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Dry-inoculation methods for low-moisture foods

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ABSTRACT

Background: The knowledge of the thermal resistance of target pathogens in food matrices is a prerequisite for the design of effective control treatments. It is also desirable, or even necessary, to validate the treatments using appropriate surrogates for the target pathogens. To obtain the thermal death kinetic information for both the target pathogens and their surrogates or validate the effect of new thermal treatments using surrogates, bacteria of interests (pathogen or surrogate) must be introduced to the food matrices at an adequate concentration to obtain survivor curves. A major challenge for the inoculation of the bacteria in low-moisture foods (LMFs) is that the inoculation could result in changes to the physical characteristics of the food matrices. For example, alteration of the microstructures and particular size could lead to different moisture absorption and desorption behaviors of treated foods in thermal treatments.

Scope and approach: The safety of LMFs is an emerging concern in the food industry. Extensive research only took place over the past ten years, and dry-inoculation has risen as a promising tool for developing efficient treatments to control pathogens in LMFs. This paper provides a general review of the methodologies for LMFs inoculation. It summarizes the recently published work in the developments of dry-inoculation methods and compares the advantages and limitations of different LMFs inoculation methods.

Key findings and conclusions: Dry-inoculation is a more suitable approach for LMFs inoculation, which offers an attractive alternative to wet-inoculation. Dry-inoculation methods require a short preparation time, and the inoculum has a long shelf-life, minimal influence on the physio-chemical properties of the food matrices, and is easier to transport.

1. Introduction

Isothermal tests are commonly used to determine the thermotolerance of bacteria at a constant temperature. The thermotolerance of bacteria is characterized by *D*-value, which is the time required to reduce the number of bacteria by 1 log (or 90%) at a given temperature, and by *z*-value, which is the number of degrees of temperature has to be increased to reduce the *D*-value by 1 log. Heat distribution and heat penetration studies have been widely used in the industry to record the temperature-time history of treated foods and develop new thermal processes. However, to produce safe products, the inactivation performance of a thermal process should be validated by artificially inoculating the foods with microorganisms of interest. Specifically, the thermal resistance (*D*-/*z*-values) of a pathogen or surrogate determined in an isothermal study, combined with the temperature-time history obtained from a heat distribution/penetration study, are used to calculate the accumulated lethality of target microorganism during a thermal process. This information can be utilized to predict the microbial lethality and provide guidance for proper thermal process designs to deliver microbial safe products (Bigelow, Bohart, Richardson, & Ball, 1920; Ray & Bhunia, 2007; Stumbo, 2013).

Low-moisture foods (LMFs) are food products with a water activity of 0.65 or lower (Sánchez-Maldonado, Lee, & Farber, 2018). Outbreaks caused by contaminated LMFs are attracting widespread attention to the food industries, regulators, and consumers (Scott et al., 2009). Until now, there is no standard inactivation processing for controlling potential pathogens in most LMFs since they were considered as safe foods. However, there were many pathogen outbreaks and food recalls recently

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associated with LMFs, including pistachios (FDA, 2016b), flours (FDA, 2016a, 2019a, 2019b), nut butter (FDA, 2018d, 2018e), milk powder (Carrasco, Morales-Rueda, & García-Gimeno, 2012), cereals (FDA, 2018f), chips (FDA, 2018c) and crackers (FDA, 2018a), and spices (FDA, 2017; 2018b, 2018g, 2019c). Reduction of pathogens in low water activity (a_w) environments (such as LMFs) is challenging since pathogens are more resistant to heat compared with those in aqueous environments (Bari et al., 2009; Commission, 2015; He, Guo, Yang, Tortorello, & Zhang, 2011; Keller, VanDoren, Grasso, & Halik, 2013; Liu, Tang, Tadapaneni, Yang, & Zhu, 2018; Podolak & Black, 2017; Rossana; Villa-Rojas, Zhu, Marks, & Tang, 2017). Therefore, studies of the thermal resistance of bacteria in a low-moisture environment are of paramount importance, which can provide guidance for LMFs food manufacturers to design safe processing conditions and identify proper surrogates for process validation.

Ideally, the introduction of bacteria into the food matrices should not influence the thermal resistance for either pathogen or surrogate in an isothermal study. However, the inoculation of LMFs using bacteria culture, especially in liquid form, would change the microstructure and physicochemical properties of LMFs. Different physical characteristics of the food matrices may result in variation of *D*- and *z*-values of the bacteria, which in turn may alter the design of the thermal process and possibly make it less effective at inactivating the desired pathogen. Currently, there are two groups of methods to inoculate bacteria in LMFs: wet-inoculation uses inoculum in an aqueous form while dry-inoculation employs inoculum prepared using either a carrier (such as chalk powder, sand, glass beads, etc.) or drying techniques (freeze-, spray, vacuum-drying). The biggest issue with wet-inoculation lies in the addition of moisture into the food matrix when a wet culture is used. The extra moisture may change some quality attributes and produce problems, such as caking, stickiness and clumping (Aguilera, del Valle, & Karel, 1995; Chuy & Labuza, 1994; Kimber, Kaur, Wang, Danyluk, & Harris, 2012; Palipane & Driscoll, 1993).

Dry-inoculation methods offer an alternative for LMFs inoculation (Blessington, Theofel, & Harris, 2013; Liu, Xu, Xie, Zhu, & Tang, 2019). Dry inoculums allow a more uniform distribution of microorganism in LMFs, with minimal impact on the properties of the food matrices (Hoffmans & Fung, 1992), resulting in less quality loss for moisture-sensitive LMFs, such as onion/garlic powder, sugar granulate, milk powder, etc.

This review article seeks to address and provides a summary of the most recent studies on dry-inoculation methods for LMFs. The advantages and limitations of dry-inoculation methods are then presented. Going forward, the rationale for using dried inoculum will be discussed. Finally, the authors will share their opinions on future applications and improvements using dry-inoculation methods for isothermal tests in addressing food safety concerns.

