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Review

Recent development in low-moisture foods: Microbial safety and thermal process

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ABSTRACT

Foodborne outbreaks and recalls of pathogen-contaminated low-moisture foods (LMFs, foods with water activity at 25 °C < 0.85) have led to numerous scientific studies on bacterial persistence, as well as newly developed industrial interventions. Conducting microbial tests of LMFs, lab tests, or validation studies in pilot plans requires complete information on protocols and parameters that need to be aware of—in particular, understanding how factors influence the thermal resistance of bacterial pathogen in LMFs is critical in designing any thermal processes. This review provides detailed information on the general protocols of microbial studies of LMFs: from pertinent pathogen identification to microbial validation studies. In particular, it reviewed the detailed procedures (e.g., lawn-harvest method), analytical protocols (e.g., recovery and enumeration of pathogens in LMFs), and specialized tools that have been utilized (even widely accepted) in laboratory-based microbial studies of LMFs. It also summarized the factors that influence the microbial validation studies. This article could support the intervention of existing pasteurization processes in the LMF industry, promoting the microbial safety of LMFs.

1. Introduction

Low-moisture foods (LMFs) have water activity at 25 °C ($a_{w,25°C}$) <0.85 (Food and Drug Administration (FDA), 2014a). Under this condition, most bacteria cannot grow to cause illness, spoilage, or produce toxins (The Association of Food Beverage and Consumer Products Companies, 2009; Scott, 1957). However, the inability of bacteria to reproduce at a low $a_{w,25°C}$ does not mean that they cannot survive in LMFs. Pathogenic bacteria such as *Salmonella enterica*, *Listeria monocytogenes*, and *Cronobacter* can remain viable in LMFs for months or even years and cause illness when consumed (Burnett, Gehm, Weissinger, & Beuchat, 2000; Limcharoenchat, James, & Marks, 2019; Zhu, Song, &Tsai, 2021).

Pathogens in LMFs pose a potential health risk, especially for sensitive individuals such as the elderly (Ly, Parreira, & Farber. 2019). Over the past decade, foodborne illness outbreaks from LMFs have been associated with *Salmonella* spp. (found in wheat flour, peanut butter, chocolate, milk powder, crackers, almonds, infant cereals, spices, and dried fungus), *Bacillus cereus* (rice, nuts, herbs, and spices), *Cronobacter sakazakii* (powdered infant formula), *Clostridium* spp. (herbs and spices), Shiga toxin-producing *Escherichia coli* strains (flour, walnuts, almonds, rice, and seeds), and *Staphylococcus aureus* (rice, seeds, nuts, and almonds) (McCallum et al., 2013; Medus et al., 2009; CDC, 2019; Rodríguez-Urrego et al., 2010; Van Doren et al., 2013). In processing and handling of any contaminated LMFs, serious food safety risk might occur when the cross-contamination happens and transfers pathogens to ready-to-eat foods (no additional inactivation step) or high-moisture foods under adequate temperature and pH range (so the bacteria will grow) (Reji, & Aantrekker, 2004; Podolak et al., 2010).

The food industry urgently needs interventions to effectively control those pathogens in LMFs. Thermal processing is one of the most effective methods for inactivating undesirable microorganisms in foods (Silva et al., 2012). Yet there has been a general lack of fundamental understanding related to quantitative relationships between thermal resistance of pathogens in LMFs and intervention parameters. LMFs originated from agriculture products have different levels of bacterial contamination, depending on the harvest methods (collecting from ground vs. mechanical harvesting) and the drying practices (sun drying

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on open mats vs. in-door drying in a sanitary environment) (Podolak et al., 2010). It is, therefore, essential to conduct risk assessments to determine the appropriate pasteurization requirements that take into consideration the initial bacterial counts and the intended uses of the final products. For instance, the 5-log reduction of target pathogen in LMFs was recommended for most dry products (FDA, 2009, 2011a,b), yet higher log reductions are required for dried sprouted seeds (Keller et al., 2018).

