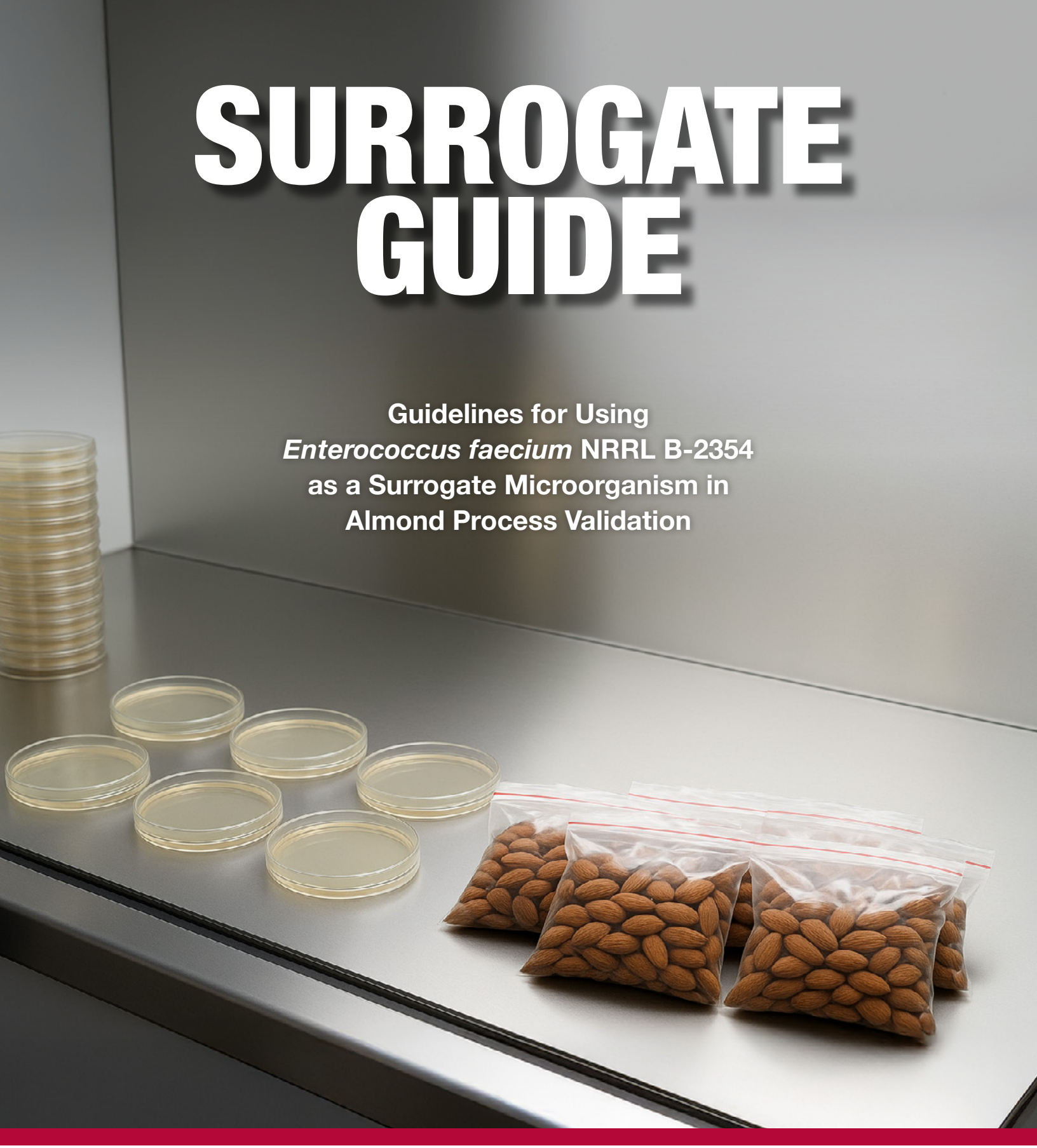
SURROGATE GUIDE

Guidelines for Using *Enterococcus faecium* NRRL B-2354 as a Surrogate Microorganism in Almond Process Validation



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SECTION 1

SURROGATE – BACKGROUND AND BIOSAFETY CONSIDERATIONS

1.1 SELECTION OF BACTERIAL SURROGATE FOR VALIDATION OF THERMAL PROCESSES FOR ALMONDS

Enterococcus faecium NRRL B-2354 has been identified as a suitable bacterial surrogate for *Salmonella* Enteritidis phage type (PT) 30 and other salmonellae (Jeong et al., 2011; Jeong et al., 2017; Zhu et al., 2021), *Escherichia coli* O157:H7, and *Listeria monocytogenes* (Moussavi et al., 2015) in validation studies of thermal treatment processes for almonds. Studies that compare the survival of a surrogate organism to one or more target pathogens in almonds under different treatment processes can be found in Appendix 1. The goal of almond process validation studies is to determine if the treatment technology and equipment can achieve the mandated minimum 4-log reduction of *Salmonella* in California-grown almonds (Federal Register, 2007: Almonds grown in California, 7 CFR part 981).

The mandated minimum 4-log reduction of *Salmonella* in almonds is supported by risk assessments published by the University of California, Davis (Lambertini et al., 2012) and the U.S. Food and Drug Administration (FDA; Santillana Farakos et al., 2017) that estimated a mean risk of less than one case of salmonellosis per year in the United States when a minimum 4-log reduction is applied. In addition, in a letter provided by FDA to the Almond Board of California (ABC), dated 10/13/2017, the following was stated: "FDA would not object to a future 403(h) notification featuring a validated process that achieves a minimum 4-log reduction in *Salmonella* given that the published risk assessment (Santillana Farakos et al., 2017) estimates that a 4-log reduction of *Salmonella* is practically equivalent to the presently accepted 5-log reduction in terms of protecting public health" (Appendix 2).

Through studies funded by ABC, and others, the use of *E. faecium* NRRL B-2354 as a surrogate has been deemed appropriate for use in almond process validation studies for the following types of processes:

- **Dry heat processes** (e.g., dry roast, brine and pre-wet dry roast, dry roast flavoring, dry plasticizing)
- **Moist air or steam processes** (ambient or vacuum)

E. faecium NRRL B-2354 may also be an appropriate surrogate for alternative thermal processes (e.g., infrared, microwave, and radio frequency heating) or other nonthermal processes. However, it is also possible that other surrogates may be more appropriate. Before any surrogate is used in validation of an alternative process, studies must be conducted and data gathered to demonstrate appropriate resistance of *E. faecium* NRRL B-2354 (or other surrogate) compared with *Salmonella* Enteritidis PT 30 (or other relevant equally or more resistant strains of *Salmonella* or other pathogens of concern) on almonds for the specific process. These data should be shared with ABC prior to conducting validation studies.

