Guidelines for using *Enterococcus faecium* NRRL B-2354 as a surrogate microorganism in pistachio process validation

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1 IDENTIFICATION OF THE APPROPRIATE PATHOGEN OF CONCERN IN PISTACHIOS

Outbreaks of salmonellosis and *Escherichia coli* gastroenteritis have been linked to consumption of tree nuts (Harris et al., 2018a) and both pathogens should be considered in a hazard assessment of nuts or nut-containing products (FDA, 2018). Based upon current information (outbreaks, surveys, recalls, and storage survival), *Salmonella* was identified as the more likely biological hazard for inshell pistachios and pistachio kernels (Harris et al., 2018a, 2018b, 2018c; Yada and Harris, 2018). The almond industry identified a single strain of *Salmonella* isolated from almonds (*Salmonella* Enteritidis phage type [PT] 30) as the target pathogen for thermal treatments (blanching, oil roasting, dry roasting, and steam pasteurization) of almonds. The thermal resistance of *Salmonella* Enteritidis PT 30 was compared to five other strains of *Salmonella*, including pistachio isolates *Salmonella* Montevideo and *Salmonella* Senftenberg; no systematic differences were observed among the *Salmonella* isolates across multiple treatments (Moussavi et al., 2017). These data suggest that *Salmonella* Enteritidis PT 30 could be used to evaluate the thermal tolerance of other organisms on pistachios. Five strains each of *E. coli* O157:H7 and of *Listeria monocytogenes* inoculated onto inshell pistachios were screened for resistance to heat in hot water and hot oil treatments. All strains evaluated were less heat resistant than *Salmonella* Enteritidis PT 30.

The goal of process validation studies is to determine the level of *Salmonella* reduction achieved by a treatment technology and associated equipment. Risk assessments have been performed to determine the appropriate target lethality for treatments of pistachios grown in the United States, and have consistently determined that a 4-log reduction would result in an estimated mean risk of less than one case of salmonellosis per year in the United States (Lambertini et al., 2017; Santillana Farakos et al., 2018).

2 SURROGATE CULTURE – BACKGROUND AND BIOSAFETY CONSIDERATIONS

A surrogate selected for process validation studies in food processing and pilot plant facilities must be nonpathogenic to humans. *Enterococcus faecium* NRRL B-2354 has been used in the food industry as a nonpathogenic test organism for many decades; previously under various other names and strain designations, including *Micrococcus freudenreichii* ATCC 8459, *Pediococcus* sp. NRRL B-2354, and *E. faecium* ATCC 8459 (Jeong et al., 2011; Kopit et al., 2014). A study 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).

Research conducted at the University of California, Davis, and Michigan State University—funded by the California Pistachio Research Board, the Center for Produce Safety, and the Specialty Crop Block Grant Program at the U.S. Department of Agriculture (USDA) has demonstrated that there are no systematic differences in the thermal resistance of *E. faecium* NRRL B-2354, *Salmonella* Enteritidis PT 30 and other salmonellae (including *Salmonella* Montevideo and *Salmonella* Senftenberg, isolated from pistachios) on inoculated pistachios exposed to various thermal treatments (Casulli et al., 2016, 2017; Moussavi et al., 2017).

These data support the use of *E. faecium* NRRL B-2354 as a surrogate organism for *Salmonella* in pistachio process validation studies for the following types of processes:

- **Dry heat processes** such as dry roast including pre-wetting or brining prior to dry roasting
- **Moist air or steam processes**

The strain *E. faecium* NRRL B-2354 can be obtained through the culture collection of the USDA National Center for Agricultural Utilization Research (NCAUR) for no charge via the online ordering system for strains in the public access catalog: http://nrrl.ncaur.usda.gov/. If this strain is not available, *E. faecium* ATCC 8459 from the American Type Culture Collection may be substituted, as this organism has been shown to be equivalent to *E. faecium* NRRL B-2354 (Kopit et al., 2014).

