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

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SECTION 1:

SURROGATE CULTURE – BACKGROUND AND BIOSAFETY CONSIDERATIONS

Enterococcus faecium NRRL B-2354 has been identified as a suitable surrogate organism for *Salmonella* Enteritidis Phage Type 30 and other *Salmonellae* in validation studies of thermal treatment processes for almonds. 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).

Through studies funded by the Almond Board of California (ABC), 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,** such as dry roast, brine and pre-wet dry roast, dry roast flavoring, dry plasticizing, etc.
- **Moist air or steam processes** (ambient or vacuum) (Jeong et al., 2011)

Enterococcus faecium NRRL B-2354 may also be an appropriate surrogate for alternative processes, such as infrared heating, microwave, radio frequency heating and others. However, before the surrogate is used in validation of other types of processes, studies must be conducted and data gathered to demonstrate appropriate resistance of *E. faecium* NRRL B-2354 compared with *Salmonella* Enteritidis Phage Type 30 on almonds for the specific process. In addition, studies comparing the resistance of *E. faecium* NRRL B-2354 and *Salmonella* Enteritidis Phage Type 30 (or other pathogens of concern) should be conducted before using *E. faecium* NRRL B-2354 as a surrogate for products other than almonds. Furthermore, protocols and guidelines established for use of this surrogate on almonds should not be considered appropriate for other products without additional scientific data to support such application.

The strain *Enterococcus faecium* NRRL B-2354 is available from the culture collection of the USDA National Center for Agricultural Utilization Research (NCAUR). The ABC Technical Expert Review Panel (TERP) recommends the use of this strain for almond process validation.

NRRL B-2354 cultures can be obtained through NCAUR for no charge via the online ordering system for strains in the public access catalog: http://nrrl.ncaur.usda.gov/.

A surrogate selected for process validation studies in food processing and pilot plant facilities must be nonpathogenic to humans. *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, *Pediococcus* sp. NRRL B-2354 and *E. faecium* ATCC 8459. A recent review (Kornacki, 2012) indicates the usefulness of *E. faecium* NRRL B-2354 as a surrogate. Furthermore, a study funded by the Almond Board of California examining the genomic and functional characteristics of *E. faecium* NRRL B-2354 has shown that this strain is a safe surrogate, 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 step-wise preparation and handling procedures for using *E. faecium* NRRL B-2354 for an almond process validation study, including inoculum preparation and the storage and transport of inoculated almond samples.

2.1 MATERIALS

ALMOND KERNELS

- Nonpareil variety, grade U.S. No. 1, size 27/30* (*If this size is not available, contact ABC.)
    - To ensure a low background microbial load on samples, use almonds pasteurized by treatment with propylene oxide (PPO) and with <300 ppm PPO residue
    - Moisture content of the kernels must be 4.0–5.5% prior to inoculation
    - Temperature of the kernels should be 21–24° C (70–75° F) prior to inoculation

CULTURE

- *E. faecium* NRRL B-2354

EQUIPMENT

- Plastic petri dishes (standard and 150-mm diameter)
- Pipettes
- Test tubes
- Glass spreaders
- Magnetic stir plate and bars
- Incubator at 35° C (95° F)
- Refrigerator at 4° C (40° F)
- Polyethylene (PE) sample bags, medium size (710 mL/24 oz.)
- PE sample bags with zipper closure, large size (30×30 cm/16×16 in.)
- Filter paper sheets (46×57 cm; P8 grade)
- Metal drying rack
- Plastic storage bin with lid, sterile
- Metal mesh tray
- Laboratory oven, convection/forced air
- Laboratory paddle blender (e.g., Stomacher lab blender or equivalent)
- Containers to hold inoculated nuts for treatment (e.g., thermostable bags or baskets)

MEDIA

- Tryptic soy agar (TSA)
- Tryptic soy broth (TSB)
- 0.1% peptone water
- Butterfield's phosphate buffer (BPB)

