

Method for Dissolution Profile Determination of Probiotic Bacteria from Controlled Release Solid Oral Dosage Forms via Direct Enumeration Fluorochrome Staining

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Summary 4',6-diamidino-2-phenylindole (DAPI) fluorochrome stained probiotic bacteria dissolution samples were used to directly enumerate and determine release profiles from controlled release solid oral dosage forms. Combined with indirect agar plate counting techniques, this method allows for quantification of viable bacterial proportion surviving dosage form manufacture and simulated gastric exposure. **Introduction** In recent years, the use of solid oral dosage forms (tablets and capsules) has emerged as an efficient and effective means to deliver probiotic bacteria to the human gastrointestinal tract. Traditionally, disintegration testing and viable enumeration of the dosage form are used as the primary means for quality control of the manufacturing process and evaluation of dosage form performance. Such methods only evaluate viable bacteria and physical integrity of the dosage form. This novel method of using a USP Type II dissolution apparatus and direct enumeration via fluorochromes permits the quantification of total bacterial load (viable and non-viable) delivered and the determination of the release rate of bacteria from the dosage form. Furthermore, when employed in combination with viable counting techniques, it allows for the quantification of the viable bacterial proportion surviving dosage form manufacture and simulated gastric exposure. **Experimental Methods** Controlled release dosage forms containing *Bifidobacterium bifidum* and *Lactobacillus acidophilus* were evaluated using a USP Type II (paddle) dissolution apparatus, in 1000mL dissolution media at 50 rpm and 37 degrees C. Simulated transit through the gastric environment was performed by exposure to 0.1N HCl at pH 1.2, followed by dosage form removal and transfer to buffered peptone sustenance media at pH 7.4. Samples of media were removed for both viable and total bacterial release enumeration at two hour intervals over 12 hours. Samples destined for fluorochrome staining were inactivated with formaldehyde and treated with Tween 80 for particle dispersal. Samples for viable enumeration were removed aseptically and subject to standard plate count analysis on MRS and RCM agar (48-72 hours incubation at 37 degrees C at both anaerobic and 5% carbon dioxide atmospheric environs). Inactivated samples were vortexed, diluted, and then exposed to DAPI fluorochrome stain in absence of light for 10 minutes. The resulting 10mL samples were vacuum filtered through pre-stained Acid Black 25mm (0.2um) absolute polycarbonate filters. Filters were then fixed on slides and directly enumerated using UV microscopy. **Results and Discussion** Direct enumeration of DAPI stained samples effectively quantified cumulative release of bacteria from the controlled release dosage form over time. This direct-count technique permitted the calculation of both the 12-hour release profile and the total bacterial load delivered from the dosage form. Standard indirect plate count viable enumeration in conjunction with inactivated DAPI enumeration demonstrates viable bacteria release throughout the dissolution period. **Conclusion** Direct count enumeration of probiotic bacteria using a USP Type II dissolution apparatus and fluorochrome staining is an effective method for quantifying the release of bacteria from solid oral dosage forms. Indirect count enumeration, when combined with this direct-count method, allows for the determination of viable proportion of bacterial loads remaining after dosage form manufacture and after simulated gastric exposure.

Introduction

The objective of this project was to develop a method for the enumeration of probiotic bacteria released from a controlled release dosage form. Traditional methods of enumeration – disintegration and agar plate counts – are insufficient when evaluating controlled release dosage forms due to their inability to measure release over time. The controlled release nature of the dosage form prevents excessive losses in bacterial viability during gastric residence and is capable of delivering lyophilized bacteria in a sustained fashion throughout the gastrointestinal tract. Due to the contents of the dosage form being lyophilized, viable bacteria, a method of analysis was required which would evaluate the release characteristics of the dosage form in both simulated gastric and intestinal environments, and allow for the enumeration of both viable and non-viable bacteria released. While other methods of staining bacterial samples exist, they often stain excipients in the dosage form (such as the crystal violet stain used in Petroff-Hauser method) making enumeration very difficult. Thus, a method was developed using a USP Type-II dissolution apparatus to evaluate the release characteristics of the dosage form, wherein the samples removed from the vessels at given time points were enumerated using traditional plate counting techniques and also treated with a 4',6-diamidino-2-phenylindole (DAPI) fluorochrome stain and evaluated using UV-microscopy. The plate counting technique enumerated viable bacteria while the DAPI technique enumerated the fraction of total bacterial load (viable and non-viable) released from the dosage form. As the method was devised to evaluate a controlled-release dosage from capable of preventing excessive die-off in the gastric environment, a surrogate method of dissolution was devised incorporating two distinct media used to simulate gastric and intestinal environments: 0.1N HCl and pH 7.4 peptone sustenance media. The method was optimized to provide

an efficient means of DAPI enumeration through calculation of a statistically meaningful number of fields visible the microscope ocular, as well as creating slides stable in ambient light, samples capable of long-term storage, and a means for photographic recording of the slides.