E. faecium NRRL B-2354 also may be an appropriate surrogate for alternative thermal processes such as infrared heating (Venkitasamy et al., 2017), microwave, radio frequency heating, and others. However, before the surrogate is used in validation of other types of processes, studies should be conducted and data gathered to demonstrate appropriate resistance of *E. faecium* NRRL B-2354 compared with *Salmonella* Enteritidis PT 30 on pistachios for the specific process. Studies comparing the resistance of *E. faecium* NRRL B-2354 and *Salmonella* Enteritidis PT 30 (or other pathogens of concern) should be conducted before using *E. faecium* NRRL B-2354 as a surrogate for products other than pistachios. Furthermore, protocols and guidelines established for use of this surrogate on pistachios should not be considered appropriate for other products without additional scientific data to support such application.

3 INOCULUM AND INOCULATED PISTACHIOS – PREPARING, HANDLING AND STORING

The following guidelines describe the materials and step-wise preparation and handling procedures for *E. faecium* NRRL B-2354 in validating thermal processes for pistachios, including how to prepare inoculum and how to store and transport the inoculated pistachio samples. Separate validations are necessary for inshell pistachios and pistachio kernels. These methods are largely based on those described by the Almond Board of California for use with almonds. The process takes a minimum of 8 days and as many as 10 days from initial streaking of the culture to obtaining dry pistachios at a target moisture content with confirmed target levels of *Enterococcus*. Laboratory studies with *E. faecium* were performed using size 21/25 pistachios; however, the size of pistachios used for validation should be representative of product most commonly run in the target process.

3.1 Materials

Pistachios – inshell or kernels

- Grade U.S. Extra No. 1 pistachios
  - Temperature of the pistachios should be 21–24°C (70–75°F) prior to inoculation
  - Initial moisture content of the pistachios should be <7%; moisture and water activity should be measured and recorded

Culture

- *Enterococcus faecium* NRRL B-2354

Equipment

- Plastic petri dishes (standard and 150-mm diameter)
- Pipettes, sterile
- Pipettor
- Test tubes or microcentrifuge tubes
- Glass spreaders or L-shaped plastic spreaders, sterile
- Plastic loops, sterile
- Falcon tubes
- Magnetic stir plate and bars or Vortex
- Biosafety cabinet or laminar flow hood
- 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; P5 grade)
- Metal drying rack
- Plastic storage bin with lid, sterile
- Metal mesh tray or aluminum mesh
- Tape
- Laboratory paddle blender (e.g., Stomacher lab blender or equivalent)
- Containers to hold inoculated nuts for treatment (e.g., thermostable bags or baskets)

Sanitizer

- 70% ethanol

Media

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

3.2 Inoculum preparation

Timeline

DAYS 1–5: Prepare inoculum

DAYS 5–6: Inoculate pistachios, and then dry inoculated pistachios at room temperature for 1 to 3 days to the final target moisture content (see section 3.3 *Inoculation and drying*) and determine that the inoculation level is >7.0 log CFU/g (see section 4.2 *Recovery and enumeration of inoculated microorganisms*)

DAYS 7–14: Begin validation trials with inoculated pistachios

Inoculum preparation

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

The amount of pistachios to be inoculated is determined by the experimental design: for example, 25 or 50 g per sample × number of sampling points × number of replicates at each sampling point. Typical validation studies will use >2,400 g of inoculated pistachios, including samples for measuring moisture, water activity, and inoculum level (before and after drying), and for traveling controls.

The total inoculum volume needed depends on the amount of pistachios to be inoculated. Make an appropriate number of 25-ml inoculum preparations and then pool as described below.

For each 25 ml of inoculum preparation:

DAY 1

Streak culture (active or frozen) onto standard (100 mm) TSA plates

Incubate at 35 ± 2 °C for 24 ± 2 hours

DAY 2

Transfer cells from isolated typical colonies 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 24 ± 2 hours

Place large TSA plates (5 plates/400 g, 150 × 15 mm) on bench top to dry overnight

DAY 4

Spread thoroughly mixed liquid culture from day 3 (1 ml/plate) over large TSA plates to produce a bacterial lawn after incubation

Incubate at 35 ± 2 °C for 24 ± 2 hours

DAY 5

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 for a total volume of 25 ml per 5 petri dishes

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

Pooled inoculum may be held for up to 0.5 hour, with stirring, while pistachio samples are being inoculated.