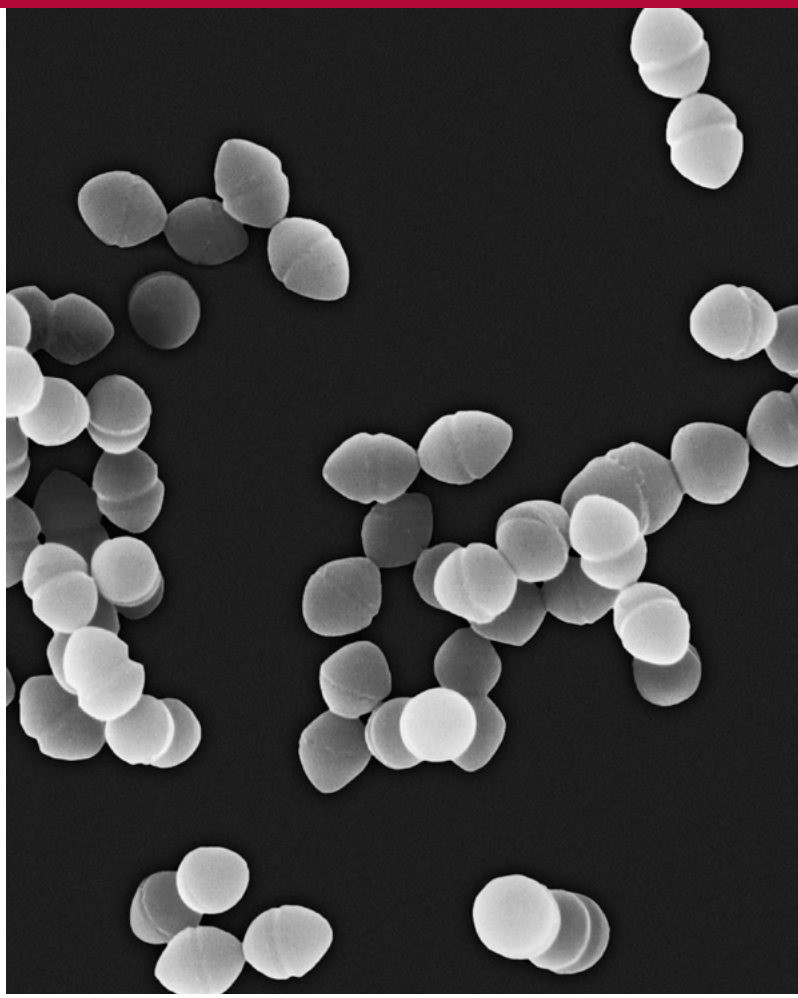
NOTE:
Protocols and guidelines established for use of *E. faecium* NRRL B-2354 on almonds should not be considered appropriate for other products without additional scientific data to support such application.

1.2 SOURCING *E. FAECIUM*

E. faecium strain NRRL B-2354 is available from the culture collection of the USDA National Center for Agricultural Utilization Research (NCAUR). *E. faecium* NRRL B-2354 can be obtained through NCAUR for no charge (as of July 2025) via the online ordering system for strains in the public access catalog: https://nrrl.ncaur.usda.gov/. *E. faecium* NRRL B-2354 was deposited with the American Type Culture Collection (ATCC) in 1960 as *E. faecium* ATCC 8459 and is available from this organization. *E. faecium* ATCC 8459 is considered a clonal relative of strain NRRL B-2354 (Kopit et al., 2014) and may be used when strain NRRL B-2354 is not available.

The whole genome sequences of both *E. faecium* NRRL B-2354 and ATCC 8459 are available:

- NRRL B-2354: Kopit et al., 2014; BioSample SAMN02604149 https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_000336405.1/
- ATCC 8459: BioSample SAMN02596948, SAMN06009036, SAMN00809109 https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_000407385.1/
- Four-strain cluster of *E. faecium*: https://www.ncbi.nlm.nih.gov/pathogens/tree/#Enterococcus_faecium/PDG000000071.725/PDS000059073.2?accessions=PDT000011582.3



1.3 BIOSAFETY CONSIDERATIONS

A surrogate selected for process validation studies in food processing and pilot plant facilities must be nonpathogenic to humans (Hu and Gurtler, 2017). *E. faecium* NRRL B-2354 has been used in the food industry as a nonpathogenic test organism for many decades, also previously under various other names and strain designations including *Micrococcus freudenreichii* ATCC 8459 and *Pediococcus* sp. NRRL B-2354. A review of the literature indicates the usefulness of *E. faecium* NRRL B-2354 as a surrogate in a range of products (Kornacki, 2012). Furthermore, a study funded by ABC, examining the genomic and functional characteristics of *E. faecium* NRRL B-2354, has shown that this strain is a safe surrogate, classified as a biosafety level 1 (BSL1) organism appropriate for use in process validation (Kopit et al., 2014).

SECTION 2

INOCULUM AND INOCULATED ALMONDS – PREPARATION, HANDLING AND STORAGE

The following guidelines describe the materials and stepwise procedures for using *E. faecium* NRRL B-2354 in an almond process validation study, including preparation of the inoculum and the preparation, handling, storage, and transport of inoculated almond samples.

2.1 MATERIALS

ALMOND KERNELS

- Nonpareil variety, whole natural (skin on) grade U.S. No. 1, size 27/30*

  **If Nonpareil No. 1 27/30 almonds are not available or if other sizes or varieties are more relevant to the process being validated, the process authority should contact ABC in advance of conducting the validation study.*
- Hand sort the almonds to remove broken and chipped/scratched nuts.
- The microbial load (aerobic plate count) of the uninoculated kernels should be ≤3 log CFU/g. This could be achieved by using non-roasted almonds that have received a treatment that reduces background microbial populations without substantially changing the physical characteristics of the almond kernel surface (e.g., not roasted or blanched). Examples of such treatments include:
  - Propylene oxide (PPO) treatment (almonds with <300 ppm PPO residue)
  - Steam treatment
- Moisture content of the kernels must be 4.0–5.5% prior to inoculation.
- Kernels should be at ambient temperature, 22 ± 2°C (72 ± 4°F), prior to inoculation.