This article aims to identify the critical aspects that should be considered in LMF safety and validation study protocols, focusing on how different intrinsic and extrinsic parameters influence pathogen resistance in LMFs. Since most early studies on thermal inactivation of pathogens in LMFs directly followed the approaches used in developing and validating thermal processes for high moisture foods, the well-established protocols for thermal inactivation of pathogens in high-moisture foods were first reviewed. Based on these protocols, the critical elements of microbial safety and process controls in thermal processes were generally outlined. The literature on thermal inactivation of pathogens in LMFs was surveyed, and how the data are used in developing and validating thermal pasteurization of LMFs was discussed. This review contributes to the current knowledge of microbial studies and thermal processes of LMFs, as it could support the methods of conducting microbial studies and promote the microbial safety of LMFs.

1.1. Microbial study of LMFs: Salmonella contamination in almonds

Concerns regarding the safety of LMFs were exacerbated by the *Salmonella* outbreak in raw almonds grown in California during the winter of 2000–2001 (Chan et al., 2002). This first *Salmonella* outbreak in almonds had caused 168 laboratory-confirmed cases of salmonellosis and, most importantly, led to a series of almond product recalls in North America and a review of current practices in the almond industry. In 2007, the Almond Board of California (ABC) recommended *E. faecium* B2354 as a *Salmonella* surrogate and the validation of the blanching, dry roasting, and oil roasting processes in whole almonds (ABC, 2007a,b,c). The original guidelines for using *E. faecium* B2354 were extended in 2014 (ABC, 2014) with expanded content (from 3 to 12 pages) on the microbial methods for growth, heat resistance, recovery, enumeration, and validation of a surrogate strain on almond surfaces. This collaborative action is an outstanding example of how the industry reacts to foodborne pathogen outbreaks and recalls. These guidelines spearheaded the development of relevant industrial guidelines for pistachios and peanuts (FDA, 2011a,b).

The ABC guidelines are only applicable for controlling *Salmonella* in nut kernels, which have many limitations regarding alternative pathogens or LMFs. For instance, a heat resistance test is required to verify the status of *E. faecium* on almond surfaces. Dried inoculated almonds (25 g) are spread on a metal mesh tray and placed in a convection/forced air oven (a gravity oven is not allowed) at 138 °C for 15 min. After the heat treatment, *E. faecium* (as the surrogate for *Salmonella*) with $\leq$ 2.5-log reduction was considered acceptable (ABC, 2014). These guidelines do not apply to other LMFs with different particle sizes and shapes, such as date pastes and flours. The heating method is unsuitable for pastes and powders as the materials will cake or create dust inside the convection oven at 138 °C. Moreover, the guidelines do not specify air circulation rate, and nor consider that the moisture content of almond kernel would change with different cavity design and air circulation rates inside a convection oven. The cooling and recovery steps of *E. faecium* after heat treatment were also not specified. But the microbial laboratory protocols (such as the agar lawn growth method that uses agar plate to culture bacteria) are widely accepted and applied in different LMFs as standard operation procedures (ABC, 2014). Derived from the ABC guidelines mentioned above, alternative guidelines were published regarding baking (OpX leadership Network, 2016) and LMFs in general (Anderson & Lucore, 2012) to provide practical assistance to food facilities of any size.

Although different guidelines have been developed to ensure the microbial safety of a limited number of LMFs, from risk assessment to final validation, more information is needed to fill the knowledge gaps regarding the wide range of LMFs, their physicochemical properties, and possible thermal treatments/technologies.

A general procedure for the microbial validation of the thermal processing of foods is presented in Fig. 1. Microbial validation studies of LMFs shared the same procedures but vast different real-life situations with those of high-moisture foods. This section reviews the critical steps in the thermal pasteurization of LMFs that differ from those of high-moisture foods.