2.2 PREPARATION TIMELINE

Days 1–5: Prepare inoculum

Days 5–6: Inoculate almonds, assess initial inoculation levels, and determine heat resistance

Days 7–14: Begin validation trials with inoculated almonds. Note: Use inoculated almonds within 14 days after inoculation unless >7.0 log CFU/g is confirmed (see Section 3.2) and additional heat resistance testing confirms adequate resistance (see Section 2.5)

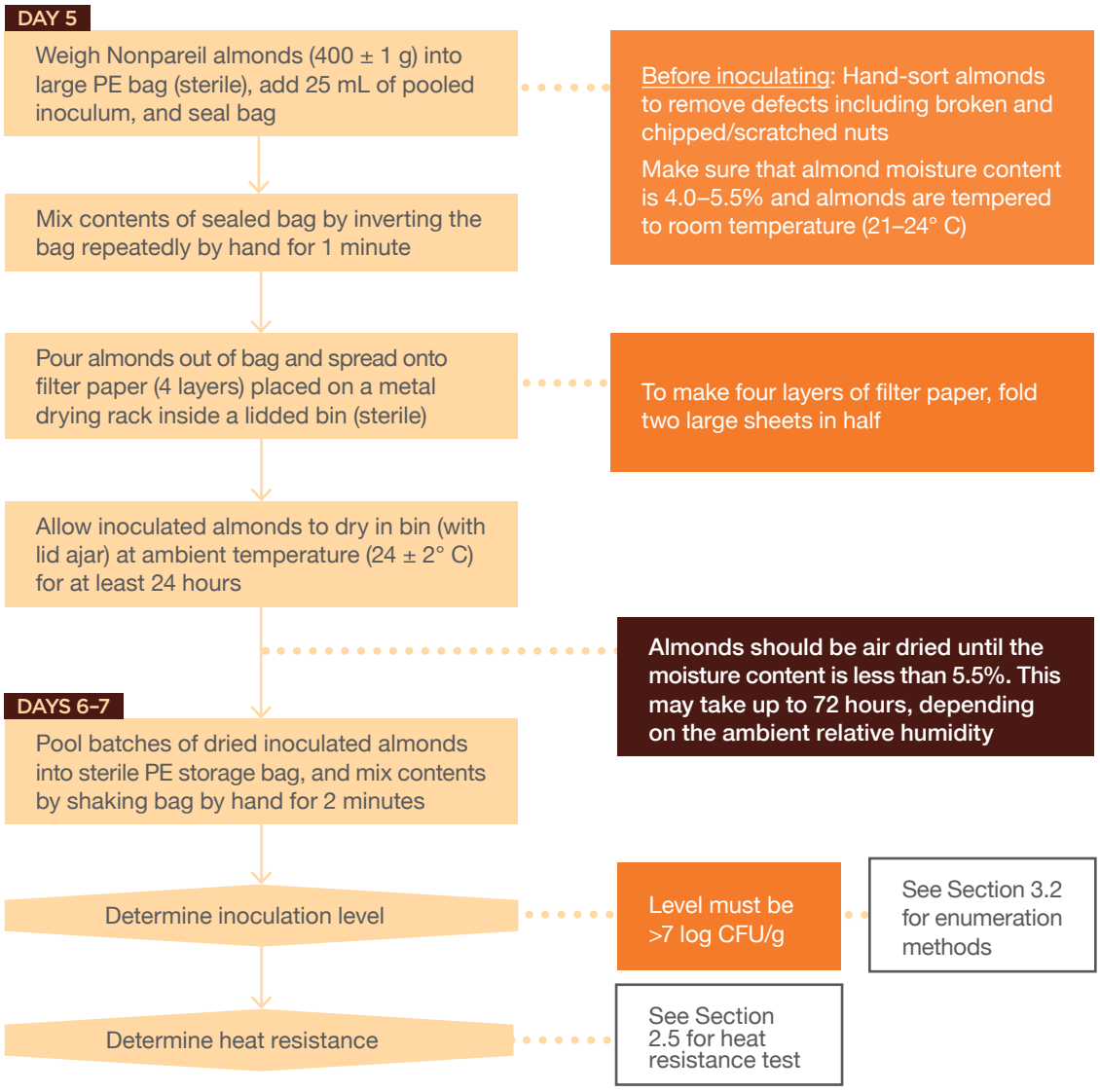
2.3 INOCULUM PREPARATION

The following procedure will yield a 25-mL suspension of cells, which is a sufficient volume to inoculate one 400-g portion of almonds.

The amount of almonds to inoculate is determined by the experimental design: 50 g per sample × number of sampling points × number of replicates at each sampling point. Typical validation studies will utilize >2,400 g of inoculated almonds including traveling controls and heat resistance test samples. The total inoculum volume needed depends on the amount of almonds to be inoculated. Make an appropriate number of 25-mL inoculum preparations and pool as described below.