Experimental Methods

Materials and Supplies

- 0.1% peptone media, 0.5% NaCl (per Difco, sterilized via autoclave, 15psi, 121° C)
- 0.1N HCl
- Dissolution Paddles and Vessels (sterilized via autoclave, 15psi, 121° C)
- 1L graduated cylinder (sterilized via autoclave, 15psi, 121° C)
- Filter wrap (Sterilized via autoclave, 15psi, 121° C)
- 70% Isopropyl Alcohol
- Transfer materials – plastic spoons, spatulas, scoops
- Stomaching instruments – dissolution retractors, spatulas, plastic spoons
- Sterile pipettes and glass sample tubes
- 99mL peptone buffer dilution bottles
- MRS and RCM agar plates
- Nitrocellulose filters (0.2 µm) pre-stained w/ Acid Black stain
- Backing filters (nitrocellulose – 1.0µm)
- Filter column assembly
- Vacuum pump
- Inactivation agent stock solution: 10% Formaldehyde (wt.vol.), filtered at 0.2µm
- Ultrapure Water (Sterilized via autoclave, 15psi, 121° C)
- DAPI stain stock solution (100µg/mL, prepared fresh)
- 10% Tween 80 surfactant stock solution
- Microscope with UV light ballast fitted with a narrow band 365/366nm UV filter and calibrated ocular graticule
- Non-fluorescing immersion oil

Dissolution Studies:

Dissolution studies were performed in 900mL 0.1N HCl at 50 RPM and 37° C for 2 hours in a type II apparatus. After exposure to the simulated gastric environment, the tablets are carefully transferred to dissolution apparatus vessels containing 1000mL 0.1% peptone media. The transfer is performed with plastic implements disinfected with 70% Isopropyl alcohol. Once the transfer is complete, release studies are conducted at 50 RPM and 37° C. Every 2 hours thereafter, 11mL samples of are removed into sterile glass tubes. Once the final dissolution sample has been removed, the infinity time point is prepared by stomaching or otherwise physically disrupting the tablet within the vessel using a spatula and increasing the paddle speed to 250 RPM for 1 hour. Maximum dispersal is achieved with the addition of 10mL of 10% Tween 80 surfactant stock solution (0.1% final concentration).

Sample preparation and storage:

Immediately upon removal, 100µL samples are aliquoted directly to agar media selective for either aerobic (MRS) or anaerobic lactobacilli (RCM) prior to inactivation. The remaining sample volume is then supplemented with 1mL 10% Tween 80 surfactant and vigorously vortexed prior to an addition of 1mL inactivation stock solution (with a final formaldehyde concentration of 1%). The samples are then stored at 4° C for DAPI analysis.

Stock solution preparation:

It is recommended that the DAPI stock solutions and 10% Tween 80 surfactant stock solution be prepared freshly within 24 hours of use. The DAPI stock solution must be protected from light at all times.

DAPI preparation:

100µL DAPI stock solution is added to the samples, bringing the sample to a final concentration of 1µg/mL. Samples are then lightly vortexed (due to the presence of formaldehyde) and allowed to stain in the absence of light for 10 minutes. The filter manifold is concurrently prepared by placing a 1.0µm nitrocellulose backing filter (pre-wetted in sterile ultra pure water) on the filter manifold base, followed by the placement of a pre-wetted 0.1µm nitrocellulose filter (pre-stained with Acid Black) on top of the backing filter. The filter columns are then affixed to the manifold and samples are filtered using a vacuum pump. Filters are fixed by gently laying the pre-stained filter on of a drop of non-

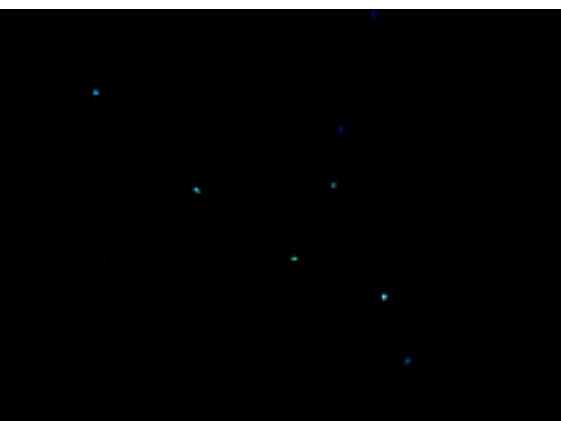


Figure 1. DAPI stain of a sample taken after two hours exposure to gastric environment



Figure 3. DAPI stain of a sample taken after 12 hours exposure to intestinal environment

