Heliyon 6 (2020) e03419

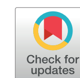
Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Customized antimicrobial efficacy tests offer superior evaluation of growth inhibitor efficacy for liquid microbial products



John P. Gorsuch [*], Zachary Jones

BiOWiSH Technologies, 2724 Erie Avenue Suite C, Cincinnati, OH, 45208, USA

ARTICLE INFO

Keywords:
Microbiology
Antimicrobial effectiveness test
Bacillus
Liquid microbial products
Bacterial contamination

ABSTRACT

Antimicrobial effectiveness tests are common methods used to assess the risk of microbial contamination in pharmaceuticals and cosmetics. These assays may be inappropriate for endospore-based microbial products which often carry a similar – if not greater – risk of microbial contamination. In the present study, we compared the antimicrobial efficacy assessment provided by United States Pharmacopeia Chapter <51> Antimicrobial Effectiveness Testing with a modified test which utilized a customized bacterial challenge. The customized challenge inoculum comprised an assemblage of 12 bacterial strains (both pathogens and spoilage organisms) isolated from the product's end-use geography. Results suggest that some microbial inhibitor systems which pass industry standard antimicrobial effectiveness tests may fail when challenged with a customized bacterial assemblage. In order to provide the best possible assessment of microbial inhibitor systems for liquid *Bacillus* products, we suggest that new antimicrobial effectiveness tests be developed for this product class which include the addition of field-relevant contaminants in addition to the industry standard pathogen challenge.

1. Introduction

Microbial contamination is cause for concern in a variety of consumer products. The pharmaceutical and cosmetic industries utilize Antimicrobial Effectiveness Tests (AET) to determine the vulnerability of a given product to microbial contamination. A variety of AET are used throughout the industry, each with its own suggested challenge organisms and pass/fail criteria. Among these methods are the United States Pharmacopeia (USP) Chapter <51> AET, the European Pharmacopeia (EP) Chapter 5.1.3 Efficacy of Antimicrobial Preservation assay, the International Organization for Standardization (ISO) Method 11930 AET and the schülke KoKo test (SKT). The specifics of these tests are presented in Table 1.

AET methods share a common underlying principle, which is the introduction of a high-concentration microbial challenge into the product of interest and the use of growth-based plate counting assays to quantify shifts in the contaminant population over time. Ideally the product of interest will present bactericidal and fungicidal properties, with success in this area defined differently for each assay. The species present in the contaminant challenge generally include standard serotypes of known human pathogens. Organisms shared among the USP, EP, ISO and SKT assays include approved serotypes of *Pseudomonas aeruginosa, Staphylococcus aureus, Candida albicans* and *Aspergillus niger/brasiliensis* (Table 1). AET may also include known spoilage organisms in their contaminant challenge, and the selection of such organisms is often based upon experience with the product under consideration. Only the SK assay requires the inclusion of additional spoilage organisms as a matter of course. The EP assay recommends the inclusion of such organisms but does not mandate their use.

In addition to pharmaceuticals and cosmetics, microbially-based products such as liquid fertilizer amendments may also be vulnerable to microbial contamination. It may seem counterintuitive to require bactericidal properties in a product which contains high concentrations of bacteria as a deliberate ingredient; however, many of these products consist of *Bacillus* endospores which are metabolically dormant and resistant to a variety of chemical stressors (Cutting, 2011). These properties render endospores resistant to many of the microbial inhibitor systems commonly used in the industry, and in most cases product application rates are low enough that an inhibitor system would experience substantial dilution in the end use application. It is especially advantageous for liquid *Bacillus*-based products to be formulated with a microbial inhibitor system, as they often contain excipients which may support the growth of contaminating microbes. Suspensions of *Bacillus* endospores often include considerable amounts of residual cell fragments

* Corresponding author.
E-mail address: jgorsuch@biowishtech.com (J.P. Gorsuch).

https://doi.org/10.1016/j.heliyon.2020.e03419
Received 22 August 2019; Received in revised form 29 December 2019; Accepted 12 February 2020
2405-8440/© 2020 The Author(s). Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Table 1. Antimicrobial effectiveness tests used in the pharmaceutical and cosmetic industries. Each test has its own specific challenge assemblage, with significant overlap in pathogen species composition and serotype requirements (Siegert, 2012, European Pharmacopeia Chapter 5).

