, specificity, and operative range, as explained following.

F1

Linearity and Equivalence of Results

The plotted values in the calibration curve were fit to a least-squares regression, and the coefficient of determination (R^2) was calculated. Linearity was determined using the chi-square (x^2) goodness-of-fit test model to evaluate the relationship between CFU and DT data obtained from the PCM and the AMM, respectively. These results were also analyzed using a Poisson-based statistical method for probability of detection (POD) (6, 7, 18). The observed POD is "the number of test method positive results divided by the number of portions tested, with a 95% confidence interval" (6). The predicted POD was calculated using the formula: $POD_{(predicted)} = 1-e^{-c}$, where e is the base of the natural logarithm and c is the number of input CFUs per vial, at a given test threshold based on the standard reference method. Furthermore, the observed POD for the AMM was calculated at each test threshold (<10 CFUs, 10–100 CFUs, and >100 CFUs) by dividing the number of positive results by the number of vials tested.

Accuracy

The accuracy of the data was assessed through the percentage of recovery, taking into account that the accuracy of a valid AMM must capture ≥70% of the microorganisms recovered via the conventional method (PCM). The accuracy percentage was measured as "the ratio of CFU/sample values derived from vial DTs (using the least-squares regression model obtained in the linearity section) to the corresponding mean plate count from the parallel sampled dilution across the test range" (25). The coefficient of correlation (CC) obtained from the calibration curve was considered to be a measure of accuracy. In the same way, DT values and their log10 equivalent CFU counterparts were statistically analyzed using the Pearson goodness-of-fit test based on a Poisson distribution.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ were determined from the calibration curve and the suitability of the method, using a bioburden threshold of <10 CFUs (for both strains). For the PCM, the LOD was calculated "as the mean CFU/

sample recovered from the plate count results based on the fewest number of recoverable microorganisms" (25). So, the LOD and LOQ were determined using data for both antacid suspensions (three different lot numbers for each one), on two different days, and for both microorganisms. The values obtained in the experiments ($n = 72$) were then averaged to determine the mean and standard deviation values. For the AMM, the LOD and LOQ were determined for the automated system data, corresponding to the same bioburden thresholds (<10 CFU) used for the PCM. For this purpose, DT values for these vials were converted to CFUs per sample using the equations derived from the calibration curve (25). Mean and standard deviation values were also determined. Thus, LOD and LOQ for both methodologies (PCM and AMM) were calculated using the following equations: LOD = 3.3*SD/m and LOQ = 10*SD/m, where SD is the standard deviation and m is the slope of the linear regression obtained for each calibration curve.

The calibration curves for both microorganisms were saved into the Soleris equipment's software. So, the DTs routinely generated by the Soleris equipment for the analysis of the antacid oral suspension are automatically compared with the corresponding calibration curve for *C. albicans* and *A. brasiliensis*. Therefore, for a DT, its equivalent result in colony-forming units will be generated, permitting establishment of limits of microbiological acceptability.

Precision, Robustness, and Ruggedness Testing

Dilutions of fresh microorganisms prepared for the selected bioburden threshold (<10 CFUs, 10–100 CFUs, and >100 CFUs) were used to determine the suitability of the method. In this way, mold and yeast were recovered via the PCM and the AMM, using both antacid suspensions (three different lot numbers for each one), on two different days, and for both microorganisms. To measure the operators' variability, three operators were used to determine the suitability of the method (preservative neutralization) using three microbial concentrations (≤10 CFUs, 10–100 CFUs, and ≥100 CFUs). For each concentration, three replicates were made, each of them with two repetitions. This procedure was done separately for *A. brasiliensis* and *C. albicans*.

Standard deviation and coefficient of variance then were determined from the DT data obtained with the alternative method. Finally, an analysis of variance (ANOVA) was performed to assess the influence of different operational variables on the DT values. All statistical analyses were performed using licensed Minitab software version 20.1.0.0.