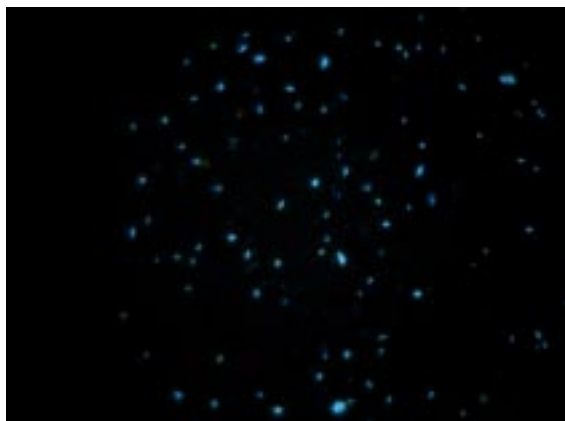


Figure 2. DAPI stain of a sample taken after 8 hours exposure to intestinal environment

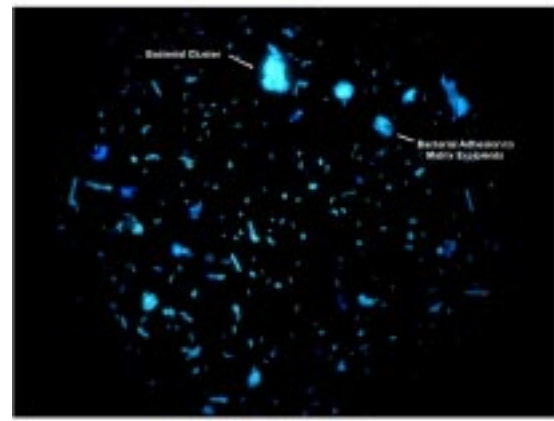


Figure 4. Infinity time point DAPI sample illustrating the presence of bacterial clusters and bacterial adhesion to matrix excipients

fluorescing immersion oil on the center of a microscope slide. A second drop of immersion oil is then evenly dispersed across the filter surface and a cover slip is then gently placed on top of the filter.

DAPI analysis:

DAPI slides are analyzed with the use of a microscope under UV light filtered at 365nm. An ocular grid is fixed in one of the eyepieces to allow for consistent enumeration of fluorescing bacteria; the ocular should be calibrated such that the surface area of the grid can be accurately calculated. A statistically significant amount of fields are enumerated and the number of aggregated clusters and individual bacteria are documented. The average bacterial count per grid is multiplied by the number of grids contained within the filter surface area; the resulting value is then multiplied by a dilution factor to yield an estimated bacterial load. The dilution factor is comprised of the dissolution volume adjusted for sample removal, addition of surfactants and inactivation solution, and any serial dilutions prior to filtration.

Results and Discussion

Many considerations must be taken when developing a method such as that described here. Maintenance of a testing environment favorable to viable enumeration, accurate calculation of dilution volumes and consistent techniques of enumeration are essential to obtaining accurate results which will allow for the determination of a release profile of fractional bacterial load from the dosage form.

DAPI staining:

The DAPI staining process requires optimization, as its effectiveness is dependant on many factors influenced by the content of the formulation and genetic composition of the organisms therein. The DAPI fluorochrome binds to A+T rich DNA sequences and therefore can have varying effectiveness on different bacterial strains. The DAPI fluorochrome may also bind non-specifically to excipients within the controlled release matrix. This phenomenon was initially intrusive to the analysis of the DAPI slides as the fluorescence emitted from the polymer created overwhelmed that of the organisms of interest. For this reason, adjustments in DAPI stain concentration, exposure durations and UV-ballast levels are often necessary for optimization of the method.

It is also highly recommended that glass sample and storage tubes be used; it was noted that when polypropylene sample tubes were used to store DAPI samples overnight, enumeration was significantly reduced due to cellular adherence to the container surface.

Calculation of Dilution Volumes:

As with any examination involving quantification of lyophilized probiotic bacteria, careful design must be given to the sampling and dilution regimen to prevent the processing of samples that lie out of the range of resolution. This is particularly relevant to any method that entails the quantification of bacterial load released from a controlled release dosage form, as the fraction of load is cumulative over time. Because the method also involves simultaneous evaluation of viable and non-viable organisms after exposure to a gastric environment, the dilution volumes for viable and non-viable samples may differ by one log or more. It is recommended that in the optimization of this method for a specific controlled release formulation, multiple viable samples be removed and plated at varying dilution volumes: e.g., if the total viable bacterial load is estimated at 1x10⁹ colony forming units (cfu) per dosage form, plating of both 1x10⁴ and 1x10⁷ cfu samples is likely to be helpful in determining an optimal dilution volume. It follows that as the duration of dissolution increases, the dilution volume at later time points will necessarily be greater than the volume necessary to enumerate viable cells immediately after exposure to a gastric environment: e.g., considering the above dosage form, it might be expected that if 1x10⁻⁶ dilutions show an acceptable number of cells immediately after transition from a simulated gastric environment, 1x10⁻⁷ will likely be the required to show agar plate counts within an acceptable range at the later dissolution time points.