3.3 Inoculation and drying

The following inoculation procedure (Figure 1) is for one 400-g portion of either inshell pistachios or pistachio kernels. To prepare a larger amount of inoculated pistachios, separately inoculate 400-g batches of pistachios and pool after drying, as described below (Figure 1).

- The final target moisture content of the inoculated pistachios should be at or less than the moisture content typically observed in the facility where the validation will take place. The firm should make these data available to the process authority requesting the pistachios. Fresh raw pistachios are typically between 3.5 and 7% moisture.
- The level of *E. faecium* must be >7.0 log CFU/g (as determined in section 4.2).
- Heat resistance testing is recommended by the Almond Board of California for validation using almonds inoculated with *E. faecium*. However, at this time, insufficient data are available to set a corresponding limit for inoculated pistachios.

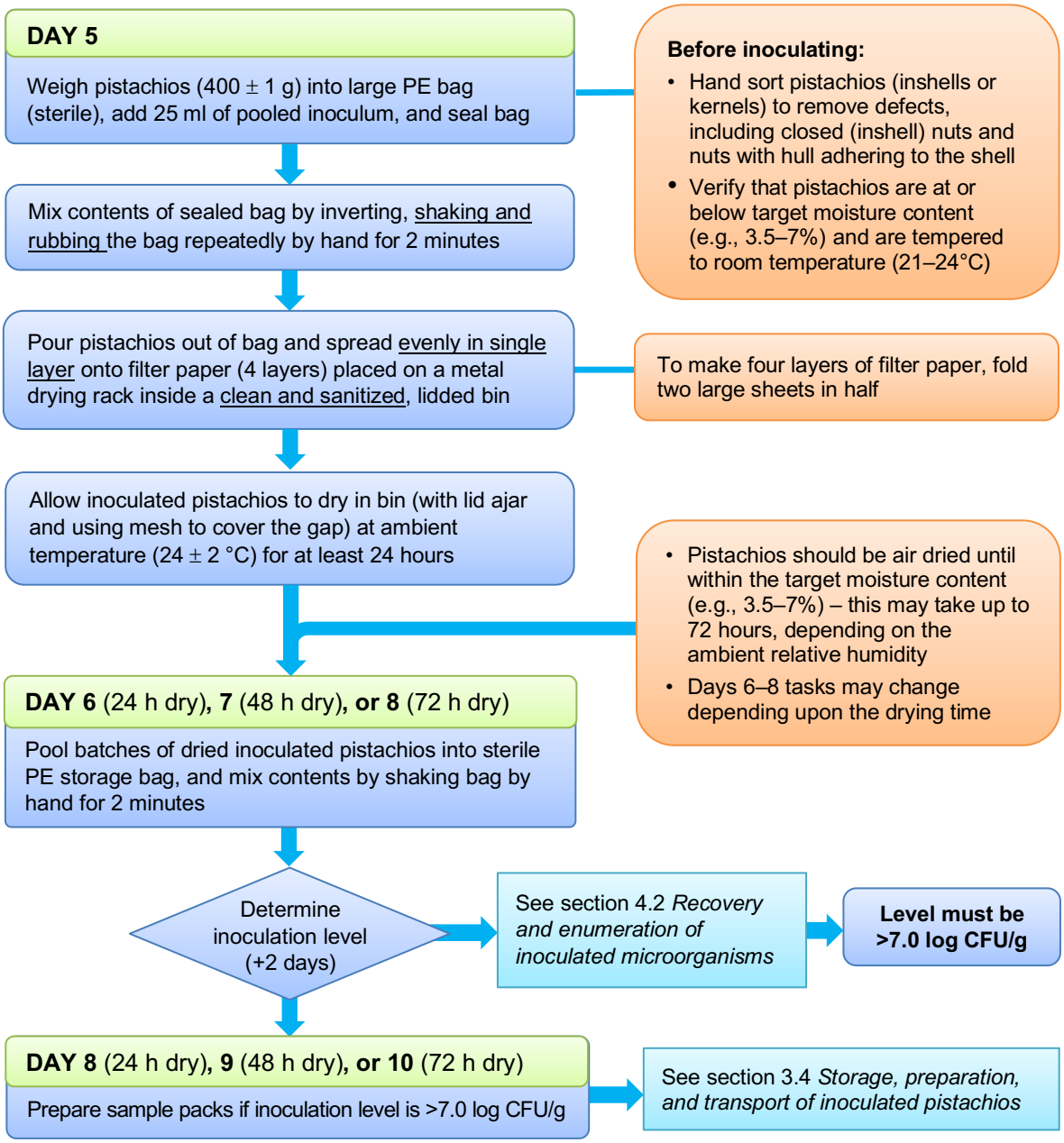

Figure 1. Schematic for the inoculation and drying of pistachios with *E. faecium* for challenge testing.

3.4 Storage, preparation, and transport of inoculated pistachios

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

STORAGE

Store inoculated pistachios at 4 ± 1 °C and use in validation trials for up to 14 days after inoculation – however, the level of *E. faecium* must be >7.0 log CFU/g at the time of the validation testing.

PREPARATION OF INOCULATED SAMPLE PACKS

Loosely pack pistachios (25 to 50 g portions) into mesh bags, baskets, or other suitable container that can be incorporated into processing line

- Prepare enough sample packs for each validation run to cover all conditions and treatments to be tested
- Also prepare triplicate inoculated sample packs to serve as “traveling” controls

TRANSPORT

Transport and handle dried inoculated samples and inoculated traveling controls in the identical manner on each day of the validation trial.

4 USE OF SURROGATE IN VALIDATION

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

4.1 Challenge testing with inoculated pistachios

For details on determination and assessment of critical processing parameters (Casulli et al., 2018), see additional guidance documents on specific processes (i.e., flatbed roaster, rotary roaster).
A general outline for conducting a challenge test in a commercial roasting operation is shown below in Figure 2.