Specificity for Exclusivity Testing

Specificity testing was carried out on 5 pathogenic bacteria commonly analyzed in the pharmaceutical industry, namely *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, and *Bacillus subtilis*. The strains were reactivated from frozen stocks in TSA or other selective media as appropriate for particular organisms and incubated for 48 h at 30°C–35°C. After the growth time was completed, isolated colonies were taken and resuspended in 0.9% saline solution until they reached a McFarland standard of 2 (equivalent to 1.0×10^8 CFU/mL). Then a serial dilution was performed to assure work was performed with the dilutions at a count of 10–100 CFU/mL. One milliliter of the dilution (count of 10–100 CFU/mL) was placed directly into each DYM-109C vial in triplicate, and then the vials were incubated for 72 h at 23.5°C in the Soleris equipment.

Specificity for Inclusivity Testing

C. albicans and *A. brasiliensis* were reactivated from frozen stocks in SDA and incubated for 48 h at 20°C–25°C. After the growth time was completed, isolated colonies were taken and resuspended in 0.9% saline solution until they reached a McFarland standard of 2 (equivalent to 1.0×10^8 CFU/mL). Then a serial dilution was done to assure work was done with these dilutions at a count of 10–100 CFU/mL. For both strains, 1 mL was placed directly into each DYM-109C vial, and they were incubated for 72 h at 23.5°C in the equipment.

Results and Discussion

AQ: 2

Linearity and Equivalence of Results

The equivalence of results between two different quantifiable methods is a measure of the similarity among the test results obtained by each one. So, taking into account that the PCM and the AMM yield quantifiable values of different units (CFUs *vs.* DT), an equivalence of results must be done. For the alternative method, all the data were converted into the period of time of 72 h

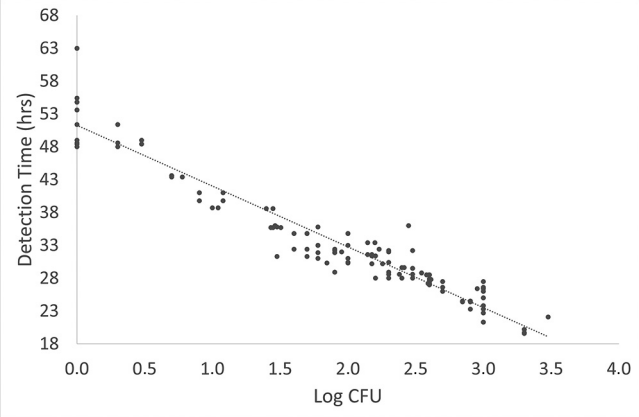

Figure 2

Calibration curve for *A. brasiliensis*. Linearity of the alternative method (DT) versus the standard reference method (Log CFU). DT, detection time.

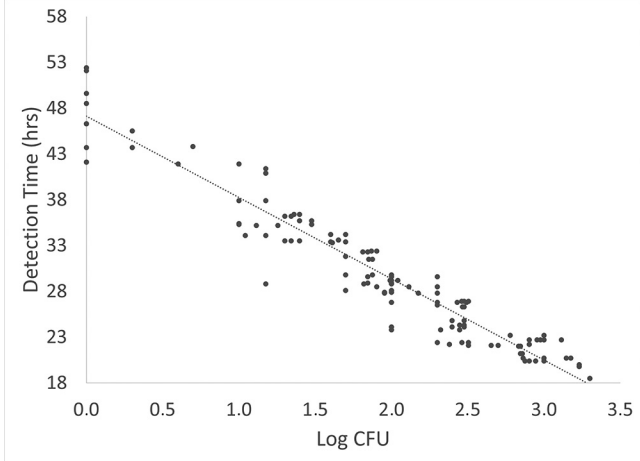

Figure 3

Calibration curve for *C. albicans*. Linearity of the alternative method (DT) versus the standard reference method (Log CFU). DT, detection time.

at 23.5°C, and the equivalent results from the PCM were obtained at Day 7 of incubation at 23.5°C. In the present study, through the construction of calibration curves and the POD model, a quantitative and a semiquantitative equivalence of results were done, respectively.