SURROGATE

- *E. faecium* NRRL B-2354 or *E. faecium* ATCC 8459

MEDIA AND DILUENTS

- Tryptic soy agar (TSA)
- Tryptic soy broth (TSB)
- 0.1% peptone (for ABC procedure)
- Butterfield's phosphate buffer (BPB; for FDA BAM procedure)

EQUIPMENT AND SUPPLIES

- Plastic petri dishes (standard 100 mm & large 150 mm diameter)
- Inoculating loops (10 µl)
- Pipettes
- Pipettor
- Test tubes or microcentrifuge tubes
- L-shaped glass or plastic spreaders
- Falcon tubes
- Magnetic stir plate and stir bars
- Vortex or similar mixer
- Incubator at 35°C (95°F)
- Refrigerator at 4°C (40°F)
- Polyethylene (PE) sample bags (sterile), medium size (e.g., 710 ml [24 oz.])
- PE sample bags with zipper closure (sterile), large size (e.g., 30×30 cm [16×16 in.])
- Filter paper sheets (46×57 cm)
- Metal drying rack
- Sterile bin with lid (optional)
- Metal mesh tray
- Laboratory oven, convection/forced air
- Laboratory paddle blender (e.g., Stomacher lab blender or equivalent)
- Sterile containers to hold inoculated almonds for the treatment being validated (e.g., mesh bags, baskets, or other suitable containers with dimensions that can be incorporated into the processing line)
- Sanitizer (e.g., 70% ethanol)

2.2 EXPERIMENTAL TIMELINE

Days 1–5: Prepare inoculum (Figure 1; section *2.3 Inoculum preparation*)

Days 5–8: Inoculate, dry, and store almonds (Figure 2; section *2.4 Inoculation procedure, drying and storage of inoculated almonds*)

Days 6–36: Begin validation trials with inoculated almonds — Note: Inoculated almonds must be used in validation trials within 28 days after placing the inoculated and dried almonds in refrigerated storage (day 0).

2.3 INOCULUM PREPARATION

The procedure described in Figure 1 will yield one 25-ml suspension of cells, which is a sufficient volume to inoculate one 400 g portion of almonds.

DAY 1

Streak culture (active or frozen) onto TSA plates
Incubate at 35 ± 2°C for 24 ± 2 hours

DAY 2

Transfer cells from an isolated typical colony into TSB (10 ml)
Incubate at 35 ± 2°C for 24 ± 2 hours

DAY 3

Transfer loop of broth culture into TSB (10 ml)
Incubate at 35 ± 2°C for 18 to 24 hours

DAY 4

Spread overnight culture (0.5 ml/plate) over large TSA plates (150 × 15 mm); prepare 5 plates (minimum) for every 25 ml of inoculum needed.
Incubate at 35 ± 2°C for 24 ± 2 hours

For best results, use a sufficient and consistent volume of TSA in the petri plates (~80 ml/large plate)

DAY 5

Check plates for evidence of uniform lawn growth and absence of microbial contaminants. Discard any plates with signs of contamination. Add 6 ml of 0.1% peptone to each plate, loosen bacterial lawn with a sterile spreader, and use a sterile pipette to collect cells into a sterile container (add additional peptone as needed to achieve a total volume of 25 ml per 5 petri dishes).

Before inoculating almonds, pool all 25-ml inoculum preparations and mix thoroughly for at least 1 minute (e.g., using a magnetic stir bar and stir plate)

Inoculum may be held for up to 0.5 hours, with stirring, while almond samples are being inoculated

FIGURE 1

Schematic of the inoculum preparation method

The weight of inoculated almonds needed for a process validation is determined by the experimental design. For example: 50 g per subsample (technical replicate) × number of technical replicates × number of replicate trials. Be sure to include enough subsamples to cover all conditions and treatments to be tested in the validation study. Typical validation studies will use ≥2,400 g of inoculated almonds, which includes samples for measuring moisture (and water activity, if done), for determining levels of *E. faecium* in inoculated almonds, for testing heat resistance, and the traveling controls (minimum three).

The total inoculum volume needed depends on the total weight of almonds to be inoculated. Prepare an appropriate number of 25-ml inoculum preparations and pool as shown in Figure 1 (e.g., 150 ml of inoculum will be needed to inoculate six 400-g portions [2,400 g] of almonds). This would represent a single biological replicate (see Section 4 *Definitions*).

2.4 INOCULATION PROCEDURE, DRYING AND STORAGE OF INOCULATED ALMONDS

The inoculation procedure shown in Figure 2 is for one 400-g portion of almond kernels. Dry the inoculated almonds at ambient temperature (22 ± 2°C [72 ± 4°F]) for 1 to 3 days to a final target moisture content of 4.0 to 5.5%. In circumstances where the intent is to validate a process with carrier almonds that have a moisture content of <4.0%, the final moisture content of the inoculated almonds may also need to be lower. In these cases, the process authority or laboratory should contact ABC in advance of conducting the validation study. To prepare a greater weight of inoculated almonds, separately inoculate 400-g batches of almonds and pool after drying. Store the dried inoculated almonds in sealed moisture-barrier containers or moisture-barrier plastic bags at 4 ± 1°C. This is day 0 of storage.

FIGURE 2

Schematic for almond inoculation, drying and storage of inoculated almonds

2.5 VERIFICATION OF INOCULATION LEVELS AND HEAT RESISTANCE

Verification of inoculation levels and heat resistance confirmation can be done concurrently. Determine that the inoculation level on the dried almonds is ≥7.0 log CFU/g (see section *3.3 Recovery and enumeration of surrogate*).

Verify that the heat resistance criterion of <2.5 log reduction is met (Figure 3). For the heat resistance test, spread 25 g of dried surrogate-inoculated almonds in a single layer on a sterile metal mesh tray. Heat the almonds in a convection or forced air oven at 280°F (138°C) for 15 min. Enumerate the surrogate on the heated inoculated test samples (section 3.3). The heat resistance is determined by subtracting the log value of survivors (heated samples) from the log value of unheated inoculated controls.

Note that these tests will require 2 days for enumeration of the surrogate on the inoculated controls and heated almonds.

If the inoculum level is ≥7.0 log CFU/g and a reduction of <2.5 log is determined in the heat resistance test, properly stored almonds may be used in validations within 28 days from day 0 of storage (Figure 2). Almonds should be stored at 4 ± 1°C in sealed moisture-barrier plastic bags to maintain moisture levels.