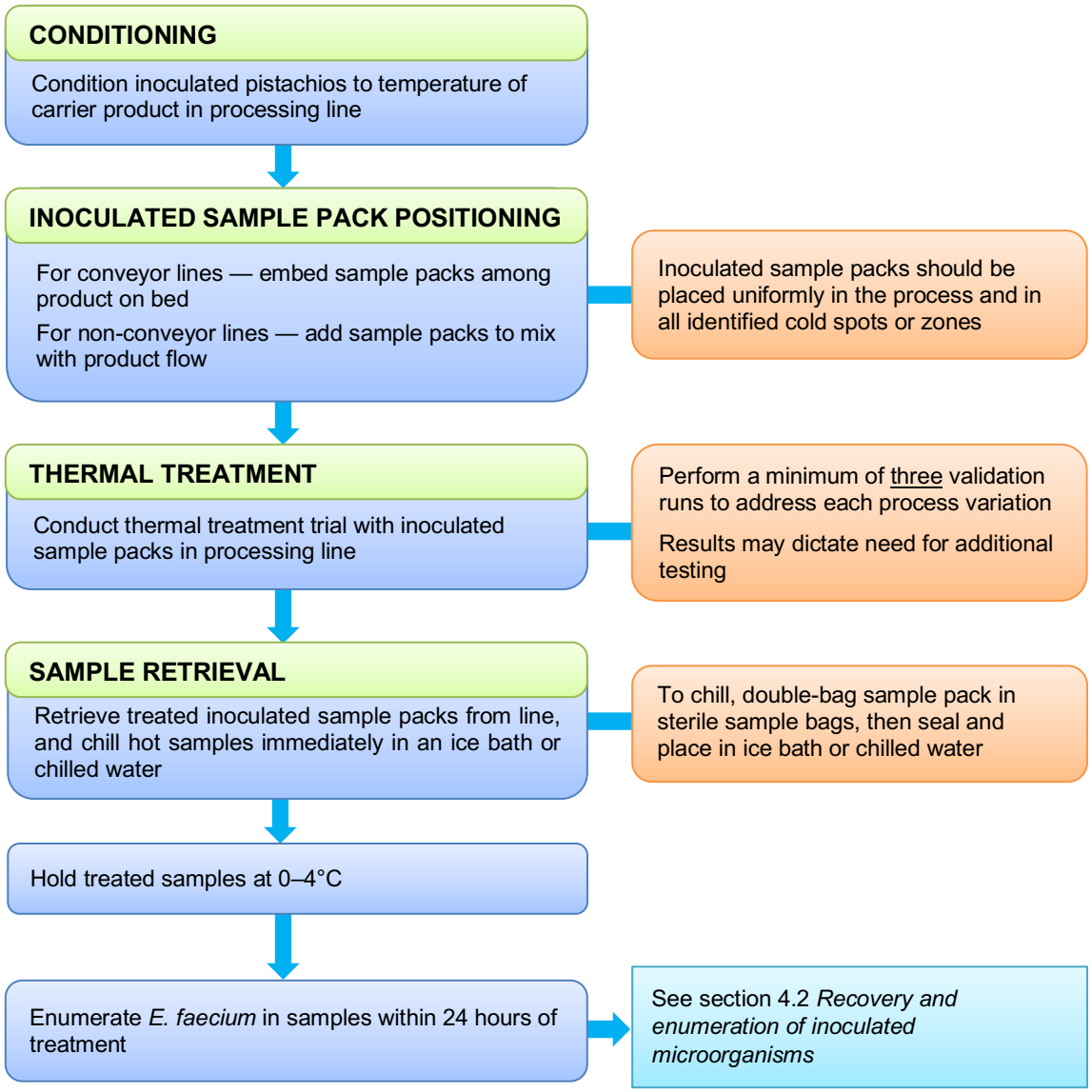

Figure 2. Schematic for challenge testing in a pistachio roaster using *E. faecium*-inoculated product.

4.2 Recovery and enumeration of inoculated microorganisms

Recover and enumerate inoculated *E. faecium* on samples (inoculated and uninoculated [control] pistachios) by following the pistachio protocol or the procedure in the *FDA Bacteriological Analytical Manual* (BAM) (Andrews and Hammack, 2003). Both methods are outlined below in Figure 3.