For the quantitative equivalence of results, calibration curves for *C. albicans* and *A. brasiliensis* were derived by plotting DT values with their respective equivalents on Log CFU, as shown in Figure 2 and Figure 3. Linear-regression analysis yielded the relationship between DTs and Log CFU values, as shown in Table I. The linearity observed in *C. albicans* and *A. brasiliensis* (0.9202 and 0.925, respectively) was consistent with USP requirements ($R^2 \geq 0.9025$) (24). Supporting these results, a X^2 test demonstrated a statistical association between the microbial concentration (CFUs) and the DT values ($P \leq 0.05$, Table I). Thus, the ability of the alternative system (linearity) to produce signs that depend on the microbial threshold is a key parameter for successfully achieving the AMM's validation. These results yielded evidence that the Soleris technology can be considered to be an alternative quantitative method for the detection of yeasts and molds through the construction of calibration curves that allow the establishment of numerically equivalent results between the accounting data from the standard reference method and the Soleris alternative method ($R^2 \geq 0.9025$ and X^2 $P \leq 0.05$).

In order to support the results obtained for the calibration curves, a semiquantitive equivalence of results was also carried out. A Poisson-based POD model using 95% confidence intervals was used to establish a statistical equivalence of results between the methodologies. This was performed for data obtained for three test microbial thresholds, including different operational variables (varying antacid suspension suppliers, lot numbers, and days). The results obtained from the $POD_{(observed)}$ and $POD_{(predicted)}$ for *C. albicans* and *A. brasiliensis,* are summarized in Table II. It commonly is accepted that if the $POD_{(predicted)}$ falls within the lower and upper 95% confidence limits of the $POD_{(observed)}$, the result is equivalent; otherwise, it is not (6, 7, 9, 18, 25). Therefore, the predicted CFU values obtained for both microorganisms (*C. albicans* and *A. brasiliensis*) at different thresholds (<10 CFUs, 10–100 CFUs, and >100 CFUs) were

Table I
Linearity Data Obtained for the Calibration Curves of Each Microorganism.

Test Organism	Linear Regression	Coefficient of Determination(R^2)	Chi-square Parameter(X^2)
A. brasiliensis	y = −9.2527x + 51.263	0.9250	$P \leq 0.05$
C. albicans	y = −8.8802x + 47.128	0.9202	$P \leq 0.05$

Table II
Equivalence of Results Obtained for the Alternative Method vs the Reference Plate Count Method.

Test Organism	Soleris Test Threshold	Mean CFU	Mean $POD_{(predicted)}$	Number of Vials		LCI	Mean $POD_{(observed)}$	UCI	Result
				Positive	Tested				
A. brasiliensis	<10 CFU	5	0.85	72	72	0.8	1	1	Equivalent
	10–100 CFU	123	1	72	72	0.8	1	1	Equivalent
	>100 CFU	1861	1	72	72	0.8	1	1	Equivalent
C. albicans	<10 CFU	3	0.84	72	72	0.8	1	1	Equivalent
	10–100 CFU	108	1	72	72	0.8	1	1	Equivalent
	>100 CFU	1817	1	72	72	0.8	1	1	Equivalent

CFU: colony-forming units; LCI: lower confidence interval; POD: probability of detection; UCI: upper confidence interval.

within the upper and lower confidence intervals, showing an equivalence of results between the AMM and the PCM.