Method and Industry of Use	Contaminant Challenge Composition	Initial Measurement	Initial Reduction Requirement	Challenge Frequency
USP Chapter 51 (Pharmaceuticals)	***Pseudomonas aeruginosa*** ATCC 9027, ***Escherichia coli*** ATCC 11229, ***Staphylococcus aureus*** ATCC 6538, ***Candida albicans*** ATCC 10231, ***Aspergillus niger*** ATCC 16404	7 days	3-log reduction for bacteria, 2-log reduction for fungi	Single challenge
EP Chapter 5.1.3 (Pharmaceuticals)	***Pseudomonas aeruginosa*** ATCC 9027; NCIMB 8626; CIP 82.118, ***Staphylococcus aureus*** ATCC 6538; NCTC 10788; NCIMB 9518; CIP 4.83; ***Candida albicans*** ATCC 10231; NCPF 3179; IP 48.72, ***Aspergillus niger*** ATCC 16404; IMI 149007; IP 1431.83	6 h for bacteria, 24 h for fungi	2-log reduction at 6 h for bacteria, 1-log reduction at 24 h for fungi	Single challenge
ISO 11930 (Cosmetics)	***Pseudomonas aeruginosa*** ATCC 9027; NCIMB 8626; CIP 82.118, NBR 13275; KCTC 2513, ***Staphylococcus aureus*** ATCC 6538; NCTC 10788; NCIMB 9518; CIP 4.83; NRBC 13276; KCTC 3881, ***Escherichia coli*** ATCC 8739; CIP 53.126; NCIMB 8545; NBRC 3972; KCTC 2571; NCTC 12932, ***Candida albicans*** ATCC 10231; NCPF 3179; IP 48.72; NBRC 1594; KCTC 7965, ***Aspergillus niger*** ATCC 16404; IMI 149007; IP 1431, NBRC 9455, KCTC 6196	7–14 days (category A and B respectively) for bacteria, 7–14 days (category A and B products, respectively) for yeast and 28 days for fungi	≥3-log reduction for bacteria, ≥ 1-log reduction for fungi	Single challenge
SKT (Cosmetics)	***Enterobacter gergoviae*** ATCC 33028, ***Escherichia coli*** ATCC 11229, ***Klebsiella pneumoniae*** ATCC 4352, ***Pseudomonas aeruginosa*** ATCC 9027, ***Pseudomonas fluorescens*** ATCC 17397, ***Pseudomonas putida*** ATCC 12633, ***Kocuria rhizophila*** ATCC 9341, ***Staphylococcus aureus*** ATCC 6538, ***Candida albicans*** ATCC 10231, ***Aspergillus niger*** ATCC 16404, ***Penicillium pinophilum*** ATCC 36839	7 days	3-4 log reduction (category A and B products respectively) for bacteria and a 2–3 log reduction (category A and B products respectively) for fungi	Weekly challenges for 7 weeks

and macromolecules (including nucleic acids, cell wall material and intracellular proteins) left over from the sporulation process (Setlow, 2018). In the absence of a microbial inhibitor system, such materials may render a liquid *Bacillus*-based product a prime habitat for the proliferation of a contaminating microorganism.

A manufacturer wishing to vet a potential microbial inhibitor system for a liquid *Bacillus*-based product may submit such a formulation to a third-party laboratory for AET according to one of the standard methods described above. In the United States the default method for AET testing is often the USP Chapter <51> AET, which challenges the product of interest with three pathogenic bacteria (*Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* 11229), one yeast (*Candida albicans* ATCC 10231) and one fungus (*Aspergillus niger/brasiliensis* 16404) (Vu et al., 2014). Although this challenge assemblage may be robust enough to provide adequate efficacy assessments for the pharmaceutical and cosmetic industries, the end use application for a liquid *Bacillus*-based product is often more biologically active than the end use application for pharmaceuticals and cosmetics. For example, a liquid fertilizer amendment may be used outdoors in close contact with soil in such biodiverse geographies as southeast Asia and South America. Because of the high likelihood that a liquid *Bacillus*-based fertilizer amendment will come into contact with diverse microbial contaminants, we hypothesize that a challenge assemblage comprised only of standard pathogen serotypes may not provide a sufficiently robust efficacy assessment for microbial inhibitor systems.

In the present study, we examined whether standard AET such as USP Chapter <51> are appropriate tools for evaluating antimicrobial treatment efficacy in a liquid *Bacillus*-based fertilizer amendment used in biodiverse geographies. We identified an assemblage of field-relevant challenge organisms by isolating contaminating microbes from unprotected product samples exposed to standard use conditions at field sites in southern Vietnam and western China. These organisms were used to conduct a customized AET, the results of which were compared to results of industry standard AET conducted according to USP Chapter <51> by an independent, third-party testing laboratory. Results from the two methods were compared to determine whether the standard method provided a comparable efficacy assessment to the customized method. As microbial products gain popularity and their use extends into more diverse geographies, robust tools will be needed to adequately assess the likelihood of microbial contamination to ensure both the consistency of product performance and the safety of the end user.