HEAT RESISTANCE TEST

Step 1: Spread 25 g of dried inoculated almonds in a single layer on a sterile metal mesh tray

Step 2: Heat in convection/forced air oven at 280°F (138°C) for 15 minutes

Step 3: Enumerate surrogate on unheated inoculated control samples and heated inoculated test samples (Figure 6)

Acceptable heat resistance: <2.5 log reduction

Heat resistance is determined by subtracting the log value of survivors (heated samples) from the log value of unheated inoculated controls

FIGURE 3

Determining heat resistance of *E. faecium* on inoculated almonds

SECTION 3

USE OF SURROGATE-INOCULATED ALMONDS IN A VALIDATION STUDY

The following guidelines describe steps to be used in challenge testing with surrogate bacterium *E. faecium* NRRL B-2354 in an almond process validation study as well as the procedures for recovery and enumeration.

3.1 PREPARATION OF SAMPLE PACKS AND TRANSPORT OF INOCULATED ALMONDS

Either before or after transportation to the processing facility, aseptically pack the inoculated almonds (50 g) loosely into sterile containers (e.g., mesh bags, baskets; Figure 4) suitable for the processing line that is being validated.

- Contact ABC prior to conducting a validation study if 50-g sample packs are not feasible or if there is a need to use an alternative approach such as using loose, dyed almonds. Additional data may need to be provided to support alternative approaches.

Prepare sufficient sample packs to cover all sampling points for the validation as determined by the experimental design. A minimum of three inoculated sample packs should be included as "traveling" controls. Sample packs should be placed inside sealed sterile moisture-barrier plastic bags or other protective secondary container. Sample packs should be stored under refrigerated conditions and transported on ice or surrounded by cold ice packs (0 to 4°C) to and from the validation site. Transport and handle the inoculated test and traveling controls in an identical manner during each day of the validation trial.

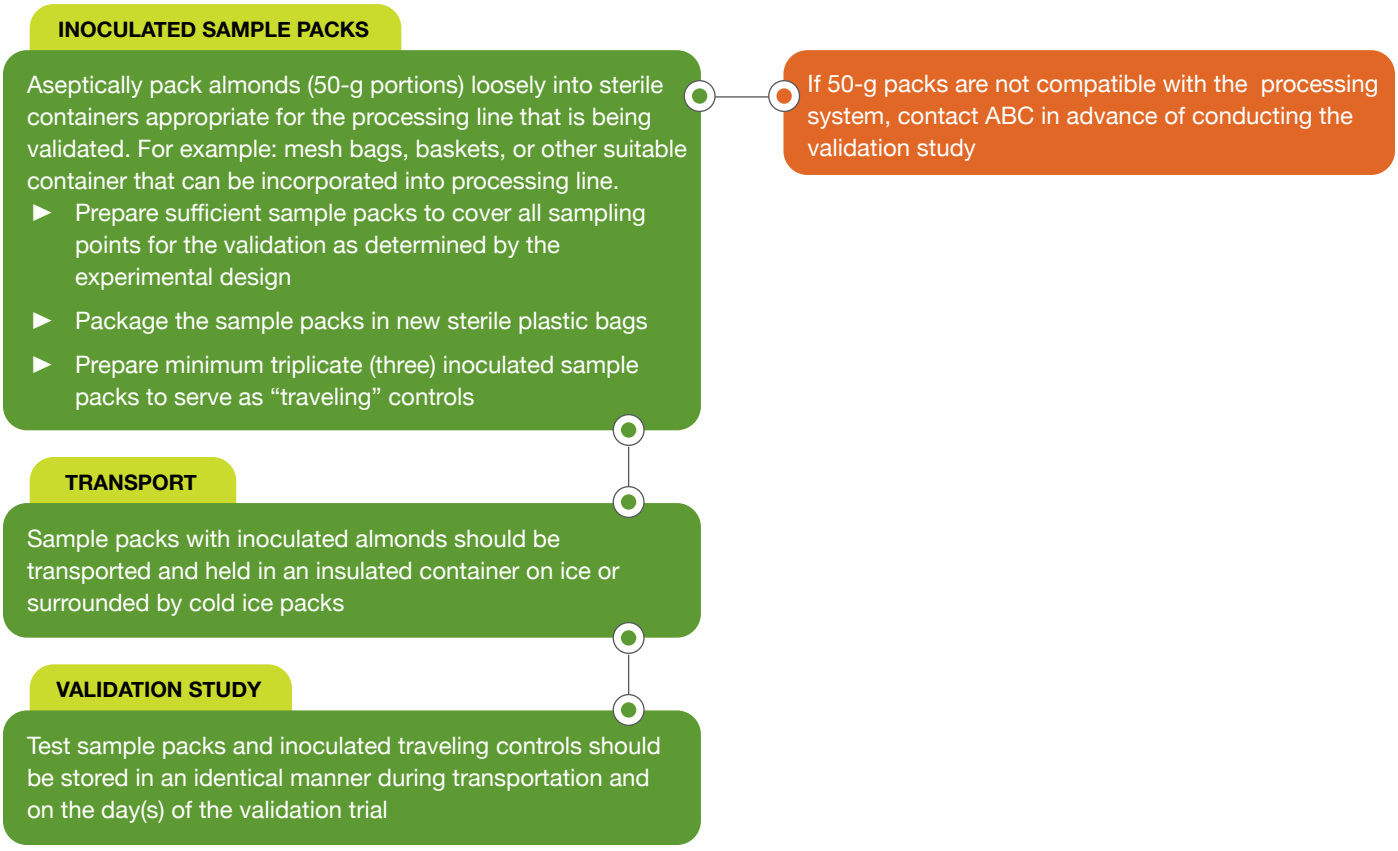


FIGURE 4

Preparation and transportation of almond sample packs

3.2 CHALLENGE TESTING WITH INOCULATED ALMONDS

This section will address some of the considerations for validation of a thermal process.

Details on determination and assessment of critical processing parameters and reporting requirements can be found in ABC guidance documents for specific processes. To conduct meaningful process validation trials for thermal processes, it is important to map the temperature of the processing line or product containers to identify potential cold spots before running validation trials.

Conduct microbial challenge testing at identified cold spots and under conditions that will always be exceeded during normal operation (Figure 5). For example: For dry roaster validation, conduct validation testing with lower temperature set points. For normal production, increase set points and establish temperature-critical factors that exceed the maximum values reached during validation testing.

Validation trials must be done for each line when more than one processing line exists.

Remove inoculated almond sample packs from the transport container and condition them to a temperature not greater than the temperature of the test carrier almonds. Embed the inoculated almond sample packs in the product flow at the identified cold spots. Sample packs should be retrieved from the processing line as described in the appropriate process-specific guidance document. Quickly cool the almonds by placing each sample pack into sterile labeled bags, sealing the bags, and then placing in an insulated container surrounded by ice or cold ice packs. Samples should be held in an insulated container on ice, or surrounded by cold ice packs, or under refrigeration and *E. faecium* should be enumerated within 24 hours of treatment (preferred) or up to 72 hours after treatment.