2. Inoculation procedure for isothermal tests

2.1. Isothermal tests

High-temperature processing (above 100 °C), such as baking, extrusion, spray drying, frying, and roasting, are important operations in the production of some LMFs, such as cookies, extruded cereals, infant formula, chips, and peanut butter (Zhang, Zhang, & Adhikari, 2020). However, little is known about their adequacy as pathogen control steps, as their objective is to produce desirable changes in sensory attributes. On the other hand, some other LMFs, such as dried fruits, spices, are produced by drying techniques (e.g. tunnel drying, sun drying, etc.) at mild operation temperatures (below 65 °C) (Elijah, Philomena, Joseph, Charles, & Paschal, 2020; Li et al., 2019; Rathore & Panwar, 2010). Regulatory requirements and new preventive controls landscape for LMFs have been changed by Food Safety Modernization Act (FSMA) recently, now the safety of LMFs is more focused on prevention rather than reaction (Barach & Dunaif, 2017). To ensure the microbial safety of LMFs, there is an increasing need for adding an extra "killing" step for those types of foods before packaging or distribution. The determination of *D*-/*z*-values using isothermal tests is important since this information can combine with the results from heat distribution and penetration studies (Fig. 1) and ultimately predict the microbial inactivation during a thermal process for validation purposes (Bianchini et al., 2014; Shah, Asa, Sherwood, Graber, & Bergholz, 2017; Verma et al., 2018; Wei, Lau, Stratton, Irmak, & Subbiah, 2019).

Isothermal tests determine the inactivation rate of a given microorganism or cocktail of them, inoculated in a food matrix, after exposure to a certain temperature for a determined amount of time. In most foods, the native bacteria population is relatively low (less than 100 CFU/g) or unknown. Because the native bacteria would not be considered enough to interfere with the thermal inactivation and thus, food is usually not treated to inactivate those bacteria. To make the native microbiome insignificant, food samples are normally inoculated with a sufficient number of bacteria (usually wet culture) to a desired high level (normally to 10^6–10^9 CFU/g) (Balasubramaniam, Ting, Stewart, & Robbins, 2004). Then, the inoculated foods are subjected to heat treatments at a certain temperature for a determined time interval (Forghani et al., 2019; Wiertzema et al., 2019).

Isothermal tests can be used to determine the thermal resistance of microorganisms, such as *D*- and *z*-values. In brief, inoculated samples are treated at a constant temperature, and the time (in minute) needed to kill 90% of bacterial cells is determined as the *D*-value (Ray & Bhunia, 2007). *D*-value indicates the rate of a microorganism that can be inactivated during a certain condition. The *D*-value of a certain bacteria is temperature-dependent and can be calculated by equation (1) at a given temperature:

$$logN_t = logN_0 - \frac{t}{D} \quad (1)$$

where N_0 is the initial population number, N_t is the number of survivors at time t, D is the decimal reduction time, $(1/D)$ refers to the slope of the thermal inactivation curve. *D*-values can be obtained from survivor curves when the log of the population is plotted against the treatment time.

z-value is the temperature increase that corresponds to a 10-fold reduction of the *D*-value. *z*-value is a measure of the relative impact of different temperatures on the thermal inactivation rate of microorganisms, with smaller values indicating greater sensitivity to increasing temperature (Stumbo, 2013). The *z*-value is determined by plotting the logarithms of at least two *D*-values against temperature by the formula:

$$z = \frac{T_2 - T_1}{logD_1 - logD_2} \quad (2)$$

where T is temperature and D is the *D*-value.

2.2. Inoculation procedure

Bacteria inoculation is essential for both isothermal test and validation study. Inoculation can influence the thermal inactivation rate, which affects the modeling of the cumulated lethality achieved in a thermal process that uses the temperature distribution data. To determine an accurate *D*-/z-values in isothermal tests, 3–4 log reduction in bacterial numbers after treatments are recommended, and at least three *D*-values at different temperature levels (better to within 10 °C) is needed for an accurate *z*-value. Since most of the microbial detection limitation is about 10^2 CFU/mL, the initial level of inoculation should be at least 10^{5-6} CFU/mL (Villa-Rojas, 2015). In general, two main approaches have been widely used for inoculation in the case of LMFs, and Fig. 2 illustrates the general inoculation procedures.

In general, the inoculation procedure includes three main steps, these are culture preparation, inoculation, and equilibration. The culture preparation usually takes 3–4 days to resuscitate bacteria from the