2. Materials and methods

2.1. Isolation and provisional identification of field-relevant microbial contaminants

Samples of a liquid *Bacillus*-based fertilizer amendment (BiOWiSH® Crop Liquid™) formulated without a microbial inhibitor system were exposed to standard use conditions in southern Vietnam and western

China. Potentially contaminated product samples were identified based on malodor, discoloration, and gas production. Samples were shipped to the United States where they were serially diluted and plated (dilutions and spread-plate assays carried out as previously described in Gorsuch et al., 2019) on selective media. MacConkey's (MAC) agar (Carolina Biological Supply, Burlington, NC) was used for the selective isolation of G- organisms. MAC plates were incubated at 37 °C for 48 h. After the indicated incubation period, plates were counted and examined for distinct colony morphologies. One isolated colony of each identified type was streaked for isolation on a plate of MacConkey's agar. Once an organism was determined to have been isolated in pure culture, a plate was streaked and sent for 16s rRNA gene sequencing at an independent, third-party testing laboratory (Nelson Laboratories, Salt Lake City, UT). Assembled 16s gene sequences were provided to the authors by Nelson Laboratories, and provisional identifications were confirmed by the authors using NCBI BLAST. Assembled sequences were deposited into NCBI GenBank, and accession numbers are listed in Table 3. Each isolate sent for provisional identification was assigned an alphanumeric code and stored in glycerol stock at -80 °C by adding 0.5mL of an overnight broth culture to 0.5mL of sterile, 50% glycerol solution.

2.2. Preparation of customized bacterial challenge inoculums

A total of 12 unique bacterial strains isolated as described above were selected for use in customized challenge studies. Organisms were brought out of cold storage and streaked onto plates of MAC agar. Plates were incubated as described above until isolated colonies were visible, then used to start overnight broth cultures in capped, 500mL Erlenmeyer flasks containing 250mL of sterile Tryptic Soy Broth (TSB, Carolina Biological Supply, Burlington, NC). Flasks were allowed to culture at 37 °C for 24 h, at which point a 10mL sample of turbid broth was collected with a sterile serological pipette and dispensed into a sterile, 15mL conical centrifuge tube. Broth tubes were chilled at 4 °C for 10 min before being pelleted in a centrifuge for 10 min at 5,000 RCF. Supernatant was decanted, and cell pellets were rinsed with sterile phosphate buffered saline (PBS) at pH 7.2 (±0.2) before being resuspended in sterile PBS. Inoculums for each bacterial species were then combined into a single "master" inoculum (dosed equivalently by volume) that was used as the microbial challenge in the customized AET assays as described below.

2.3. Growth profiling of contaminating microbes in liquid Bacillus spore suspensions

In order to determine a reasonable sampling schedule for customized AET, growth of the microbial inoculum described in Section 2.2 above was modeled in a sample of liquid *Bacillus* endospore suspension ($n = 3$) formulated without a growth inhibitor over the course of 28 days. Inoculum was dosed to bring the initial contaminant concentration of the sample to 5.0×10^5 CFU/mL. Samples were collected for plate counting as described above at 0 days, 2 days, 10 days, 21 days and 28 days. *Bacillus* populations were also measured by plating on Tryptic Soy Agar (TSA) (Carolina Biological Supply, Burlington, NC) after pasteurization at 80 °C for 20 min as described in previous work (Gorsuch et al., 2019) to determine the impact, if any, of contaminant proliferation on endospore dormancy.

2.4. Industry standard antimicrobial effectiveness tests

Six formulations of liquid *Bacillus*-based fertilizer amendment containing candidate antimicrobial inhibitor systems (proprietary) were prepared for use in this study. Product samples were shipped to Q Laboratories (Cincinnati, Ohio) for AET according to USP Chapter <51> with $n = 3$ separate submissions per product type. This method involves splitting the product sample into five separate aliquots, each of which is dosed with a single-pathogen challenge. Starting contaminant concentration in challenged products is between 10^5 and 10^6 cells/mL (Vu et al., 2014). Culturable contaminant concentration was assessed at 14 days and again at 28 days by plating counting each challenge organism on selective medium.

2.5. Customized antimicrobial effectiveness tests

Customized AET ($n = 3$ separate assays per product) were conducted on identical samples of the product formulations sent for standard AET in Section 2.4 above. Assays were conducted as described above with several modifications (Table 2). To simulate a field-relevant contamination event in which multiple contaminants may be introduced simultaneously, product samples (40mL aliquots stored in 50mL conical tubes) were dosed with 100μL of bacterial challenge inoculum (described in Section 2.2 above) rather than examining each organism in pure culture. Final contaminant concentration of challenged products was targeted at 10^7 CFU/mL. Challenge tubes were placed in conical tube racks and allowed to incubate without shaking at 37 °C to simulate product storage conditions in Vietnam during the summer. Contaminant concentration was assessed initially and again at 48 h, at which point the product was scored as passing (minimum 2-log reduction in bacterial concentration) or failing.