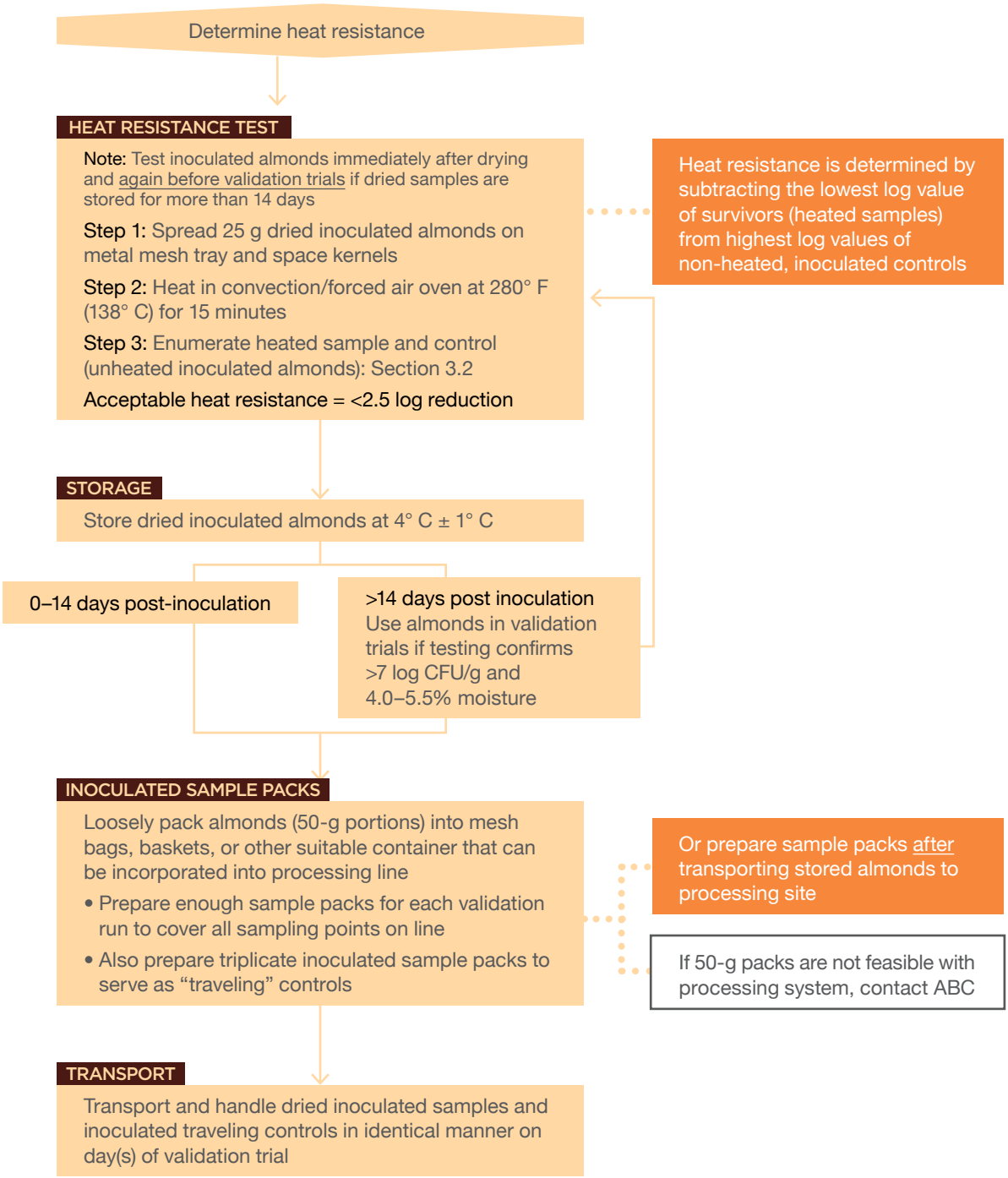
2.4 INOCULATION PROCEDURE AND DRYING

The following inoculation procedure is for one 400-g portion of almond kernels. To prepare more inoculated almonds, separately inoculate 400-g batches of almonds and pool after drying as described below.