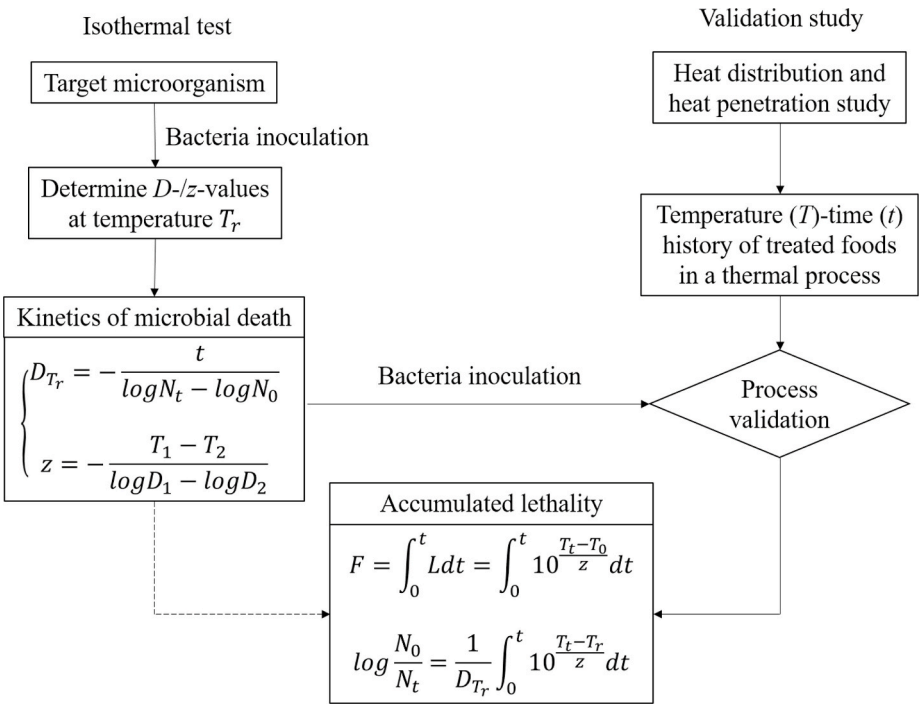

Fig. 1. Inclusion of inactivation kinetics and thermal penetration studies in the design of inactivation processes.

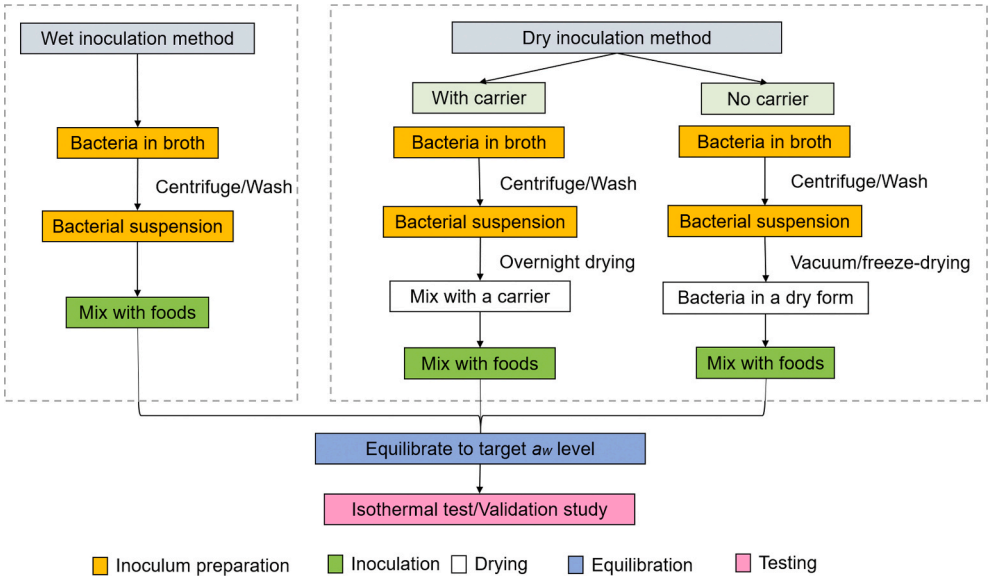

Fig. 2. General procedures used in wet and dry-inoculation methods for LMFs.

frozen stock by multiple transfers in the culture broth. Then, bacteria were harvested by lawn or broth methods (Villa-Rojas, 2015).

For wet-inoculation methods, it is difficult to mix the wet culture with LMFs homogenously without a localized change in physical properties. Typically, a determined amount of wet culture is firstly mixed with a small amount of food sample, called a "seed" inoculum, which is then mixed with a larger food sample (Liu et al., 2019; Villa-Rojas, 2015). To ensure proper inoculation concentration throughout the sample, 1 g samples of the food are randomly taken to be plated and enumerated, this is called a homogeneity test (Hildebrandt et al., 2016). If the homogeneity test results have a narrow standard deviation (<0.5 CFU/g), the sample was appropriately inoculated and ready to be used. After inoculation, the sample needs to be equilibrated to the target a_w before performing the isothermal test. A few days or even weeks are needed to bring back the a_w of inoculated samples to the original or target a_w levels.

Dry inoculation methods have two approaches, the use of a carrier (Blessington et al., 2013; Hildebrandt et al., 2017; Liu et al., 2019; Nurul Hawa Ahmad et al., 2019) or drying the bacteria (Xu et al., 2017). The carrier method uses a similar approach as the wet inoculation, but instead of inoculating a food sample, it inoculates an inert carrier such as chalk (Enache et al., 2015; Hildebrandt et al., 2017; Liu et al., 2019). The inoculated carrier is pre-dried overnight or until it reaches extremely low a_w (less than 0.5) (Fig. 2). The inoculated carriers will be used as the "seed" inoculum and inoculate the food sample.

The second dry-inoculation method uses dried bacteria to directly

inoculate the food samples (Hoffmans & Fung, 1992; Xu et al., 2017). In this scenario, the wet culture will be dehydrated by freeze-, vacuum-, or spray-drying and then mixed with the food matrices. Protective agents (such as skim milk, sucrose, trehalose, etc.) increase the chances of microbial survival and therefore are commonly added to the wet culture before drying (Fig. 2).

3. Dry-inoculation methods for LMFs

Dry-inoculation methods have shown several advantages in LMFs inoculation over wet-inoculation. Dry-inoculation methods have been successfully applied in wheat flour (Xu et al., 2017), non-fat milk powder (Liu et al., 2019), nut kernels (Blessington et al., 2013), sugar granulates (Beuchat, Mann, Kelly, & Ortega, 2017), and spices (Hildebrandt et al., 2017). Table 1 summarizes the published studies using dry inoculum for LMFs.

3.1. Dry inoculum with a carrier

Dry inoculation has been used in a wide variety of LMFs (From Table 1). For example, Hoffmans and Fung (1992) used chalk as a carrier to prepare *Salmonella* typhimurium inoculated low-moisture poultry feed for the extrusion apparatus. A shelf-life study was also conducted for ten other strains by using the same procedure and the results showed that these dry inoculums were very stable.