Accuracy

In order to measure the accuracy of the AMM, DT values were used to determine log CFU by the previously described linear regression (Table I). The mean accuracy log CFU derived from the alternative method DT was ≥70% of the parallel plate count for each bioburden threshold (<10 CFUs, 10–100 CFUs, and >100 CFUs). The percentage of recovery results obtained for both microorganisms is shown in Table III. So, for *C. albicans*, the percentage of recovery was 118%, 106%, and 118% at <10 CFUs, 10–100 CFUs, and >100 CFUs bioburden threshold, respectively. For *A. brasiliensis*, the percentage of recovery was 121%, 117%, and 105% at <10 CFU, 10–100 CFU, and >100 CFU bioburden threshold, respectively (Table III). The Pearson goodness-of-fit test was chosen to assess the proximity of the results obtained by the AMM and those observed for PCM for each microorganism. In this way, the automated system was able to predict CFUs from DT values using a 95% of confidence interval ($P \geq 0.05$, Table III). Supporting these results, *C. albicans* and *A. brasiliensis* showed a CC of 0.96 and 0.95, respectively, fulfilling USP requirements (CC >0.95) (24). So, a high CC is a reliable indication of the accuracy that the quantifiable data acquired via the PCM (in CFUs) can be calibrated to DT units generated by the alternative method.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ for *C. albicans* and *A. brasiliensis* were determined for the PCM as well as for the AMM. The LOD and LOQ were calculated using the standard deviation of data obtained for the fewest number of recoverable microorganisms (<10 CFUs) and the slope of the corresponding standard curve(s). The LOQ was 3 CFUs for the PCM and 4 CFUs for the AMM (Table IV). The LOD for the PCM and the AMM was 1 CFU/sample (Table IV). Thus, the LOD and LOQ of the AMM for *C. albicans* and *A. brasiliensis* were statistically similar to

Table III
Percentage Recovery Obtained for the Alternative Method vs the Reference Plate Count Method

Test Organism	Soleris Test Threshold	Mean Soleris Counts	Mean Log CFU	Recovery (%)	Pearson	Coefficient of Correlation (CC)
C. albicans	<10 CFU	0.66	0.55	118%	$P \geq 0.05$	0.96
	10–100 CFU	2.14	2.00	106%	$P \geq 0.05$	
	>100 CFU	3.84	3.25	118%	$P \geq 0.05$	
A. brasiliensis	<10 CFU	1.28	0.94	121%	$P \geq 0.05$	0.95
	10–100 CFU	2.47	2.00	117%	$P \geq 0.05$	
	>100 CFU	3.48	3.18	105%	$P \geq 0.05$	

Table IV
Limit of Detection (LOD) and Quantification (LOQ) for the PCM and AMM at the Lowest Microbial Threshold (<10 CFU) Before Total Decay of Response.

Test Organism	PCM, CFU/sample			AMM, CFU/sample			Fisher's exact test
	LOD	LOQ	SD	LOD	LOQ	SD	
A. brasiliensis	1	3	3	1	5	4	$P \geq 0.05$
C. albicans	1	3	3	1	4	4	$P \geq 0.05$

AMM, alternative microbiological method; LOD, limit of detection; LOQ, limit of quantification; PCM, platelet count method; SD, standard deviation.

those of the PCM, according to Fisher's test ($P > 0.05$). According to USP <1111> requirements' acceptance criteria for the microbiological quality of nonsterile dosage for all aqueous preparations for oral use, such as the antacid oral suspension tested, there should be an absence of *Escherichia coli* (1 g or 1 mL) and a total count for yeasts and molds of <20 CFUs to fall under the microbiological specification before the product's release for sale. In this way, the AMM was shown to have the ability to detect 1 CFU during the assay time (72 h), thus ensuring the microbiological assessment quality for yeast and mold analysis as specified by the USP.

Intermediate Precision Robustness and Ruggedness

AQ: 3 In order to gain insight into the ST validation, the intermediate precision was estimated. The precision of the alternative quantitative microbiological method is the degree of agreement among individual test results when the experimental design is applied repeatedly to several samples across the range of the test (24, 31). The repeatability and the intermediate precision were estimated independently for each bioburden threshold and microorganism (*C. albicans* and *A. brasiliensis*). For this experiment, ruggedness was interpreted as the intermediate precision, a type of intralaboratory precision involving the effect of different suppliers, days, lots, and operators on the test result variability, as well as the repeatability. In order to observe the effect of these operational variables on the average DT for each bioburden level, a multifactorial ANOVA was performed.

It can be seen that for *C. albicans* and *A. brasiliensis*, the number of lots from the same supplier, operators, and different days of analysis do not have a significant effect on the mean of the DTs (ANOVA, $P > 0.05$, Table V). T5
However, a comparison among the providers showed a strong influence on the average of the DTs as well as the mean of the CFUs (ANOVA, $P < 0.05$, Table V). These statistical differences probably correspond to differences in the preservative concentration among the raw materials used by those two suppliers. However, besides the average DT differences observed for these suppliers at the

Table V
Ruggedness Validation.