2.6. Evenness of failing score distribution across methods

Evenness in the distribution of failing scores across AET methods (USP <51> method and the customized method, respectively) was compared to a hypothetical even distribution using an exact test of goodness of fit (McDonald, 2014).

Table 2. Modifications to USP Chapter <51> AET used in customized challenge studies and the purpose of each modification.

	USP Chapter <51> Procedure	Customized AET Procedure	Modification Rationale
Addition of challenge microorganisms	Five challenge organisms are added separately into five respective subsamples of the tested product.	All challenge bacteria are dosed together into the test product sample in a nominally equivalent (w/w) dosage.	Contamination in the field will likely involve an assemblage of contaminants (such as in soil) rather than a single species.
Sampling time points	Product is tested at 14 days and 28 days post-challenge.	Product is tested immediately upon challenge addition, and again after 2 days of incubation.	Rapid growth profile and plateau of customized challenge populations in *Bacillus* endospore suspensions (Figure 2) and the likelihood of rapid product usage after an initial contamination event by the end user.
Plate counting medium	MacConkey's agar (*E. coli*), Centrimide agar (*P. aeruginosa*), Manitol salts agar (*S. aureus*)	MacConkey's agar (all challenge organisms)	All challenge organisms produce robust growth on MacConkey's agar.

3. Results

3.1. Isolation and provisional identification of field-relevant microbial contaminants

A variety of organisms were isolated from contaminated Vietnamese and Chinese product samples, and microbial communities appeared to vary considerably in species composition from sample to sample (Figure 1). Results of 16s rRNA gene sequencing are presented in Table 4. Several of the isolated organisms, such as *Pseudomonas aeruginosa*, were strains of AET challenge organisms used in the industry standard methods described above; however, many of the organisms do not appear in the challenge assemblages of any standard methods.

3.2. Growth profiling of contaminating microbes in liquid bacillus spore suspensions

Field-isolated bacterial contaminants proliferated rapidly and achieved high concentrations in liquid samples of liquid *Bacillus*-based fertilizer amendment formulated without a microbial inhibitor (Figure 2). Average contaminant populations increased from 6.4×10^5 CFU/mL at the start of the experiment to 1.2×10^8 CFU/mL after 2 days of incubation. Contaminant populations remained constant at 10 days (2.18×10^8 CFU/mL) before decreasing to 6.0×10^7 CFU/mL at 21 days, where they plateaued through 28 days (3.1×10^7 CFU/mL). Populations of *Bacillus* endospores (the deliberate microbial ingredient) remained heat-stable throughout the duration of the experiment.

3.3. Industry standard antimicrobial effectiveness test

All six of the product formulations described in Section 2.4 above passed USP Chapter <51> AET conducted at an independent, 3rd party testing laboratory. An example data set from a single replicate series is presented in Figure 3. All submitted product samples passed the UPS Chapter <51> AET for *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 8739, with culturable cells of each organism dropping below the plate counting reading frame at 14 days and 28 days.

3.4. Customized antimicrobial effectiveness test

Of the six product formulations described in Section 2.4 above, three failed the customized AET assay by allowing proliferation of the bacterial challenge. An example data set from a single replicate series is presented