A study reported by Blessington et al. (2013) used sand which was coated with *Salmonella* cells as a dry carrier for almonds and walnut kernels inoculation. They concluded that the dry inoculation method is a useful, viable alternative for survival challenge studies, which effectively eliminate the need for the post-inoculation drying step.

Enache et al. (2015) used talc powder as a carrier for challenge studies. *Salmonella* Tennessee and *Enterococcus faecium* cells were harvested and inoculated onto talc powder after incubation and dried for 24 h at room temperature with a_w <0.55. The results showed that the dry inoculum prepared on talc powder was stable in terms of survival and thermal resistance in model peanut paste for at least 30 days.

Hildebrandt et al. (2017) compared a dry transfer inoculation of low-moisture spices by using silica beads and compared its relative efficacy to inoculation by using an aqueous suspension of cells. Inoculated silica beads were prepared by immersing the beads into *Salmonella* cocktail suspension and dried for 24 h at ambient room temperature. The survival of *Salmonella* during 220-day storage at ambient conditions was tested. The results showed that a dry transfer inoculation method resulted in a higher recovery of *Salmonella* than the aqueous inoculation method in clove and oregano.

In summary, carriers (such as chalk, silica beads, sand, talc powder, etc.) with a low cost and a powdered form have been used commonly. The biggest advantage of using these carriers is to reduce the cost when preparing a large amount of dry inoculum for bulk sample inoculation.

3.2. Dry inoculum with no carrier

Another approach for dry-inoculation is using dried bacteria without carriers (Stamp, 1947). Drying has been used for bacteria preservation during transportation, and it has been attempted with lots of organisms. Dried bacteria can be prepared by drying unfrozen inoculum in a vacuum desiccator or a freeze-dryer, spray dryer, or fluidized bed dryer (Hammer, 1911; Morgan, Herman, White, & Vesey, 2006; Stamp, 1947).

Perdana et al. (2013) compared the viability loss of *Lactobacillus* Plantarum WCFS1 prepared by single droplet drying, spray drying, and freeze-drying. Results showed that fast drying prevented dehydration inactivation and Weibull model can be used to predict the inactivation during all drying processes.

Larena, Melgarejo, and De Cal (2003) compared three drying methods, freeze-drying, spray drying, and fluidized bed drying on the viability of Penicillium oxalicum. 100% viability of Penicillium oxalicum was maintained after fluidized bed drying and freeze-drying. In fact, both spray drying and fluidized bed drying are widely used in large batch industry production for products, such as coffee, detergents, etc., whilst not for bacteria cells.

Table 1
Published studies using dry-inoculation methods for LMFs.

Product	Carrier	Microorganisms	Drying process	Reference
Almond meal	Talc powder	*Enterococcus faecium* NRRL-B2354	Dry in a biosafety cabinet for 24 h	Nurul Hawa Ahmad et al. (2019)
Non-fat milk powder	Silicon dioxide	*Salmonella* enterica Enteritidis PT 30, *Enteritidis faecium* NRRL B-2354	Dry overnight in a biosafety cabinet	Liu et al. (2019)
Clove powder, oregano leaves, ginger powder, ground black pepper	Silicon beads	*Salmonella* enterica serovars: Anatum 6802, Oranienburg 1839, Tennessee K4643, Enteritidis PT30,	Dry 24 h at ambient room conditions in a biosafety hood	Hildebrandt et al. (2017)
Sucrose	Sand	Five serotypes of *Salmonella* enterica: Agona strain F5567, Enteritidis strain 2415 (ATCC BAA-1045), Montevideo strain G4639, Tennessee strain K4643, Typhimurium DT104	Dry for 20–25 h at 22 °C	Beuchat et al. (2017)
Model peanut butter paste (50% fat, a_w 0.6),	Talc powder	*Salmonella* Tennessee, *Enteritidis faecium* NRRL B-2354	Dry at room temperature for 24 h to a final a_w <0.55	Enache et al. (2015)
Whole black peppercorns, cumin seeds	Silica sand	*Salmonella* enterica serovars: Tennessee K4643, Ball ARL-SE-085, d Johannesburg aRL-SE-013	Dry for 48 h to a_w 0.3	Bowman et al., 2015
Almond/walnut kernels	Sand	*Salmonella* enterica Enteritidis PT 30	Dry at 40°Cfor 24 h	Blessington et al. (2013)
Pecan nutmeats	Chalk stick	A five-serotype mixture of *Salmonella* enterica Anatum 6802, Enteritidis ATCC BAA-1045, Oranienburg 1839, Sundsvall 1659, Tennessee K4643	Chalk sticks were immersed in bacteria suspension, dried at 30 °C for 24 h, and pulverized into powder form	Beuchat and Mann (2011)
Low-moisture poultry feed	Chalk (calcium carbonate)	*Salmonella* Typhimurium, *Bacillus cereus, Clostridium perfringens, Escherichia coli, Enterobacteraerogenes, Lacobacillus plantarum, Listeria monocytogenes, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus faecalis*	Shelf-life test for 6 months Chalk was soaked in bacteria broth and dry at 37 °C for 3 days	Hoffmans and Fung (1992)
Wheat flour	No carrier	*Enteritidis faecium* NRRL B-2354	Freeze-drying at −80 °C for 48 h	Xu et al. (2017)
Nonfat dry milk, spinach powder, dry dairy mix	No carrier	20 *Salmonella* species isolated from foods	Freeze-drying	Flowers, Mozola, Curiale, Gabis, and Silliker (1987)
Meat and bone meal (a_w 0.60 to 1.0)	No carrier	*Salmonella* Typhimurium, *Salmonella* Senftenberg 775W	Dried at 40 °C under vacuum for 12 h	Riemann (1968)

Freeze-drying that removes water from the cells without viability losses has been used as an excellent method for long-term cell preservation. However, freeze-dried cultures are relatively expensive and this drying technique has volume limitations to produce in a large scale production compared with other drying techniques (Hoffmans & Fung, 1992; Larena et al., 2003). Flowers et al. (1987) used freeze-dried *Salmonella* cell pellets to inoculate dry powdered, granulated, or semi-solid products (e.g., chocolate and peanut butter) for rapid detection of *Salmonella* in foods. To make a homogenous inoculation, freeze-dried cell pellets were ground first and inoculated with a small amount of sample, and then used as a "seed" inoculum for a larger amount of inoculation. The semi-solid food samples, chocolate, and peanut butter were melted at 45 °C before inoculation.