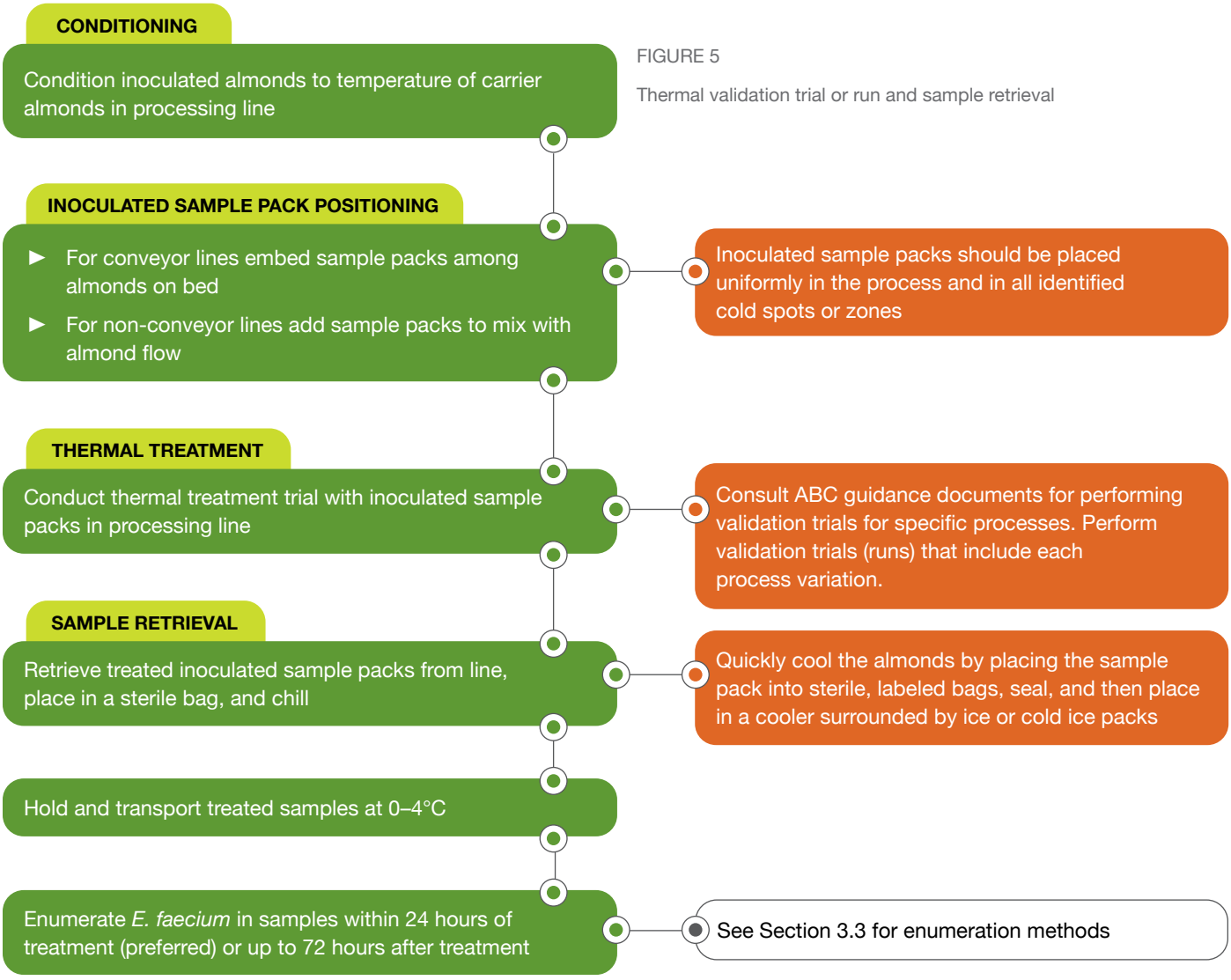


FIGURE 5

Thermal validation trial or run and sample retrieval

3.3 RECOVERY AND ENUMERATION OF SURROGATE

Recover and enumerate *E. faecium* on almonds by following the ABC protocol or the FDA BAM protocol described in the FDA Bacteriological Analytical Manual (Andrews and Hammack, 2022) (Figure 6). The use of a spiral plater as outlined in the FDA BAM (Maturin and Peeler, 2001) is acceptable but not discussed here.