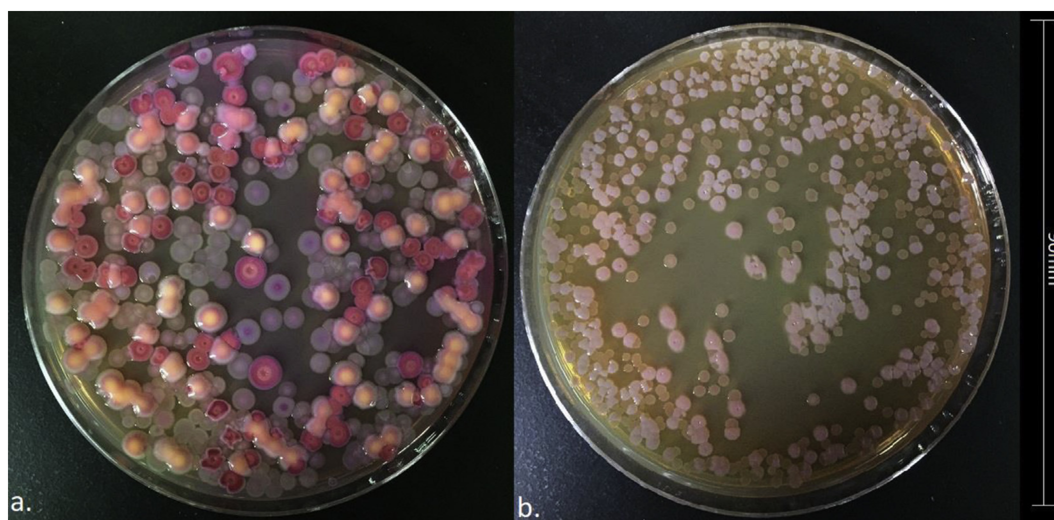


Figure 1. A variety of bacteria were isolated from samples of contaminated product, and culturable microbial communities differed in species composition. Pictured are plates of MacConkey's agar from serial dilutions of two separate contaminated product samples from southern Vietnam showing qualitative differences in colony morphology among their dominant culturable species.

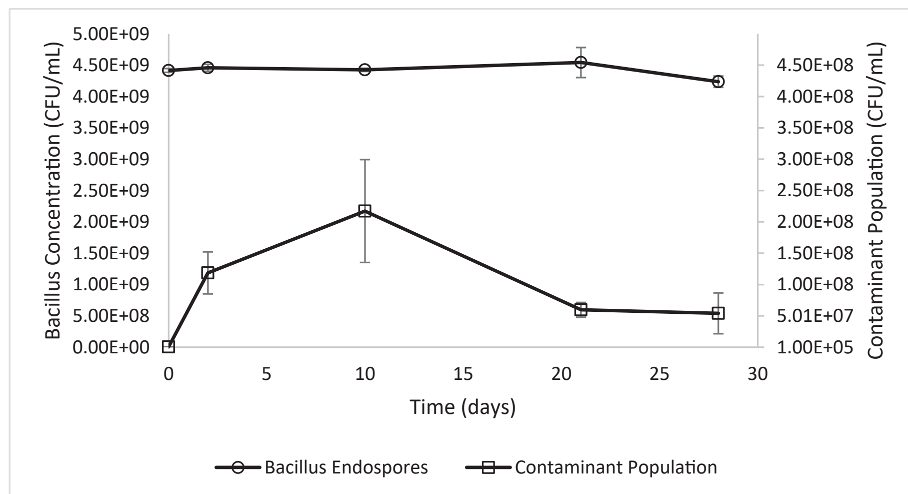


Figure 2. Growth profile of contaminating microbes in a liquid microbial product consisting of a *Bacillus* endospore suspension. Populations of contaminating microbes increased by three log units within 2 days of challenge inoculation before decreasing to a plateau at 21 days and 28 days. Populations of *Bacillus* endospores remained constant and heat-stable throughout the growth study. Error bars represent one standard deviation above and below the data point ($n = 3$ replicates and $n = 3$ countable plates per replicate for each data point). Data comprising this figure are available in Supplementary Material – Figure 2.

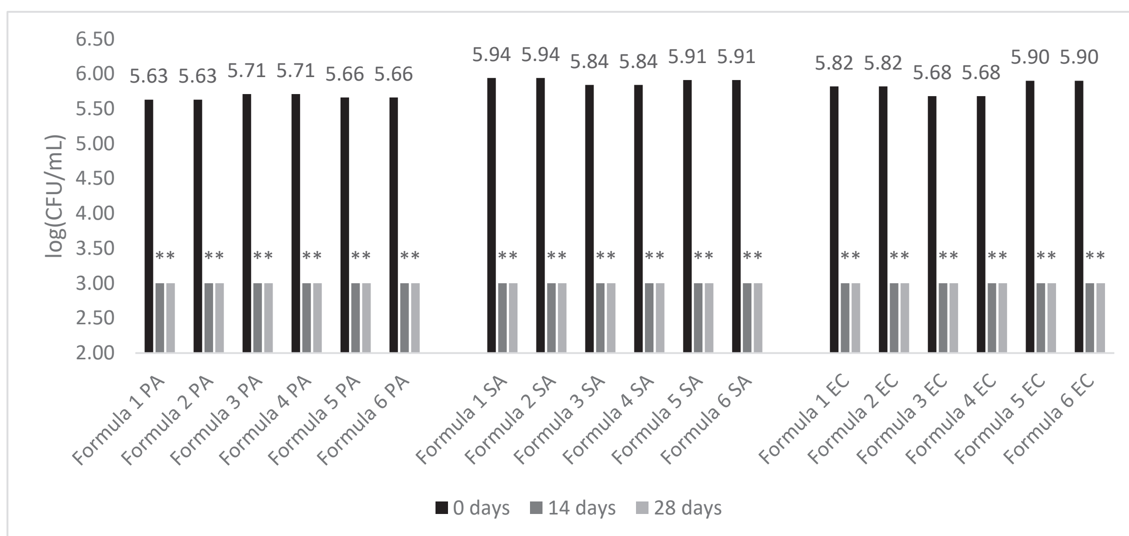


Figure 3. Results of a series of industry standard AET (USP 41 <51>) conducted by an independent testing laboratory. All product formulations passed the bacterial contaminant challenges for *P. aeruginosa* (PA), *S. aureus* (SA) and *E. coli* (EC) with a score of $<1.0 \times 10^3$ CFU/mL (*) recorded at 14 days and 28 days. These passing scores are included in the survey of scores presented in Table 4. Data comprising this figure are available in Supplementary Material – Figure 3.