Xu, Liu, et al. (2018) developed a freeze-dried surrogate inoculum for thermal inactivation studies and further applied it for validation of radio-frequency pasteurization. The protective effect of different protectants was evaluated, and the freeze-dried surrogate had a stable shelf life. The developed dry inoculum was used as a qualified surrogate of *Salmonella* for thermal processing validation.

4. Advantages of dry-inoculation methods

4.1. An easy mix with LMFs

Wet-inoculation methods normally require hand mixing or massaging until clumps are not observed, which is time-consuming for a large-scale sample preparation. A uniform distribution of wet culture in some high-sugar or high-fat LMFs (such as honey, syrup, peanut butter, and nuts paste, etc.) is even more challenging due to their high viscosity. For oil-enriched foods, the hydrophilic bacteria culture does not mix well with hydrophobic products and the mixing step might cause formation of emulsions after vertexing. On the other hand, dry inoculum, with a low level of initial a_w, can be mixed with LMFs easily and homogenously distributed in the powdered LMFs, such as flours, grains, sugars, milk powder, spices, etc.

4.2. Minimum impact on LMFs

When using a wet inoculation method, the physical properties of LMFs might be modified due to infused water which induces the powder to clump (Aguilera et al., 1995; Kimber et al., 2012; Palipane & Driscoll, 1993). Liu et al. (2019) reported that non-fat milk powder after inoculating with wet inoculum was hardened and the experimenter had to pulverize the inoculated sample by using a grinder. Even though many of the LMFs can re-invigorate by removing the moisture through evaporation during drying, the addition of moisture, for some other dry ingredients, is irreversible in terms of texture and sensory attributes. For example, high sugar-content products (such as sugar granulates, honey powder, onion powder) are very sensitive to moisture, and the introduction of moisture will let the sugar dissolve partially or completely depending on how much wet material was added. Onion and garlic powder (initial moisture content 4–5% d.b.) harden soon after absorbing water from the environment. If an isothermal test was performed with a similar powder, dry inoculum is better to be applied than wet inoculum. Also, the use of dry inoculum could prevent the release of water-soluble antimicrobials for spices, which may artificially reduce microbial numbers without processing (Bowman et al., 2015; Vanderzant & Splittstroesser, 2015; Waje, Kim, Kim, Todoriki, & Kwon, 2008).

4.3. Reduced post-inoculation drying time

LMFs after inoculating with wet inoculum should be equilibrated back to low a_w, as described in section 2.2. For wet-inoculated fat-humectant systems (such as peanut butter and chocolate liquor), the conditioning step of wet-inoculation is time-consuming since the moisture transmission in emulsions is very slow (Tiemstra & Tiemstra, 1974). For some products such as nuts, temperature and relative humidity may affect the length of post-inoculation drying and may also modify the properties of kernel surfaces (Blessington et al., 2013; Enache et al., 2015; Moussavi, Lieberman, Theofel, Barouei, & Harris, 2019). In this case, the post-inoculation drying steps should be shortened or even eliminated to reduce the influence of drying on the thermal properties of tested microorganisms.

In contrast with wet inoculation, the condition time necessary for samples inoculated with dry-inoculum is much shorter. In some cases, the food matrices can be pre-equilibrate to the target a_w before inoculation, and thus, the isothermal study can be conducted immediately or on the same day after inoculation.

4.4. High stability and consistency

Wet inoculum prepared by using different methodologies or persons behaves varied in desiccation tolerance during post-drying as well as heat resistance during thermal processing, resulting in large variations in the results from different testing locations (Wiertzema et al., 2019). For instance, D_{80}-values of *Salmonella Enteritidis* PT30 in wheat flour varied from 3.8 min to 8.4 min based on five different culture preparation protocols (Hildebrandt et al., 2016; Villa-Rojas, 2015). Even the same culture preparation method is followed, factors such as the media for enumeration, temperature vibration in the incubator, and individual technical difference can also result in the different characteristics of bacteria prepared from different batches. This discrepancy caused by methodology will cause problems when choosing the accurate *D*-value of microorganism that used as a guidance for thermal process design.

Dry inoculum has a longer shelf life comparing to wet inoculum due to the absence of water addition. Bowman et al., 2015 compared one dry inoculation method with three wet inoculation methods and found that the inoculation methods influenced the recoverability of *Salmonella,* and the dry transfer method was the most stable strategy. Similarly, population reductions of *Salmonella* Enteritidis from inoculated sand after 30 days were around 1 log CFU/g on almonds and walnuts (Blessington et al., 2013), and comparable reductions were also observed in peppercorns and cumin seeds (Bowman et al., 2015). Also, dry inoculum can be prepared in a large volume and preserved at a high concentration. Therefore, a dry inoculum can be prepared in one large batch and can be used for multiple challenges studies at different times and locations, which prepares comparable data between labs.

4.5. Suitability for LMFs contamination by dry events

LMFs may be contaminated anywhere along the production chain, and bacteria may enter LMFs in a facility and carried by wet or dry vehicles (Bowman et al., 2015; De Roin, Foong, Dixon, & Dickson, 2003; Hildebrandt et al., 2017). Bacteria show increased thermal resistance when using a dry transfer to low-a_w foods. Thus, inoculated foods prepared by the dry-inoculation method provide conservative results in the subsequent challenge studies and process validation.