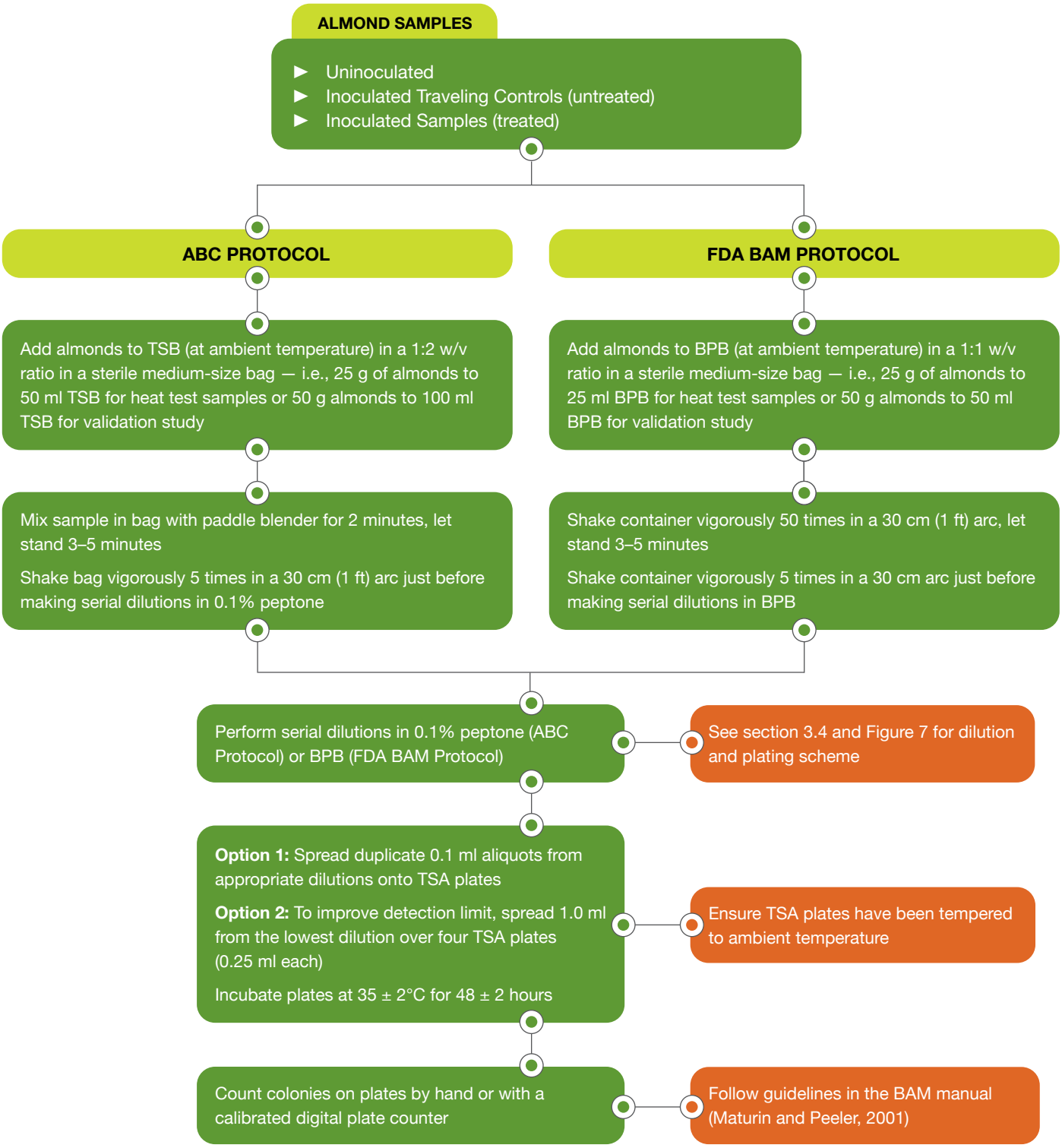


FIGURE 6

Recovery and enumeration of surrogate organism

3.4 DILUTION AND PLATING SCHEMES

Before plating, temper TSA plates for >4 hours to ambient temperature if they have been refrigerated, or prepare the plates the previous day and hold at ambient temperature overnight. Perform serial dilutions in 0.1% peptone (ABC protocol) or BPB (FDA BAM protocol). Plate, in duplicate, appropriate aliquots (0.1 ml [Option 1]) from appropriate dilutions onto TSA plates (Figures 6 and 7). After treatment, when low counts are anticipated, it is recommended that 0.25 ml of the lowest dilution be plated in quadruplicate (Option 2) to improve the limit of detection. Incubate plates at 35 ± 2°C for 48 ± 2 hours. For Option 2, 150-mm diameter TSA plates may be used but are not required.

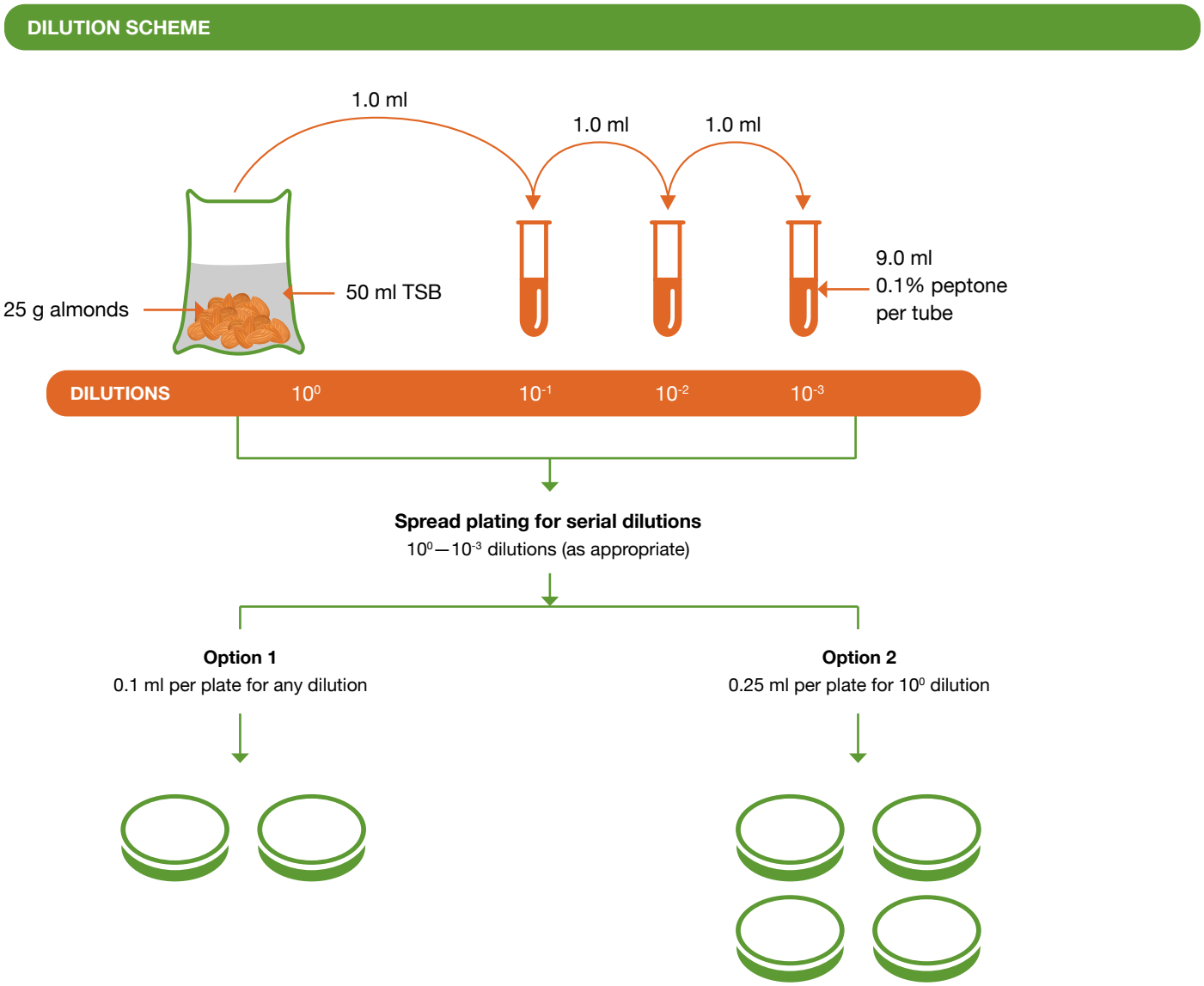


FIGURE 7

Example dilution and plating scheme using the ABC protocol. Option 1 is most appropriate for determining initial levels of surrogate on inoculated almonds and for the heat resistance test. Option 2 is often used to improve the limit of detection and is most appropriate for samples that have been exposed to a process where a significant reductions and low counts are anticipated. It may be appropriate to use both Options 1 and 2 for a single sample when outcomes are unknown.