ANOVA	*C. albicans*			*A. brasiliensis*		
	<10 CFU	10–100 CFU	>100 CFU	<10 CFU	10–100 CFU	>100 CFU
Supplier 1 *vs* supplier 2	0.008	0.027	0.000	0.000	0.000	0.000
Supplier 1; lot 1 *vs* lot 2 vs lot 3	0.908	0.792	0.000	0.496	0.383	0.000
Supplier 2; lot 1 *vs* lot 2 vs lot 3	0.085	0.555	0.000	0.150	0.503	0.000
Supplier 1 at Day 1 *vs* supplier 1 at Day 2	0.898	0.520	0.000	0.771	0.307	0.878
Supplier 2 at Day 1 *vs* supplier 2 at Day 2	0.122	0.336	0.025	0.218	0.054	0.000
Operator 1 *vs* Operator 2 *vs* Operator 3	0.673	0.584	0.149	0.711	0.677	0.101

The numbers represent the *P* values of the ANOVA test to compare the effects among suppliers, lots of the same suppliers, operators, and different days on the average DT at different bioburden thresholds for *C. albicans* and *A. brasiliensis*.
ANOVA, analysis of variance; DT, detection time.

Table VI
Precision of the Alternative Method for the Individual Test Organism

	C. albicans			*A. brasiliensis*		
	<10 CFU	10–100 CFU	>100 CFU	<10 CFU	10–100 CFU	>100 CFU
Standard deviation	3.88	4.92	3.23	4.51	3.32	1.80
Coefficient of variation	9.67	17.49	24.92	11.67	13.05	9.43

three different microbial bioburden levels tested (ANOVA, $P < 0.05$, Table V), the values from the AMM and the PCM exhibited a high degree of concordance at each microbial threshold (SD <5 and coefficient of variation 15%–35%, Table VI). It may be useful to note that the calibration curves made for yeasts and molds during the validation process included all these variations for routine assessment of yeast and molds in the antacid oral suspension (X^2 $P \leq 0.05$).

In the same way, for both the microorganisms, *C. albicans* and *A. brasiliensis*, a significant difference was observed in the DT average at a microbial bioburden level >100 CFUs ($P \leq 0.05$, Table V). Probably the variance in the inoculum preparation at high concentrations (>100 CFUs) is much greater, where minimal variations of the sample preparation can strongly alter the outcomes. It may be useful to note that the difference observed in the DT average at higher inoculum level may not be material in view of the acceptable results obtained at the lower inoculum levels and current compendial limits of 20 CFU yeasts and molds for the antacid oral suspensions evaluated. However, this inherent variability of these methods must be considered during the selection, development, and validation of alternative methods. Nonetheless, the coefficient of variation and standard deviation fell under USP requirements (SD <5 and coefficient of variation <35%, Table VI).

The robustness parameter was assessed by the manufacturer. According to the Neogen supplier's information, the detection of yeasts and molds for the Soleris equipment has demonstrated its capacity to remain unperturbed by small but deliberate variations in the method's parameters such as temperature variation (28°C ± 0.5°C), algorithm detection parameter (10 optical units ± 2 optical units), and different Soleris equipment.

Specificity

As listed in Table VII, all the pathogenic bacteria tested (*E. coli*, *S. thyphimurium*, *S. aureus*, *P. aeruginosa*, and *B. subtilis*) were unable to grow in the Soleris DYM 109 C vials (Table VII), whereas *C. albicans* and *A. brasiliensis* were able to grow as expected (Table VII). In the present study, we utilized *A. brasiliensis* and *C. albicans* as representatives for yeasts and molds, respectively. However, according to the information supplied by the manufacturer, the DYM 109 Soleris vials allow the growth of several species of yeasts and molds other than those tested in the present investigation. Thus, DYM Soleris vials allowed the growth of *A. brasiliensis*, *C. albicans*, *Penicillium roqueforti*, food-isolated molds, and *Saccharomyces cerevisiae*. At the same time, the DYM Soleris vials inhibited bacterial growth such as of *Bacillus cereus* and *Staphylococcus aureus* because of the presence of chloramphenicol and oxytetracycline, thus ensuring that the growth observed inside the vials corresponded exclusively to yeast and mold growth. These results show the high specificity of the alternative method to exclusively detect the growth of yeasts and molds.