2.5 HEAT RESISTANCE, STORAGE AND TRANSPORT OF INOCULATED ALMONDS

Follow the handling procedures for inoculated almonds (and uninoculated controls) as indicated below before starting challenge testing.



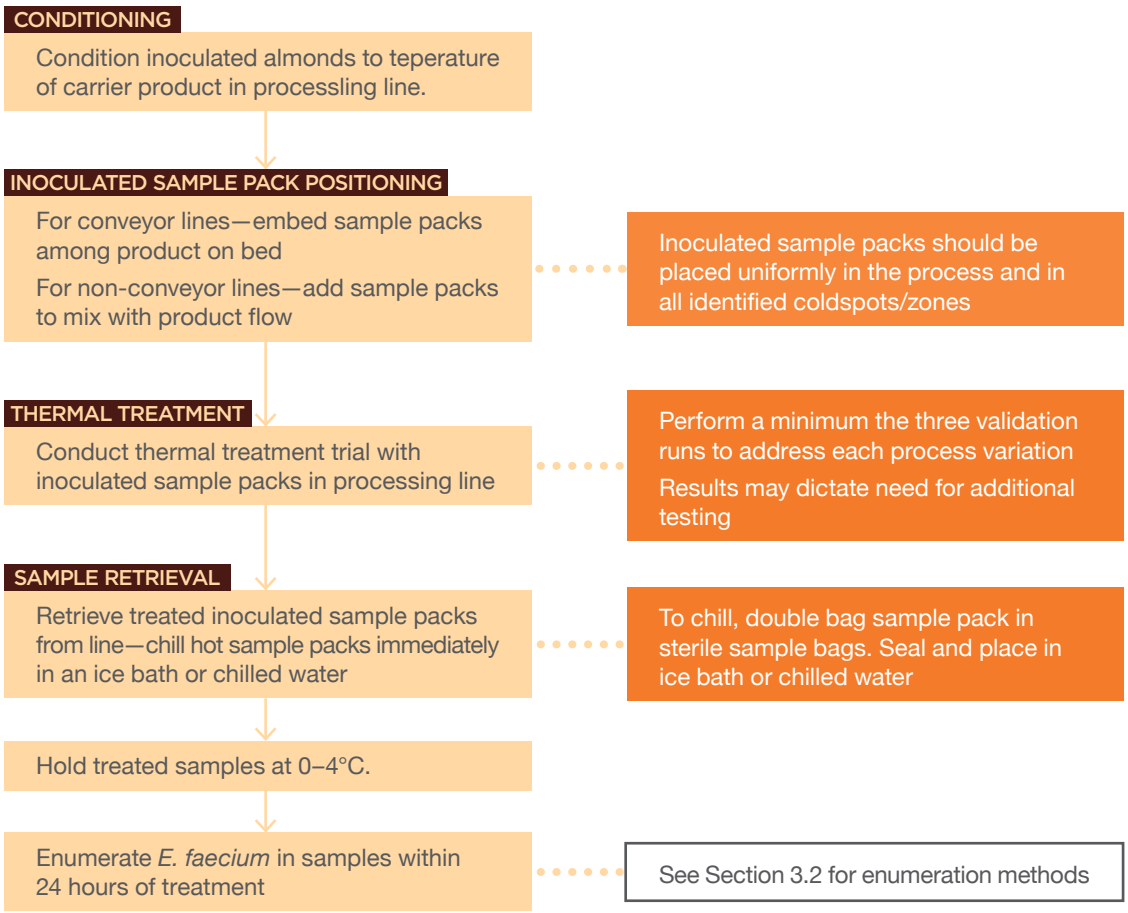
SECTION 3:

USE OF SURROGATE IN VALIDATION

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

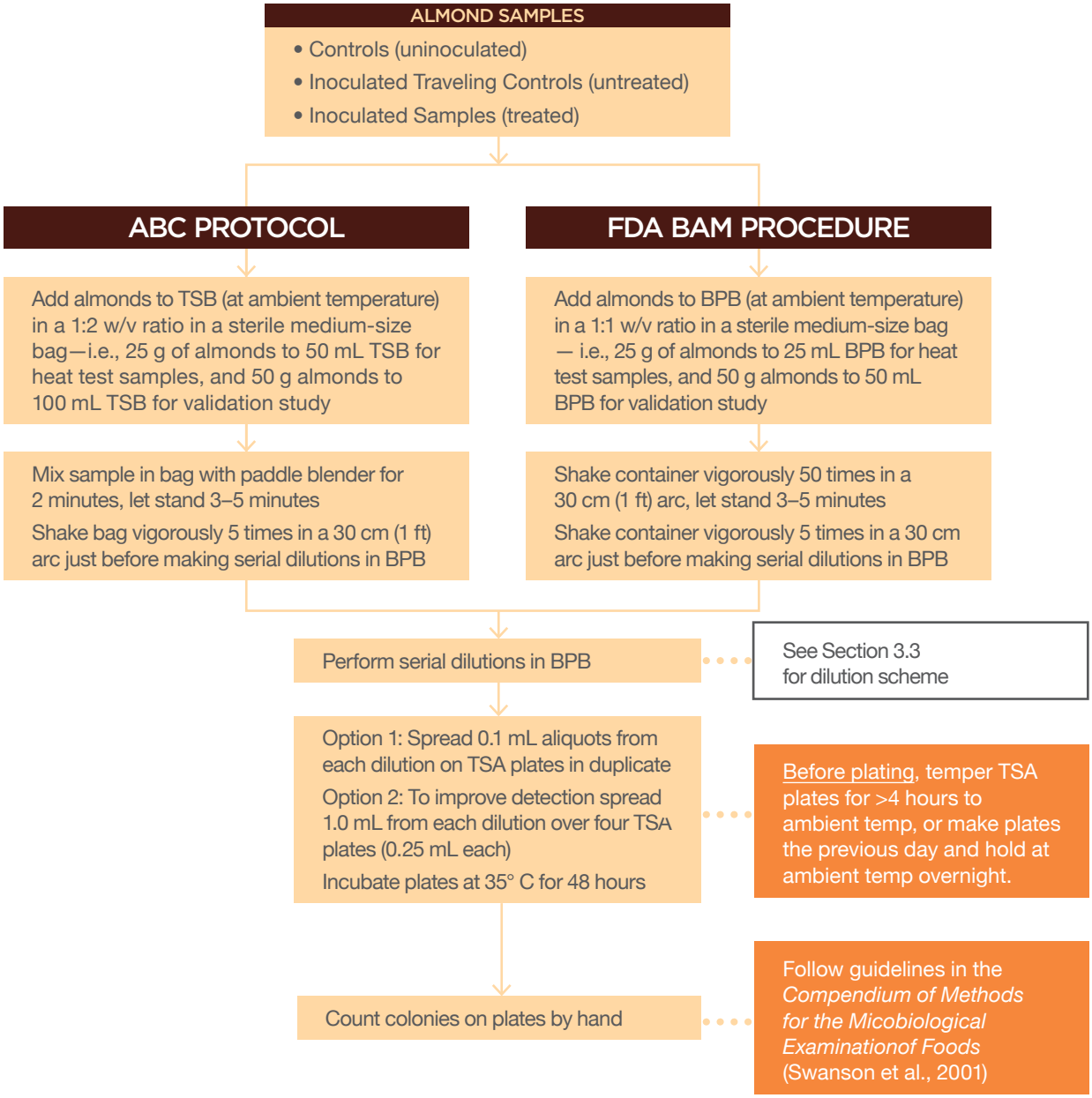
3.1 CHALLENGE TESTING WITH INOCULATED ALMONDS