3.5 DATA REPORTING

For details on reporting requirements see additional ABC guidance documents for specific processes.

Include the following items in the process validation report to be evaluated by TERP:

Within the body of the report:

- Validation test date(s) and surrogate enumeration date(s).
- Summary data for uninoculated and *E. faecium*–inoculated almonds. This should include information about moisture levels, aerobic plate count (APC) before inoculation, and *E. faecium* count after inoculation, after drying, and after the heat resistance test.
- Summary data for the validation test results (i.e., calculated reductions for each sample, average reductions, and standard deviations, where appropriate).
  - Values for each sample should be converted to $\log^{10}$ **before** calculating log reductions.
  - Both average (with standard deviation) and minimum log reduction values (see calculation examples below):
    - Log reduction = log initial counts (inoculated traveling controls) – log survivors (inoculated treated samples).
    - To calculate **average log reduction**, subtract the average log of all three of the inoculated treated samples from the average log of all the corresponding untreated inoculated samples (traveling controls).
    - To calculate **minimum log reduction**, subtract the highest log of the number of survivors among the inoculated treated samples from the lowest log of initial counts in the corresponding untreated inoculated samples (traveling controls).

Important: Average values and standard deviations are useful in interpreting results and should be included. However, for almond validation purposes, the lowest log reduction values achieved among the samples tested must meet the minimum 4-log reduction requirement.

Within the appendixes of the report:

- *E. faecium* surrogate certificate (**see Appendix 3** for an example certificate).
  - Include the following:
    - APC results for uninoculated almonds and date performed
    - Date almonds were inoculated with *E. faecium*
    - Almond moistures before inoculation and after inoculation and drying and dates analysis performed
    - Date inoculated dried almonds were put into storage at 4°C
    - Heat resistance test results and date performed (see calculation example in Appendix 5)
- All raw data of *E. faecium* counts from the validation (traveling controls and test samples) and respective log CFU/g values. See an example document for reporting microbiological data in Appendix 4 and calculation examples in Appendix 5.

SECTION 4

DEFINITIONS

Biological replicates are measurements of biologically distinct samples – e.g., when different *E. faecium* preparations are used to separately inoculate almonds.

Carrier almonds are the almonds into which the surrogate-inoculated almond packs will be placed – they should represent almonds for which the validation is being conducted.

Duplicate plates are two separate petri plates typically used for each serial dilution of each subsample. In some cases, quadruplicate plates are used.

Experimental trial or experimental run refers to a single performance of an experiment – it is a singular instance where the experimental procedure is carried out under certain specified conditions.

Replicate trial refers to the repetition of an entire experiment or a specific part of an experiment under the same or similar conditions. For example, two replicate validation trials may be conducted on the same day or on two different days.

Technical replicates are repeated measurements of the same sample. A validation study usually involves multiple technical replicates of 50-g subsamples of inoculated almonds that are exposed to the treatment.

Traveling controls are untreated inoculated samples that are transported to and from the validation site and held under the same conditions as the treated inoculated samples on each day of the validation trial.

SECTION 5

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APPENDICES

APPENDIX 1 – SURROGATE/PATHOGEN SURVIVAL STUDIES

Visit: bit.ly/4p4RKhd

or scan the QR code

APPENDIX 2 – FDA 403(H) NOTIFICATION 10/13/2017

Visit: bit.ly/4gBDRnz

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APPENDIX 3 & 4 – SURROGATE CERTIFICATE & MICROBIOLOGY REPORT

View example Surrogate Certificate and Microbiology Report templates online.

Visit: bit.ly/4nNAcVK to access the sample PDFs.

or scan the QR code

APPENDIX 5 – DATA CALCULATION EXAMPLES

Below are examples of calculations to determine $\log_{10}$ colony forming units (CFU)/g and $\log_{10}$ reduction of *Enterococcus faecium*. Refer to section 3.4 and 3.5 (pages 12 and 13) and Figures 6 and 7 for the dilution schemes. In these dilution schemes, appropriate dilution aliquots of 0.1 ml (Option 1) or 0.25 ml (Option 2) are plated in duplicate or quadruplicate, respectively, onto TSA plates (Figures 6 and 7). For dilution calculations, assume that almonds remain intact and TSB or BPB are not absorbed.

As indicated in section 3.5, both average and minimum log reductions should be reported. To calculate **average log** reduction, subtract the **average log** of all three of the inoculated treated samples from the average log of all the corresponding untreated inoculated samples (traveling controls). To calculate minimum log reduction, subtract the **highest log** of the number of survivors among the inoculated treated samples from the **lowest log** of initial counts in the corresponding untreated inoculated samples (traveling controls).

Important: Average values and standard deviations are useful in interpreting results and should be included. However, for almond validation purposes, the lowest log reduction values achieved among the samples tested must meet the minimum 4-log reduction requirement.

The examples below provide calculations using both the ABC protocol and the FDA BAM protocol. Example 1 uses Option 1 and data from a hypothetical heat resistance test. Example 2 uses Option 2 and data from a process validation where very low counts are anticipated. Example 3 uses Option 2 and data from a process validation where no colonies have been detected at any of the plated dilutions.

Initial surrogate counts on the inoculated almonds:

Example 1: 7.40 log CFU/g

Examples 2, 3, and 4 (three untreated travelling controls): 7.40, 7.80, and 7.60 log CFU/g (minimum 7.40 log CFU/g; average = 7.60 ± 0.2 log CFU/g).

EXAMPLE 1 PLATING SCHEME FOR OPTION 1

EXAMPLE 1a – for a single sample (in this case for a heat resistance test) using the ABC protocol (Figure 6; 50 g almonds and 100 ml TSB):

- Counts on duplicate plates (plated with 0.1 ml of a 10^{-3} dilution [a combined dilution factor of 10^{-4}]) are 30 and 42 colonies.
  - The average number of survivors in this sample is: (30 + 42) colonies/2 plates = 36 colonies/plate
  - **To calculate log CFU/g:** (36 [colonies]/10^{-4} [dilution factor]) × 100 ml of TSB/50 g of almonds = 720,000 CFU/g = 5.86 log CFU/g
  - **To calculate log reduction:** 7.40 log CFU/g (initial surrogate counts) – 5.86 log CFU/g (heat-treated inoculated almonds) = 1.54 log reduction.