To sum up, dry-inoculation methods are stable, easier, and faster, which is imperative for academic/industrial application in designing standard protocols for inactivation studies especially for LMFs.

5. Possible limitations of dry-inoculation methods

5.1. Thermal resistance alteration

A dry inoculum might not have comparable characteristics to a wet inoculum, resulting in different *D*-values of strains prepared by different inoculation methods. As mentioned previously, a dry inoculum is commonly prepared by prior exposure to a low-a_w environment, such as drying in the ambient at room temperature for 24 h or even longer. This drying step, allowing bacteria expose to multiple stressors, such as desiccation and starvation, can improve the survival or thermal

resistance of bacteria.

Liu, Rojas, Gray, Zhu, and Tang (2018) observed significantly increased thermal resistance of *Enterococcus faecium* using silicon dioxide as a carrier, which might be caused by the adaption of microorganisms during post-drying. For freeze-dried inoculum, the freeze-drying process might cause cell death and injury because of the changes in the physical state of the membrane lipids and protein damage or protein denaturation. Xu et al. (2017) reported that freeze-dried *Enterococcus faecium* without a protective agent had a smaller *D*-value compared to its aqueous form. However, injured cells retain their viability upon resuscitation under a nutritionally adequate environment (Bretz & Kocka, 1967). Addition of the protective agents can reduce the number of dead cells, which can protect bacterial cells during the freeze-drying process and further storage (Abadias, Benabarre, Teixido, Usall, & Vinas, 2001). Xu et al. (2017) explored the use of microbial freezing buffer and skim milk to generate freeze-dried inoculum. However, the addition of cryoprotective agents on the influence of bacterial thermal resistance should also be further discussed.

5.2. Special storage condition

Bacterial dry inoculum should be stored at a condition with limited exposure to moisture. Therefore, dried bacteria should be stored properly to have better survivability before isothermal tests. For example, freeze-dried inoculum is very sensitive to moisture and temperature. The storage conditions (i.e. isolation from moisture, oxygen, and storage temperature fluctuation) can influence the survivability (Miyamoto-Shinohara, Sukenobe, Imaizumi, & Nakahara, 2008; Sinskey & Silverman, 1970). Similarly, dry inoculum prepared with a carrier might also tend to absorb moisture from the environment if it was not packaged well. A special package condition, such as vacuum packing, is needed for long time storage. The physical properties, such as moisture content, should be monitored before tests.

5.3. Not applicable for bulk solid LMFs

Dry inoculum is suitable for powdered LMFs, and not for bulk solids, including chocolate, dry dog food kibbles, and herb leaves, since the dry inoculum in a powder form cannot be easily embedded in or attached to the surface of those products, causing poor distribution and uniformity. Inoculating the surface or immersing the solid LMFs into bacteria broth is more suitable in these circumstances. For some food blocks with less heat sensitivity, such as nut kernels, black peppercorn, a wet inoculation method is a better choice since they can be soaked in bacteria broth and then air-dried for isothermal tests with a better uniformity (ABC, 2014; Kim, Sagong, Choi, Ryu, & Kang, 2012). Also, for herb leaves, a dry inoculum is hard to uniformly attach the surface compared with liquid culture, however, the use of wet culture will cause agglomeration and stickiness when spraying the wet bacteria onto herbs. Moreover, the wet inoculum will let the bacteria expose to water-soluble antimicrobial compounds, which will generate extra lethal effects on the bacteria (Hildebrandt et al., 2017; Shelef, 1984).

5.4. Other concerns

The difference in density between bacteria and carriers will cause possible non-uniform distribution of the bacteria in the inoculated sample, resulting errors for sampling and further detection. Moreover, the addition of the carriers might change the heat transfer of the treated sample during thermal processing. Even though the amount of the carriers is typically small compared with the total weight of the inoculated sample, the addition of inoculated carriers with different ratios might affect the time required to reach the target temperature (come-up-time) during isothermal tests. The addition of carriers on altering the isothermal behaviors (a_w change with temperature) on inoculated foods is not yet known.

Liu, Tang, et al. (2018) determined the absorption isothermal curves of silicon dioxide and found a_w does not change with temperature. However, for other carriers (such as sand, glass beads, chalk, talc powder, etc.), these influential factors, such as density, isothermal properties, or inoculation radio, on the results of isothermal tests have not been explored systematically. Using directly dried inoculum is the most promising one, but the cost is high for scaling up. However, this issue might be solved by adding a cryoprotective agent to increase the mass residue after drying (Xu et al., 2017).

6. Comparison of wet-and dry-inoculation methods

Laboratory inoculation of LMFs commonly involves a suspension of the pathogen in liquid followed by a post-drying process. The advantages of using wet-inoculation method still exist: 1) easy to mix with liquids, such as buffers solutions prepared at low a_w levels, syrup, liquid honey, etc.; 2) wet culture can be prepared in a large volume and inoculated with samples in a large scale; 3) most of the culture preparation procedures are well-documented and thus, a direct comparison with the published data is available. Wet inoculation methods are widely used for inactivation or process validation using thermal and non-thermal technologies for LMFs, such as isothermal treatment (Hildebrandt et al., 2016; Liu, Rojas, et al., 2018), pulsed light-Emitting Diode treatment (Subedi, Du, Prasad, Yadav, & Roopesh, 2020), radio-frequency pasteurization (Liu, Ozturk, et al., 2018; Rossana; Villa-Rojas et al., 2017; Xu, Yang, Jin, Barnett, & Tang, 2020), cold plasma treatment (Chaplot, Yadav, Jeon, & Roopesh, 2019), extrusion (Bianchini et al., 2014), etc. A summary of advantages and disadvantaged of wet and dry-inoculation methods for LMFs are tabulated in Table 2. However, this review mainly focuses on addressing dry-inoculation methods and a comprehensive discussion of wet-inoculation methods falls outside the scope of this paper.