in Figure 4. Based in part upon the results of contaminant growth studies presented in Figure 2 above, product formulations were tested for microbial proliferation after 2 days benchtop incubation. Products which showed an increase or stasis in contaminant concentration were considered to have failed the challenge study, whereas products showing a decrease in contaminant contamination were considered to have passed the challenge. Three of the six product formulations showed decreases in contaminant population after 2 days and received passing scores, while the other three treatments showed robust contaminant proliferation and received failing scores (Figure 4).

In cases where product formulations failed customized AET, the community of culturable microorganisms at 48 h was invariably distinct from the population at 0 h. An example of this difference is presented in Figure 5. In these cases, the morphologically distinct coliform colonies were not present on MacConkey's plates at 48 h; rather, the final contaminant population was made up entirely of *Pseudomonas aeruginosa* and/or *P. taiwanensis* (provisional identification rendered via 16s rRNA gene sequencing as described above) which at T0 had comprised only a subset of the contaminant population.

3.5. 1.6 Evenness of failing scores across AET methods

The occurrence of failing scores across methods is presented in Table 4. Distribution of failing scores did not conform to a hypothetical even distribution (Exact test for Goodness of Fit, $p = 0.002$, $\alpha = 0.05$).

4. Discussion

Antimicrobial effectiveness tests are valuable tools used to evaluate the vulnerability of product formulations to microbial contamination, thus ensuring the effectiveness of the product and the safety of the end user. Though pharmaceuticals and cosmetics are the primary product types assessed by AET, microbial products such as liquid suspensions of *Bacillus* endospores are also potentially vulnerable to microbial contamination. Industrial endospore suspensions often contain excipients left over from the sporulation process including cell fragments, nucleic acids, cell wall material and intracellular proteins (Setlow, 2018), compounds which may support the proliferation of heterotrophic microbial contaminants in an unprotected liquid product. Because *Bacillus*

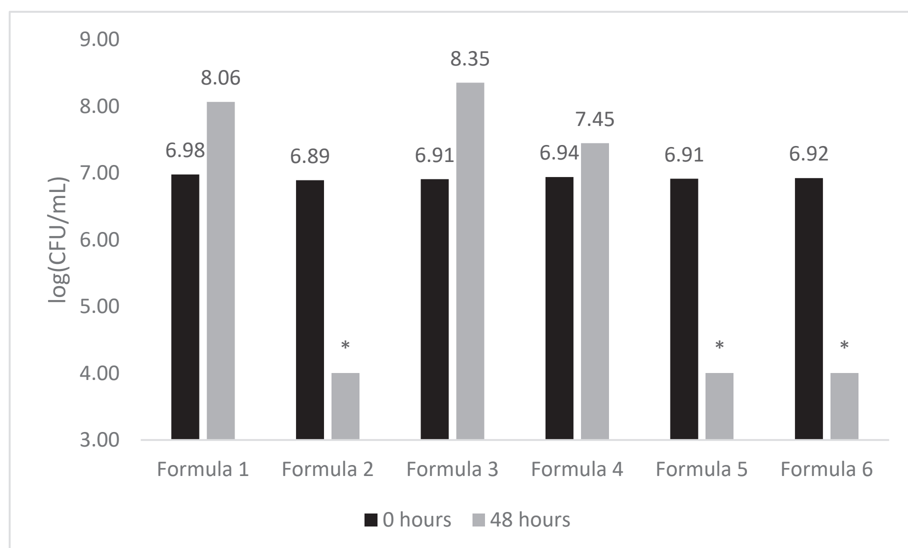


Figure 4. Results of a series of customized AET. A proliferation of contaminating microbes was observed after 2 days in Formulas 1, 3 and 4 resulting in a failing score for those products. In Formulas 2, 5 and 6 contaminant concentration had dropped below the reading frame after 2 days, resulting in a value of $<1.0 \times 10^4$ CFU/mL (*) and a passing score for those samples. These passing and failing scores are included in the survey of scores presented in Table 4. Data comprising this figure are available in Supplementary Material – Figure 4.