Operative Range

Quantitation ranges were established from the calibration curves for each microorganism. So, for *C. albicans*, there exists a high correlation (R^2 = 0.9202, CC = 0.96, and X^2 $P \leq 0.05$) between the alternative

Table VII
Results for Specificity Testing for the Soleris Yeasts and Molds Method

Microorganisms	Soleris Vial DT/ND	Plate count (CFU)
Pseudomonas aeruginosa	ND	0
Escherichia coli	ND	0
Staphylococcus aureus	ND	0
Salmonella typhimurium	ND	0
Bacillus subtilis	ND	0
Candida albicans	30.4	89
Aspergillus brasiliensis	26.1	96

ND = no growth detected withing 72 h.
DT = Growth detected withing 72 h.

and standard methods from 1 CFU to 1.9 $\times$ 10^3 CFUs. In the same way, for *A. brasiliensis*, there exists a high correlation (R^2 = 0.925, CC = 0.95, and X^2 $P \leq 0.05$) between the alternative and standard methods from 1 CFU to 2.0 $\times$ 10^3 CFUs.

Conclusions

In this study, carried out in Coaspharma laboratories, it has been demonstrated that based on *C. albicans* and *A. brasiliensis* calibration curves and on validation testing at different microbial concentrations (<10 CFUs, 10–100 CFUs, and >100 CFUs), it was possible to establish a quantitative equivalence of results, which allows demonstration that the Soleris automated growth system can be used as a replacement for the standard reference method for the quantification of *C. albicans* and *A. brasiliensis* in an antacid oral suspension. This proposed alternative method is potentially faster than the PCM for assessing yeasts and molds. This method reduced the assessment time from 5–7 days, as is usual for the reference method, to a maximum of 72 h or even less time, because the limits of detection (1 CFU) for both microorganisms could be reached in maximum times of 63 h and 53 h for *A. brasiliensis* and *C. albicans,* respectively. This fact was clearly demonstrated by the calibration curves obtained for each microorganism tested. The evidence demonstrates that this alternative automated method yields accurate quantitative results equivalent to those of the PCM (R^2 >0.9025, CC >0.95, and percent recovery >70%). Its ability to remain unaffected by different operational variables (antacid suspension, product lot, operators, day, and microbial bioburden) was evidence of its reliability and stability.

As can be observed in the calibration curves, the LODs (1 CFU) for both microorganisms can be reached in maximum times of 63 h and 53 h for *A. brasiliensis* and *C. albicans,* respectively (Figure 2 and Figure 3). These results show the good performance of the AMM in detecting the lowest microbiological contamination, ensuring an accurate microbiological assessment of the antacid oral suspension. Furthermore, the LOD and LOQ were not statistically different between the alternative and the standard method (Fisher's test P > 0.05), showing that the AMM's performance is *not inferior* to that of the PCM. All these results show that AMMs are an important tool for more quickly manufacturing pharmaceutical products such as antacid oral suspensions. However, further investigation would be required to qualify Soleris technology for other environmental isolates and recovery of these isolates from production batches at the incubation time tested (72 h), or whether more incubation time will be required to recover them. However, the evidence in foods shows that products naturally contaminated by yeasts and molds could be recovered in the 72 h of incubation time using the Soleris System (6).

The use of such alternative methodologies results in a reduction of company warehousing costs, improved efficiency in inventory control, and the ability to respond more quickly to adverse microbiological results.

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Conflict of Interest

The authors declare no conflict of interest. All the research was funded by Laboratorios Coaspharma S.A.S.

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4—Au: Should there be some unit of measurement associated with the numerical values in Table VII?