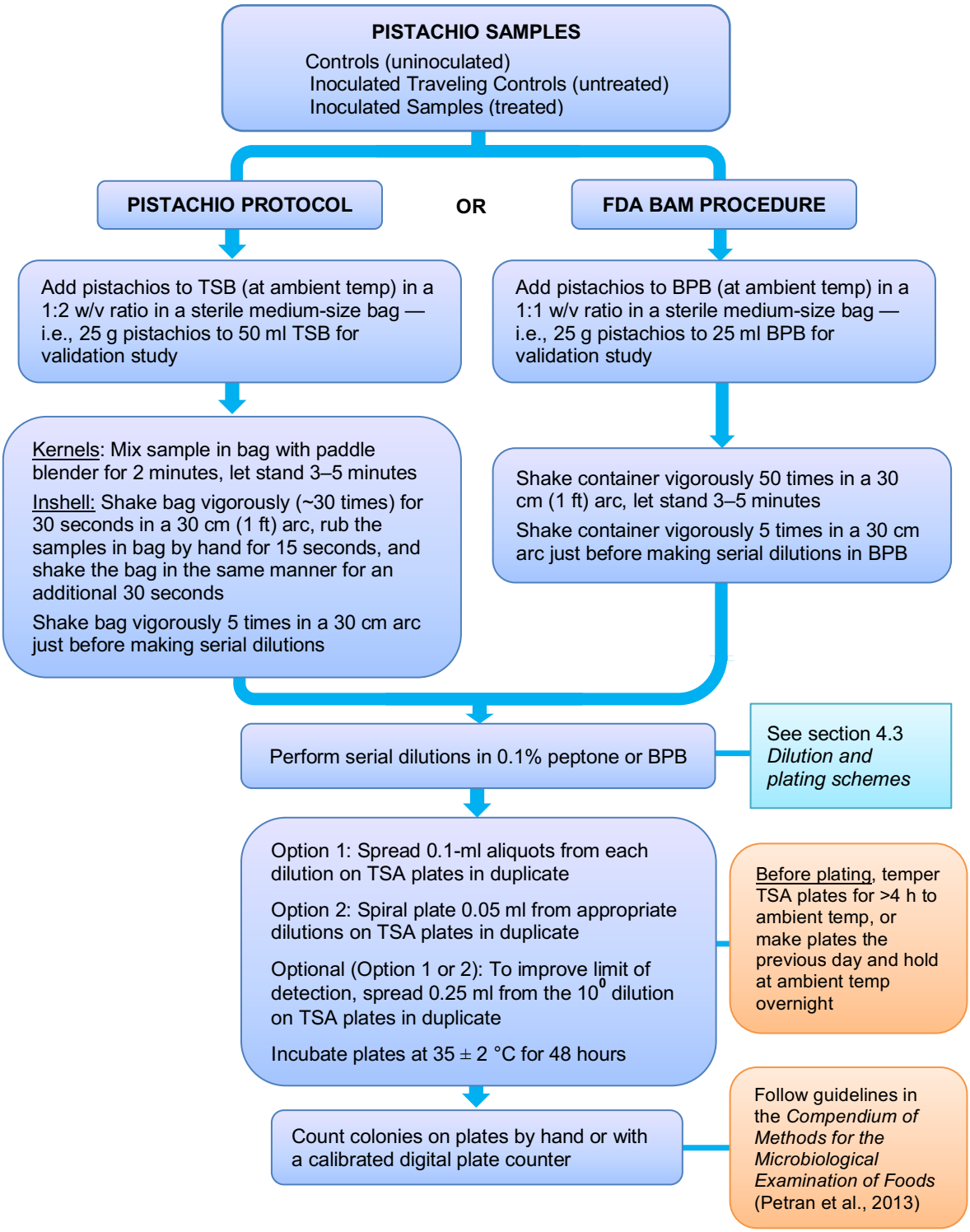

Figure 3. Schematic for the recovery and enumeration of inoculated microorganisms.