7. Rationale of using dry inoculum

7.1. Morphological changes of dried bacteria

Studying the morphology of bacteria after drying is a direct way to visualize their structure changes. *Salmonella* Enteritidis PT30 (*S.* Enteritidis) and its surrogate *Enterococcus faecium* NRRL B-2354 (*E. faecium*) were examined under scanning electron microscopy and their

Table 2
Comparison of wet and dry-inoculation methods for LMFs.

	WET INOCULATION	DRY INOCULATION	
		With carrier	No carrier
Advantages	Existed protocol for culture preparation	Easy to inoculate with powdered LMFs, easy to transport, shelf-life stable, no extra moisture	
	Can prepare in a large volume	Can prepare in a large volume	No further condition after inoculation
	Work well with solid LMFs	Cost-effectiveness	
Disadvantages	Change properties of LMFs	Not suitable for solid LMFs, such as chocolate, dry dog food kibbles, herb leaves, etc.	
		Special storage conditions	
	Extended post-inoculation drying time	Extra drying time	High cost
	Difference in properties from batches	Thermal properties altered	Hard to prepare in a large amount
	Water-soluble antimicrobial compounds	Might influence the heat transfer	Sensitive to oxygen and moisture

microstructures were compared between liquid and dry forms (Fig. 3). Bacteria in dry forms were prepared by drying on silicon dioxide (Fig. 3, B and E) or freeze-drying (Fig. 3, C and F). *E. faecium* are gram-positive cells with coccus shape and appeared in pairs and chains (Fig. 3, A). Morphology changes of *E. faecium* were neither observed after dehydrating on silicon dioxide (24 h) (Fig. 2, B) nor after freeze-drying (Fig. 3, C). This can be explained by the thick cell envelope of gram-positive cells. *S.* Enteritidis are rob shape gram-negative cells with slime layers as formed biofilm (Fig. 3, D). *S.* Enteritidis can attach to the surface of silicon dioxide and showed a smooth and flat surface compared with its liquid form (Fig. 3, E). However, the structure of S. Enteritidis cells are well-maintained after freeze-drying (Fig. 3, F). Besides, the survivability of bacteria after desiccation by both methods was very high, as reported by Liu, Tang, et al. (2018); Xu, Shah, Song, & Tang (2020).

7.2. Equilibrium among foods, bacteria, and surrounding air

Several studies have investigated the influence of a_w on the thermal resistance of bacteria in different matrices (Riemann, 1968; Syamaladevi, Tadapaneni, et al., 2016; Villa-Rojas et al., 2013; Xu, 2019; Xu, Tang, et al., 2018). More recent evidence reals that a_w is a decisive parameter influencing the thermal resistance of bacteria in LMFs (Liu, Rojas, et al., 2018; Syamaladevi, Tang, Villa-Rojas, Sablani, Carter, Campbell, 2016; Xu, Tang, et al., 2018). Notably, limited studies have published on investigating how the bacteria will adjust to its surrounding environment during conditioning or thermal treatments. It is generally accepted that a_w of a single bacteria cell cannot be directly adjusted through conditioning. Yet, it can be easily controlled by adjusting their micro-environments by changing the a_w of the food matrices. The basic mechanism underneath is that the small physical size of bacteria cells allow them to equilibrate to its surroundings in a very fast way (Syamaladevi, Tang, & Zhong, 2016). At equilibrium, the a_w of the bacteria is regarded as the same as that of a low-moisture environment. In this case, the a_w of bacteria can be controlled or adjusted once the a_w of the food environment has been controlled.

The low-moisture environment of bacteria exposed to is LMFs. The a_w of LMFs can be adjusted by controlling the relative humidity (RH) of the surrounding air, since at equilibrium, the a_w of the conditioned sample is equal to the RH of the surrounding air at a certain temperature. All the isothermal studies are based on the assumption as illustrated in Fig. 4:

$$\textbf{a_w of bacteria} = \textbf{a_w of foods} = \textbf{RH of surrounding air @ equilibrium} \quad (3)$$

The second part of the equation is based on the definition of a_w, while the first part of the equation needs to be re-considered by understanding

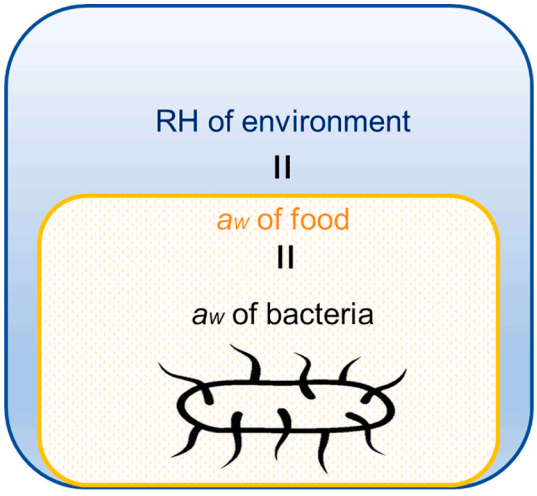

Fig. 4. A state of equilibrium is reached when the ratio between partial pressure of water in each system (air, food and bacteria) and the partial pressure of a standard state (pure water for water activity and equilibrium vapor pressure for RH) is the same.