Consideration must also be taken for the overages allotted in the die-off inherent in the manufacturing of dosage forms and the losses due to final product stability. Many manufacturing processes involve compression and heat forces, such as occur during table and capsule manufacture, which necessitate including an overage of lyophilized bacteria, such that the final product contains an acceptable number of viable bacteria sufficient to meet label claim or other testing standard. Similarly, the final product stability of any probiotic dosage form is not infinite, and thus a loss in viability will occur over time. It is important to account for both variables when calculating dilution volumes, as the non-viable bacteria will be visible during DAPI enumeration.

Enumeration:

The enumeration of bacteria from DAPI slides is only as accurate as the consistency of the counting method utilized. The distributions of bacteria along the filter surface will vary depending on the effectiveness of sample dispersal, the degree of agglomeration that resides from mechanical post-processing (grinding) of lyophilized cultures prior to the manufacture of dosage forms, and the composition of the controlled release matrix containing the bacteria.

Figures 1-4 show photographs of DAPI slides viewed through the microscope ocular. Figure 1 shows a sample removed after 2 hours in simulated gastric media; Figure 2 shows a sample removed after 8 hours total dissolution time; Figure 3 shows a sample removed after 12 hours total dissolution time; and Figure 4 shows a sample removed at the infinity time point. As can be observed, the number of bacteria visible increases with each successive time point, indicating the cumulative release over the duration of dissolution.

Each of these is evaluated differently depending on the type of information that is desired. Small clusters of bacteria on populated slides are presumed to result from the filtration process and should therefore always be enumerated. Bacteria-excipient agglomerations result from the manufacture of dosage forms and should be enumerated only when evaluating the effect of manufacture on total bacterial load. Large clusters such as that indicated in Figure 4 are presumed to be present in the bacterial constituent of the dosage form and

attempts at estimating the enumeration of such clusters should be performed only when evaluating the effect of freeze drying and downstream processing of freeze dried powder, which spans beyond the focus of the described method. It should be noted that if clusters are included in enumeration, it is important to record their frequency of distribution so that the extrapolation of such clusters could be included in the total count. Furthermore, the infinity time point sample will contain a far greater amount of bacterial clusters and bacteria-excipient agglomerates than samples taken during dissolution, as such large particles are not released until mechanical disruption of the dosage form. The enumeration of such clusters at the infinity time point can indicate the effectiveness of the mechanical disruption method, which proves invaluable in method development. As the purpose of the described dissolution method is to determine the degree of release of the bacterial constituent of the dosage form, to include the bacterial load of such clusters in the enumeration of DAPI slides would not correlate to viable counts, as such clusters would produce only a single colony on an agar plate.

Multi-organism formulations present a variety of limitations for viable enumeration that may be overcome with the use of DAPI enumeration. The large concentration differences of organisms commonly found in probiotic products often only permits accurate enumeration of the organisms that represent the majority of the lyophilized blend, due to interspecies and/or competitive inhibition and the limited resolution of agar plate counts. When evaluating controlled release dosage forms, this presents a unique problem, as interspecies and/or competitive inhibition can also falsely decrease viable enumeration from agar plate counts, which may be mistaken for death due to gastric exposure. Individual gastric viability profiles accompanying DAPI analysis can allow for the extrapolation of viable content for organisms that can be differentiated by colony morphology: e.g., a gastric viability study profile of *Streptococcus thermophilus* (S.t.) might indicate that 80% of the organisms survive gastric exposure, while an analysis of a multi-organism formulation in which S.t. comprises only 5% of the lyophilized blend might show zero viability due to competitive inhibition reducing the number of quantifiable colonies below the resolution of an agar plate count. It should be noted that such an assessment can be made only if the S.t. can be easily discerned from the other organisms in the DAPI analysis, and that the suspected inhibition is confirmed to be competitive inhibition (as opposed to interspecies inhibition) through an analysis of growth on agar media with potentially competing organisms are dispersed in equal proportions.

Conclusions

Enumeration of probiotic bacteria using a USP Type II dissolution apparatus and DAPI fluorochrome staining is an effective method for quantifying the release of bacteria from controlled release dosage forms. When combined with a traditional agar plate count enumeration, this method allows for the determination of viable proportion of bacterial loads remaining after simulated gastric exposure.

The DAPI method furthermore offers many additional benefits including the ability to produce fixed slides that can be used for retroactive analysis, the ability to analyze images of the DAPI slides, and the variety of evaluations that can be performed taking into account the plethora of information that is made available through visual observations. The use of a 4 Mega Pixel digital camera through the eyepiece fitted with the ocular graticule resulted in superb images fit for analysis at any time.

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