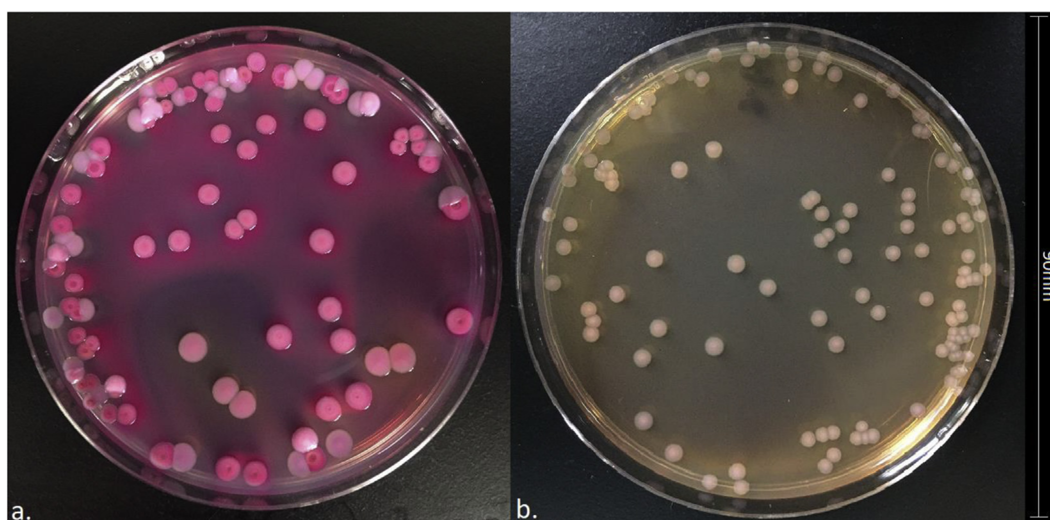


Figure 5. Differences in contaminant species composition on countable plates at 0 h (a) and at 48 h (b) in a product preparation which failed the customized AET assay (pictured) but passed the industry standard test. Culturable contaminating microbes at 48 h were exclusively *Pseudomonas aeruginosa* (provisional identification rendered by 16s rRNA gene sequencing) whereas *P. aeruginosa* made up only a subset of the population at 0 h.

endospores are resistant to a variety of chemical stressors many antimicrobial inhibitor systems would be expected to have minimal impact upon product stability or upon performance in the end use application, where products are often significantly diluted. Therefore, an evaluation of industry standard AET as efficacy assessment tools for antimicrobial inhibitor systems in liquid *Bacillus* products is necessary to either confirm their usefulness or to highlight the need for unique assays tailored to the needs of microbial product manufacturers.

In the present study, we identified an assemblage of field-relevant microbial contaminants for use in customized AET by exposing liquid *Bacillus*-based fertilizer amendment preparations without a microbial inhibitor system to standard use conditions in southern Vietnam and in western China. Samples were diluted and plated on MacConkey's agar to isolate G-bacteria, and morphologically distinct isolates were identified using 16s rRNA gene sequencing and stored in glycerol stock. Product preparations containing varying proprietary inhibitor systems were then subjected to an industry standard AET (USP Chapter <51>) at an independent 3rd party laboratory as well as to a customized AET which utilized the assemblage of Vietnamese and Chinese organisms presented in Table 3.

Our data suggest that standard AET developed for use in the pharmaceutical and cosmetic industries may not provide the most robust possible antimicrobial efficacy assessment for liquid *Bacillus* products. When subjected to USP Chapter <51> AET, all six product formulations passed the microbial challenges; however, when subjected to a customized AET based upon the product's end use conditions only three of the six product formulations passed the bacterial challenge. Failing AET scores were produced only by the customized assay for the tested product prototypes, a presentation which deviated significantly from a hypothetically even distribution of failing scores (Table 4). This suggests that the customized bacterial assemblage provided a more stringent antimicrobial efficacy evaluation than did the industry standard consortium in the tested product prototypes. In samples which failed the customized microbial challenge but passed the standard method, proliferated communities of contaminating bacteria were dominated by *P. aeruginosa* and/or *P. taiwanensis* after 48 h of incubation. This suggests a higher tolerance to the tested inhibitor systems for these isolates than is exhibited by *P. aeruginosa* ATCC 9027 or other bacteria from the USP Chapter <51> assemblage. Because products such as microbial fertilizer amendments are often used in biodiverse geographies and in close association with soil, it may be beneficial to reflect the phenotypic diversity of likely contaminants from the end-use application in AET challenge assemblages.