EXAMPLE 1b – for a single sample using the FDA BAM protocol (Figure 6; 50 g almonds and 50 ml BPB):

- Counts on duplicate plates (plated with 0.1 ml of a 10^{-3} dilution [a combined dilution factor of 10^{-4}]) are 60 and 84 colonies.
  - The average number of survivors in this sample is: (60 + 84) colonies/2 plates = 72 colonies/plate
  - **To calculate log CFU/g:** (72 [colonies]/10^{-4} [dilution factor]) × 50 ml of BPB/50 g of almonds = 720,000 CFU/ g = 5.86 log CFU/g
  - **To calculate log reduction:** 7.40 log CFU/g (initial surrogate counts) – 5.86 log CFU/g (heat-treated inoculated almonds) = 1.54 log reduction.

EXAMPLE 2 PLATING SCHEME FOR OPTION 2

(to improve detection limit when lower counts are anticipated)

EXAMPLE 2a – for a single sample (in this case from a validation) using the ABC protocol (Figure 6; 50 g almonds and 100 ml TSB):

- Counts from quadruplicate plates (plated with 0.25 ml of the initial dilution) are 5, 7, 10 and 3 colonies. Add the counts (total = 25) to determine the colonies in 1 ml (i.e., 4 × 0.25 ml = 1 ml) = 25 CFU/ml.
  - This total count (25 CFU/ml) is the count in 1 ml of TSB from the TSB/almond mixture.
  - **To calculate log CFU/g:** 25 CFU/ml × 100 ml/50 g = 50 CFU/g = 1.70 log CFU/g
  - **To calculate log reduction:** 7.40 log CFU/g (minimum count for untreated inoculated samples [traveling controls]) – 1.70 log CFU/g (treated inoculated almonds) = 5.70 log reduction.

EXAMPLE 2b – for a single sample using the FDA BAM protocol (Figure 6; 50 g almonds and 50 ml BPB):

- Counts from quadruplicate plates (plated with 0.25 ml of the initial dilution) are 10, 14, 20 and 6 colonies. Add the counts (total = 50) to determine the colonies in 1 ml (i.e., 4 × 0.25 ml = 1 ml) = 50 CFU/ml.
  - **To calculate log CFU/g:** 50 CFU/ml × 50 ml/50 g = 50 CFU/g = 1.70 log CFU/g
  - **To calculate log reduction:** 7.40 log CFU/g (minimum count for untreated inoculated samples [traveling controls]) – 1.70 log CFU/g (treated inoculated almonds) = 5.70 log reduction.

EXAMPLE 3 PLATING SCHEME FOR OPTION 2

(preferred method for presenting values when there are no colonies on any of the plates - when counts are below the limit of detection)

EXAMPLE 3a – for a single sample (in this case from a validation) using the ABC protocol (Figure 6; 50 g almonds and 100 ml TSB):

- No colonies were observed on quadruplicate plates (plated with 0.25 ml of the initial dilution: 4 × 0.25 ml = 1 ml). To determine the limit of detection, assume that 1 colony was detected on a single plate.
  - This total count (1 CFU/ml) is the count in 1 ml of TSB from the TSB/almond mixture.
  - **To calculate log CFU/g:** 1 CFU/ml × 100 ml/50 g = 2 CFU/g = 0.30 log CFU/g. Expressed as <2 CFU/g Estimated (Est.) or <0.30 log CFU/g Est.
  - **To calculate log reduction:** 7.40 log CFU/g (minimum count for untreated inoculated samples [traveling controls]) – 0.30 log CFU/g (treated inoculated almonds) = 7.10 log reduction. Expressed as >7.10 log reduction Est.

EXAMPLE 3b – for a single sample using the FDA BAM protocol (Figure 6; 50 g almonds and 50 ml BPB):

- No colonies were observed on quadruplicate plates (plated with 0.25 ml of the initial dilution). To determine the limit of detection, assume that 1 colony was detected on a single plate.
  - This total count (1 CFU/ml) is the count in 1 ml of BPB from the BPB/almond mixture.
  - **To calculate log CFU/g:** 1 CFU/ml × 50 ml/50 g = 1 CFU/g of almonds (0 log CFU/g). Expressed as <1 CFU/g or <0 log CFU/g Est.
  - **To calculate log reduction:** 7.40 log CFU/g (minimum count for untreated inoculated samples [traveling controls]) – 0 log CFU/g (treated inoculated almonds) = 7.40 log reduction. Expressed as >7.40 log reduction Est.

If the laboratory wishes to deviate from the example above, a brief explanation and justification should be provided in the report.

EXAMPLE 4 PLATING SCHEME FOR OPTION 1

(calculating surrogate reductions when counts are below the limit of detection)

EXAMPLE 4a – for a single sample (in this case from a validation) using the ABC protocol (Figure 6; 50 g almonds and 100 ml TSB):

- No colonies were observed on duplicate plates (plated with 0.1 ml of the initial 100 dilution [a combined dilution factor of 10^{-1}]). To determine the limit of detection, assume that 1 colony was detected on a single plate.
  - The average number of survivors in this sample is: (1 + 0) colonies/2 plates = 0.5 colonies/plate
  - **To calculate log CFU/g:** (0.5 [colonies]/10^{-1} [dilution factor]) × 100 ml of TSB/50 g = 10 CFU/g = 1.0 log CFU/g. Expressed as <10 CFU/g Estimated (Est.) or <1.0 log CFU/g Est.
  - **To calculate log reduction:** 7.40 log CFU/g (minimum count for untreated inoculated almonds) – 1.0 log CFU/g (treated inoculated almonds) = 6.40 log reduction. Expressed as >6.4 log Est.

EXAMPLE 4b – for a single sample using the FDA BAM protocol (Figure 6; 50 g almonds and 50 ml BPB):

- No colonies were observed on duplicate plates (plated with 0.1 ml of the initial 100 dilution [a combined dilution factor of 10^{-1}]). To determine the limit of detection, assume that 1 colony was detected on a single plate.
  - The average number of survivors in this sample is: (1 + 0) colonies/2 plates = 0.5
  - **To calculate log CFU/g:** (0.5 [colonies]/10^{-1} [dilution factor]) × 50 ml of BPB/50 g = 5 CFU/g = 0.70 log CFU/g. Expressed as <5 CFU/g Est. or <0.70 log CFU/g Est.
  - **To calculate log reduction:** 7.40 log CFU/g (minimum count for untreated inoculated almonds) – 0.70 log CFU/g (treated inoculated almonds) = 6.70 log reduction. Expressed as >6.70 log Est.

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