To run meaningful process validation trials, 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. For example: For dry-roaster validation, conduct testing with temperature set points lowered. For normal production, increase set points and establish temperature critical factors that exceed the maximum values reached during validation testing.



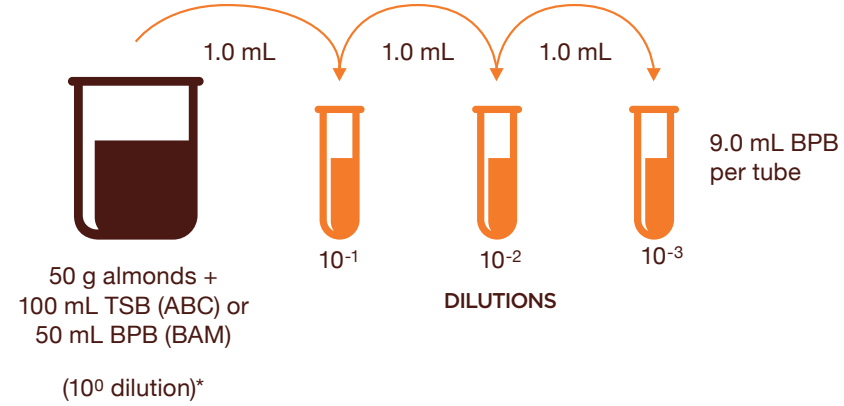
3.2 RECOVERY AND ENUMERATION OF INOCULATED MICROORGANISMS

Recover and enumerate inoculated *E. faecium* on all samples (inoculated and uninoculated [control] almonds) by following the ABC protocol or the procedure described in the *FDA Bacteriological Analytical Manual* (BAM) (Andrews and Hammack, 2003).



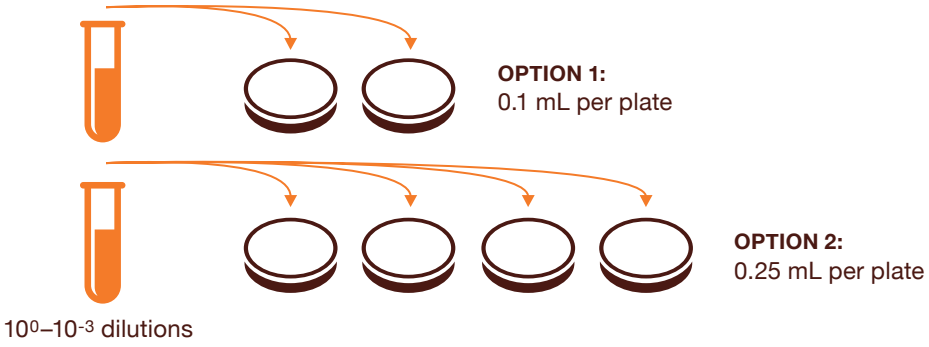
3.3 DILUTION AND PLATING SCHEMES

DILUTION SCHEME



*ABC Procedure–A correction factor of x2 is needed in calculations for 1:2 w/v dilution