Additionally, robust growth of contaminating microbes was observed in growth-modeling experiments (Figure 2) and in products which failed customized AET (Figure 3). Populations of contaminating bacteria increased by 1–2 log units within 48 h of introduction into product samples. Because microbial products such as liquid fertilizer amendments are often opened and closed repeatedly by the end user, and multiple samples are often drawn from the same container over time, the

Table 3. Results of 16s rRNA gene sequencing of bacterial isolates included in customized AET challenge assemblages. Organisms generally included in standard methods were detected as contaminants in the tested product samples, including *P. aeruginosa* and *K. pneumoniae.*

Strain ID	Provisional Identification	16s Sequence GenBank Accession Number	Country of Origin
VN1	*Pseudomonas aeruginosa*	MN943272	Vietnam
VN2	*Pseudomonas aeruginosa*	MN943273	Vietnam
VN3	*Pseudomonas taiwanensis*	MN943274	Vietnam
VN4	*Klebsiella pneumoniae*	MN943275	Vietnam
VN5	*Shigella sonnei*	MN943277	Vietnam
VN6	*Enterobacter hormaechei*	MN943279	Vietnam
CH1	*Citrobacter freundii*	MN943280	China
CH2	*Serratia marcescens*	MN943281	China
CH3	*Morganella morganii*	MN943282	China
CH4	*Pseudomonas veronii*	MN943284	China

Table 4. Evenness in the distribution of failing scores across AET methods. All failing scores for the product prototypes examined during the present study were produced by the customized AET method. This distribution of failing scores did not conform to a theoretical even distribution (Exact test for Goodness of Fit, $p = 0.002$, $\alpha = 0.01$).

Custom AET	Replicate 1	Replicate 2	Replicate 3
Formula 1	***FAIL***	***FAIL***	***FAIL***
Formula 2	PASS	PASS	PASS
Formula 3	***FAIL***	***FAIL***	***FAIL***
Formula 4	***FAIL***	***FAIL***	***FAIL***
Formula 5	PASS	PASS	PASS
Formula 6	PASS	PASS	PASS
USP <51> AET			
Formula 1	PASS	PASS	PASS
Formula 2	PASS	PASS	PASS
Formula 3	PASS	PASS	PASS
Formula 4	PASS	PASS	PASS
Formula 5	PASS	PASS	PASS
Formula 6	PASS	PASS	PASS

standard approach of enumerating contaminating microbes at 7, 14 and 28 days may not be appropriate. Should a contamination occur when the product is first opened, our data suggest that an ineffective inhibitor system could result in the robust proliferation of a pathogen such as *P. aeruginosa* within 2 days; therefore, a robust antimicrobial efficacy assessment protocol should take this potential for rapid product spoilage into account.

As *Bacillus*-based products gain popularity across a variety of industries and their usage extends into biodiverse geographies, robust tools are needed to ensure that these products are not vulnerable to contamination by pathogens and spoilage organisms. While the customized AET described in the present study is not suggested as an alternative standard method, our data call attention to the risk inherent in assuming that standard AET assays will provide adequate efficacy assessments for antimicrobial inhibitor systems in liquid *Bacillus-based* products. Therefore, it may be advantageous for the microbial product industry to collaborate on the development and validation of an alternative AET protocol to better address the industry's needs.

Declarations

Author contribution statement

John P. Gorsuch: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Zachary Jones: Conceived and designed the experiments; Analyzed and interpreted the data.

Funding statement

This work was supported by BiOWiSH Technologies.

Competing interest statement

The authors declare the following conflict of interests:

J. Gorsuch and Z. Jones are employees of BiOWiSH Technologies, which funded this research.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2020.e03419

Acknowledgements

Thanks to the staff of Nelson Laboratories (Salt Lake City, UT, USA) for providing additional sequencing data to the authors over and above standard reporting practices, allowing 16s sequences to be assigned NCBI GenBank accession numbers.

References

European Pharmacopeia Chapter <5.1.3>.Efficacy of Antimicrobial Preservation.
Gorsuch, J., LeSaint, D., VanderKelen, J., Buckman, D., Kitts, C., 2019. A comparison of methods for enumerating bacteria in direct fed microbials for animal feed. J. Microbiol. Methods 160, 124–129.
McDonald, J.H., 2014. Handbook of Biological Statistics, 3rd ed. Sparky House Publishing, Baltimore, Maryland.
Setlow, P., 2018. Observations on research with spores of Bacillales and Clostridiales species. J. Appl. Microbiol. 126, 348–358.
Siegert, W., 2012. ISO 11930 – a comparison to other methods to evaluate the efficacy of antimicrobial preservation. SOFW J. 138, 44–53.
Vu, N., Nguyen, K., Kupiec, T., 2014. The essentials of United States Pharmacopeia chapter <51> antimicrobial effectiveness testing. Int. J. Pharm. Compd. 18, 123–130.
Cutting, S., 2011. Bacillus probiotics. Food Microbiol. 28, 214–220.