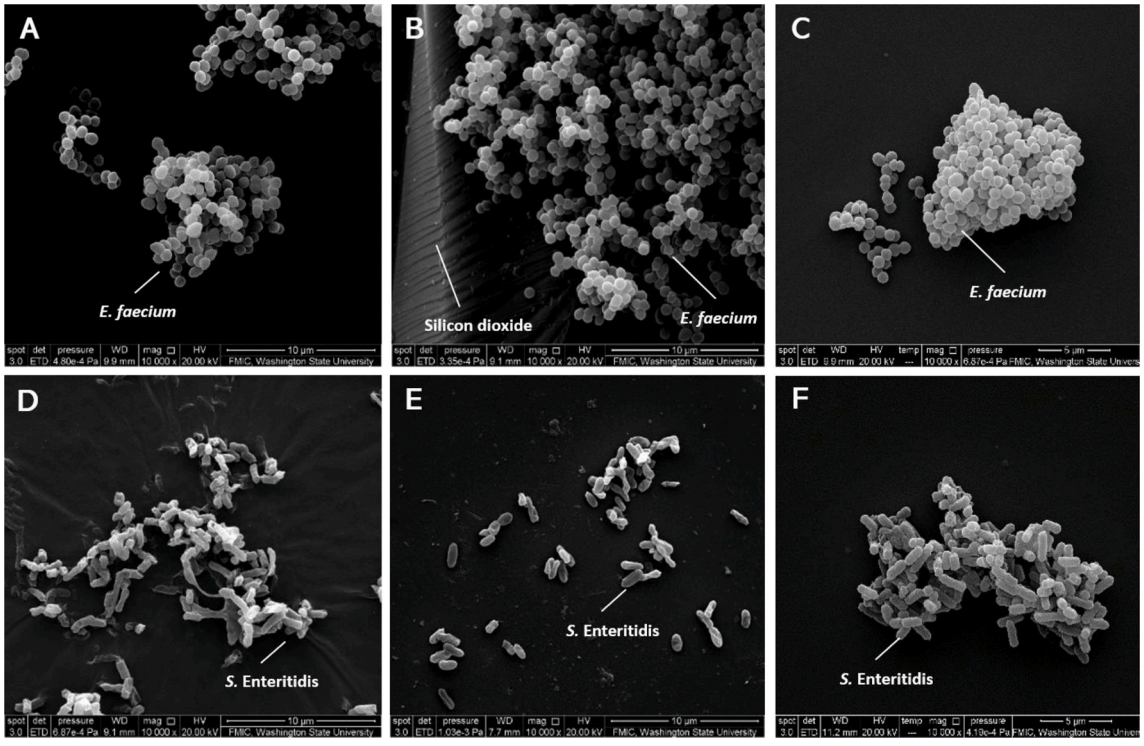

Fig. 3. *E. faecium* and *S.* Enteritidis cells under scanning electron microscopy at 10 K magnification. Scale bar = 10 μm. (A) Liquid *E. faecium,* (B) *E. faecium* dried on silicon dioxide, (C) freeze-dried *E. faecium,* (D) Liquid *S.* Enteritidis, (E) *S.* Enteritidis dried on silicon dioxide, (F) freeze-dried *S.* Enteritidis.

the discrepancy of adsorption/desorption isotherms of pure bacteria and food matrices. The isotherms of bacteria reveals how bacteria react with moisture and how their a_w will change with temperature. Most studies tend to focus on isotherms of food matrices rather than bacteria, and the adsorption behavior of pure bacteria is till poorly understood (Al-Muhtaseb, McMinn, & Magee, 2002; AquaLab; Aviara, 2020; Labuza & Hyman, 1998; Lemus, 2011).

In real industrial operations, there are two main possibilities of the interaction of foods with an exposed environment. The first category is open systems where treated foods are fully exposed to their environment (e.g. roasting, drying, and baking), the moisture content of the foods will change as the processing progresses. The other category is close systems in which foods are heated in sealed containers, where the moisture content of the foods remains constant, while the a_w within the foods always changes with temperature. In this case, the understanding of how bacteria react with the dynamic changes of moisture and a_w during a thermal process will be of vital importance to explain the different thermal resistance in different food matrices.

The isotherms of gram-positive cocci, *Enterococcus faecium* NRRL B-2354, a valid surrogate for *Salmonella* Enteritidis PT30, has been generated by Syamaladevi, Tang, & Zhong (2016). This is the first report on the water diffusion behavior of pure bacteria. The a_w of bacteria increased considerably as temperature increased from 20 °C to 80 °C and the results suggested that a single bacterium cell can equilibrate with the environmental humidity and temperature in a very fast way (within seconds). That means, during the condition period of food samples, the *E. faecium*'s a_w can be regarded as equal to the food matrix, which supports the evidence of the first part of equation (3). However, this study discussed mainly on single-layered gram-positive bacteria in a spherical shape. A systematic study on gram-negative bacteria with other shapes, such as *Salmonella* and *Listeria* has not been validated yet. Moreover, the influence of biofilms and cell clumps or aggregation during treatment have not been considered as well.

8. Conclusions

Dry-inoculation methods provide a promising approach for LMFs inoculation. Benefits that accrue from dry inoculum are easy inoculation, uniformed distribution, sustainability, and short post-inoculation drying. Further, the same batch of dry inoculum can be used for multiple challenge studies at different times, different facilities, or both. The dry inoculum does not introduce extra moisture to the inoculated products, and the inoculum is shelf-stable and easy for transportation. When the LMFs are contaminated by fine particulates, the use of dry inoculum will reproduce more accurate data compared with wet inoculum. However, if the LMFs are contaminated by wet events, the potentially increased thermal resistance of dry inoculum (due to post-inoculation drying or the addition of protective agents) might yield overly conservative results, resulting in an extra cost in food processing. The thermal resistance of dry inoculum after preparation should be tested and directly compared with that of wet inoculum before further applications. The main goal is to develop a dry inoculum with higher or at least equal thermal resistance compared to the wet inoculum.

Declaration of competing interest

There is no conflict of interest among Authors and Co-authors and have no conflict of interest to declare.

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