SPREAD PLATING FOR SERIAL DILUTIONS



3.4 DATA REPORTING

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

- All raw data of microbiological counts and respective log CFU/g values (see examples on page 11)
- Average and minimum log reduction values (see examples on page 11):
  - Log reduction = initial counts – survivors
    = log CFU/g in untreated inoculated samples (traveling controls) – log CFU/g in treated inoculated samples
  - To calculate minimum log reduction, subtract the highest log of the number of survivors in the inoculated treated samples for each process parameter from the lowest log of initial counts in the corresponding untreated inoculated samples (traveling controls).
  - Please note: Data must be converted to base 10 BEFORE any calculations are done. Although average values are useful in interpreting results, for almond validation purposes, the least log reduction values achieved must meet the minimum 4-log destruction requirement.
- Date(s) of almond inoculation, heat resistance test results and pre-/post-inoculation almond moisture
- Validation test date(s) and enumeration date(s)

3.5 DATA CALCULATION EXAMPLES

Below are two examples of calculations to determine log CFU/g and log reduction of *E. faecium*. Refer to Section 3.3 for the dilution scheme. For dilution calculations, assume that almonds are not homogenized and TSB or BPB are not absorbed by the almonds. In the examples given, assume that the lowest count for untreated inoculated samples is 7.4 log CFU/g.

EXAMPLE 1:

- Counts on two plates on which 0.1-mL samples of a 10^{-1} dilution were plated are 30 and 42 colonies. Use 36 (the average number of survivors in this sample) to calculate the minimum log reduction.
    - Since this count was obtained by plating a 0.1-mL sample of a 10^{-1} dilution of TSB or BPB in the primary TSB/almond mixture or BPB/almond mixture, respectively, the count in TSB or BPB is 100 x 36 = 3,600 CFU/mL. (If the count was from a 10^{-2} dilution, multiply by 1,000 instead of 100.)
    - To calculate log CFU/g values: If the ABC protocol (Section 3.2) was used, multiply 3,600 by 2 = 7,200 CFU/g of almonds (3.9 log CFU/g). If the FDA BAM procedure was used, the count is 3,600 CFU/g (3.6 log CFU/g); there is no conversion factor.
    - To calculate log reduction: For the ABC protocol example above, 7.4 log CFU/g (untreated inoculated almonds [traveling controls]) – 3.9 log CFU/g (treated inoculated almonds) = 3.5 log CFU/g (log reduction). If the FDA BAM procedure was used, the log reduction is 7.4 log CFU/g – 3.6 log CFU/g = 3.8 log CFU/g.

EXAMPLE 2:

- Counts from quadruplicate plates on which 0.25-mL samples of a 10^{0} dilution were plated are 5, 7, 13 and 0 colonies. Add the counts (total = 25).
    - This total count is the count in 1 mL of TSB or BPB from the TSB/almond or BPB/almond mixture (= 25 CFU/mL).
    - To calculate log CFU/g values: If the ABC protocol was used, multiply 25 by 2 = 50 CFU/g of almonds (1.7 log CFU/g). If the FDA BAM procedure was used, the count is 25 CFU/g (1.4 log CFU/g); there is no conversion factor.
    - To calculate log reduction: For the ABC protocol example above, 7.4 log CFU/g (untreated inoculated samples [traveling controls]) – 1.7 log CFU/g (treated inoculated almonds) = 5.7 log CFU/g (log reduction). If the FDA BAM procedure was used, the log reduction is 7.4 log CFU/g – 1.4 log CFU/g = 6.0 log CFU/g.

SECTION 4:

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