4.3 Dilution and plating schemes

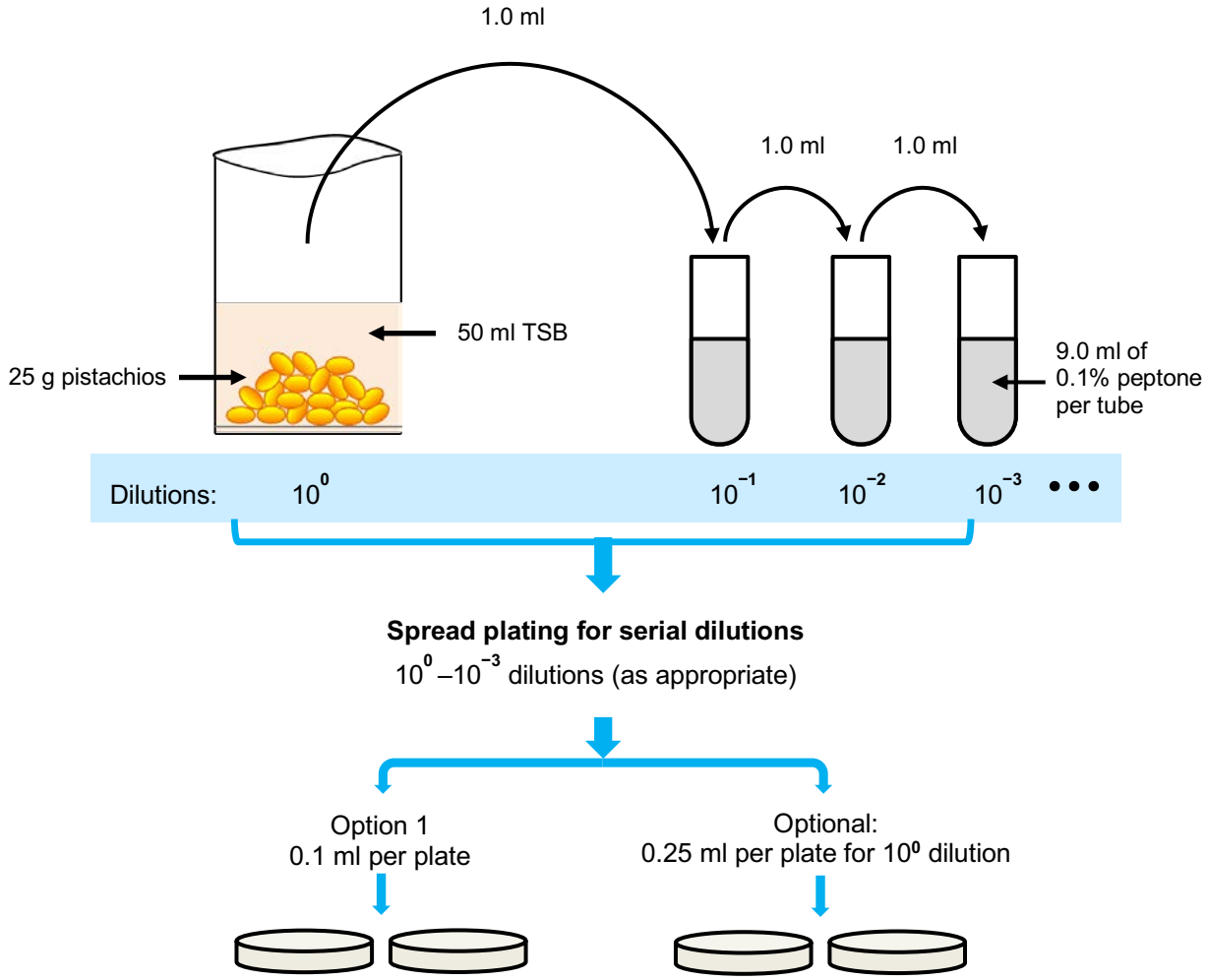

Figure 4. Example dilution scheme using the pistachio protocol.*

* Note that a correction factor of ×2 is needed in calculations for a 1:2 w/v dilution for the 10^0 dilution when using the pistachio protocol (see section 4.2 for details).

4.4 Data reporting

Include the following items in the process validation report:

- All raw data of microbiological counts and respective log CFU/g values (see examples in section 4.5 *Data calculation examples* and section 4.6 *Data reporting table example*)
- Average and minimum log reduction values (see examples in section 4.5):
    - **Log reduction = log initial counts − log survivors**

      Note: Log initial counts is the log CFU/g in untreated inoculated samples (traveling controls) and log survivors is the log CFU/g in treated inoculated samples
    - **Minimum log reduction = lowest log initial counts − highest log survivors**

      Note: Subtract the highest log CFU/g of survivors in the inoculated treated samples for each process parameter from the lowest log CFU/g of initial counts in the corresponding untreated inoculated samples (traveling controls)
    - Important: Data must be converted to log base 10 with two decimal places BEFORE any calculations are done. Although average values are useful in interpreting results, for pistachio validation purposes, the minimum log reduction values achieved must meet the reduction criteria identified in your food safety plan.
- Date(s) of pistachio inoculation and pre-/post-inoculation pistachio moisture
- Validation test date(s) and enumeration date(s)
- All process variables and conditions relevant to the process being validated

4.5 Data calculation examples

Processors are encouraged to consult appropriate statistical expertise in analyzing and interpreting validation test results. The Almond Board of California provides guidelines for simplified analysis of surrogate test results. Below are two examples of such calculations to determine log CFU/g and the log reduction of *E. faecium*. Refer to section 4.3 for the dilution scheme. Report the level of microorganism in each sample as CFU/g based on the average count of two plates. Appropriate dilutions should be plated to result in colony counts between 25 to 250 (follow guidelines in the *Compendium of Methods for the Microbiological Examination of Foods*, Petran et al., 2013). For dilution calculations, assume that pistachios are not homogenized and TSB or BPB are not absorbed by the pistachios. In the examples given, assume that the lowest count for untreated inoculated samples is 7.92 log CFU/g.

Determining level of *E. faecium*:

EXAMPLE 1:

- Counts on two plates on which 0.1-ml samples of a 10^{-1} dilution were plated are 70 and 94 colonies. Use 82 (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/pistachio mixture or BPB/pistachio mixture, respectively, the count in TSB or BPB is 100 × 82 = 8,200 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 pistachio protocol was used, multiply 8,200 by 2 = 16,400 CFU/g of pistachios (4.21 log CFU/g). If the FDA BAM procedure was used, the count is 3,600 CFU/g (3.91 log CFU/g); there is no conversion factor.
    - **To calculate minimum log reduction**: For the pistachio protocol example above, 7.92 log CFU/g (untreated inoculated pistachios [traveling controls]) − 4.21 log CFU/g (heat treated inoculated pistachios) = 3.71 log CFU/g (log reduction). If the FDA BAM procedure was used, the log reduction is 7.92 log CFU/g − 3.91 log CFU/g = 4.01 log CFU/g.

EXAMPLE 2:

- Counts from quadruplicate plates on which 0.25-ml samples of a 10^{0} dilution were plated are 11, 7, 13, and 0 colonies. Add the counts from the four plates (total = 31).
    - This total count is the count in 1 ml of TSB or BPB from the TSB/pistachio or BPB/pistachio mixture (31 CFU/ml).
    - **To calculate log CFU/g values**: If the pistachio protocol was used, multiply 31 by 2 = 62 CFU/g of pistachios (1.79 log CFU/g). If the FDA BAM procedure was used, the count is 31 CFU/g (1.49 log CFU/g); there is no conversion factor.
    - **To calculate minimum log reduction**: For the pistachio protocol example above, 7.92 log CFU/g (untreated inoculated samples [traveling controls]) − 1.79 log CFU/g (heat treated inoculated pistachios) = 6.13 log CFU/g (log reduction). If the FDA BAM procedure was used, the log reduction is 7.92 log CFU/g − 1.49 log CFU/g = 6.43 log CFU/g.

4.6 Data reporting table example

Traveling Controls (TC)

TC #	Plate A	Plate B	Average count	Dilution	CFU/g	log CFU/g
TC1	88	94	91	1000000	91000000	7.96
TC2	90	78	84	1000000	84000000	7.92
TC3	110	120	115	1000000	115000000	8.06
Average						7.98
St Dev						0.07
Minimum level						7.92

Heat Treated Inoculated Samples

Sample #	Plate A	Plate B	Average count	Dilution	CFU/g	Heat treated individual sample (B) (log CFU/g)	Minimum level of traveling control (A) (log CFU/g)	Minimum log reduction of individual sample (A−B) (log CFU/g)
1	35	50	42.5	100	4250	3.63	7.92	4.30
2	48	32	40	100	4000	3.60	7.92	4.32
3	29	25	27	100	2700	3.43	7.92	4.49
4	70	94	82	100	8200	3.91	7.92	4.01
5	30	48	39	100	3900	3.59	7.92	4.33
6	39	35	37	100	3700	3.57	7.92	4.36
7	30	50	40	100	4000	3.60	7.92	4.32
8	60	52	56	100	5600	3.75	7.92	4.18
9	29	29	29	100	2900	3.46	7.92	4.46
10	34	32	33	100	3300	3.52	7.92	4.41
					Average:	3.61	Average:	4.32
					St Dev:	0.14	St Dev:	0.14
							Minimum reduction:	4.01

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6 GLOSSARY

Colony forming units (CFU): CFU is the measure of viable bacterial cells

Replicate: Number of samples tested at the same time from the same batch of inoculated pistachios

Replication: Validation study conducted at a different time with a different batch of inoculated pistachios

Traveling control (TC): Positive control sample that has been inoculated with the test organism and is exposed to the same environmental conditions, except for heat treatment