1.2. Identification of the pertinent pathogen in LMFs

The pathogenic bacteria that have been associated with outbreaks or recalls in different LMFs from 2009 to 2019 are summarized in Table 1. For instance, high counts of *B. cereus* ($\geq 10^5$ CFU/g), *C. perfringens* ($\geq 10^3$ CFU/g), and *E. coli* ($\geq 10^2$ CFU/g) were reported in retail spices and herbs sold in the United Kingdom (Sagoo et al., 2009). Notably, spices imported into the U.S. are 1.9 times more likely to contain pathogens than other imported foods (Gurtler & Keller, 2019a). Potential pathogens (*B. cereus*, *S. enterica*, and *Cronobacter*) and spore-formers in LMFs have been reviewed by Ly et al. (2019). Among these, *Salmonella* is implicated in most outbreaks (45%) and has been the most investigated microbial hazard (Young et al., 2015). *Salmonella* exhibits high thermal resistance in low-moisture environments (Finn, Condell, McClure, Amézquita, & Fanning, 2013). It can infect people even as low as one cell, depending on age and health of the host and strain differences among members of the genus (FDA, 2012). Consequently, *Salmonella* in LMFs has been identified as a hazard by the Grocery Manufacturers Organization (Barhoumi, Maaref, & Jaffrezic-Renault, 2010) and FDA (2016c). Presently, *Salmonella* remains the most concerning foodborne pathogen to public health owing to its persistent survival in LMFs and facility surfaces (Margas, Meneses, Conde-Petit, Dodd, & Holah, 2014), as well as its attribution to the highest numbers of outbreaks (The Association of Food Beverage and Consumer Products Companies, 2009; Van Doren et al., 2013).

Several Shiga-toxigenic *E. coli* (STEC) infections were reported in salami and raw cookie dough (EFSA, 2011.; Neil et al., 2012.). For instance, a multistate outbreak with 54 cases of O26 and O121 *E. coli* infections and 21 cases of O26 *E. coli* infections were linked to flours (Crowe et al., 2017; FDA, 2019). Recalled products may have remained in homes and caused illnesses even after the recalls, owing to the extended shelf life of LMFs (FDA, 2019). In addition, infections with STEC can be more severe than *Salmonella* (salmonellosis is frequently self-limiting) because it may result in hemolytic uremic syndrome, severe kidney damage, thrombotic thrombocytopenic purpura, and other such subsequent health issues (FDA, 2012).

A recall owing to a severe and systemic form of listeriosis is also documented in Table 1. *L. monocytogenes* exhibits prolonged survival in almond kernels (Kimber, Kaur, Wang, Danyluk, & Harris, 2012), wheat flour (Taylor, Tsai, Rasco, Tang, & Zhu, 2018), and infant formula (Koseki & Nakamura, 2015). In addition, *L. monocytogenes* was documented with much higher case fatality rates (20–30%) than those of *Salmonella* serovar Newport (0.3%) and Typhimurium (0.6%) (Jones et al., 2008). Detailed information on the prevalence, survival, and thermal resistance of *L. monocytogenes* in LMFs has been recently reviewed (Taylor, Quinn, & Kataoka, 2019).

Powdered infant formula is also at risk for the presence of *Cronobacter sakazakii* (previously known as *Enterobacter sakazakii*) (Quinn, 2020). In an infant formula with an $a_{w,25°C}$ of 0.28, for one year, *C. sakazakii* only showed 0.72 $\pm$ 0.05, 1.29 $\pm$ 0.16, and 2.41 $\pm$ 0.22-log reductions (with 5- to 5.4-log initial population) at 5 °C, 22 °C, and 35 °C, respectively (Koseki & Nakamura, 2015). Compared with the reductions in *S. enterica*, *L. monocytogenes*, and *E. coli* O 157:H7 (Koseki & Nakamura, 2015), *C. sakazakii* had the highest survival following

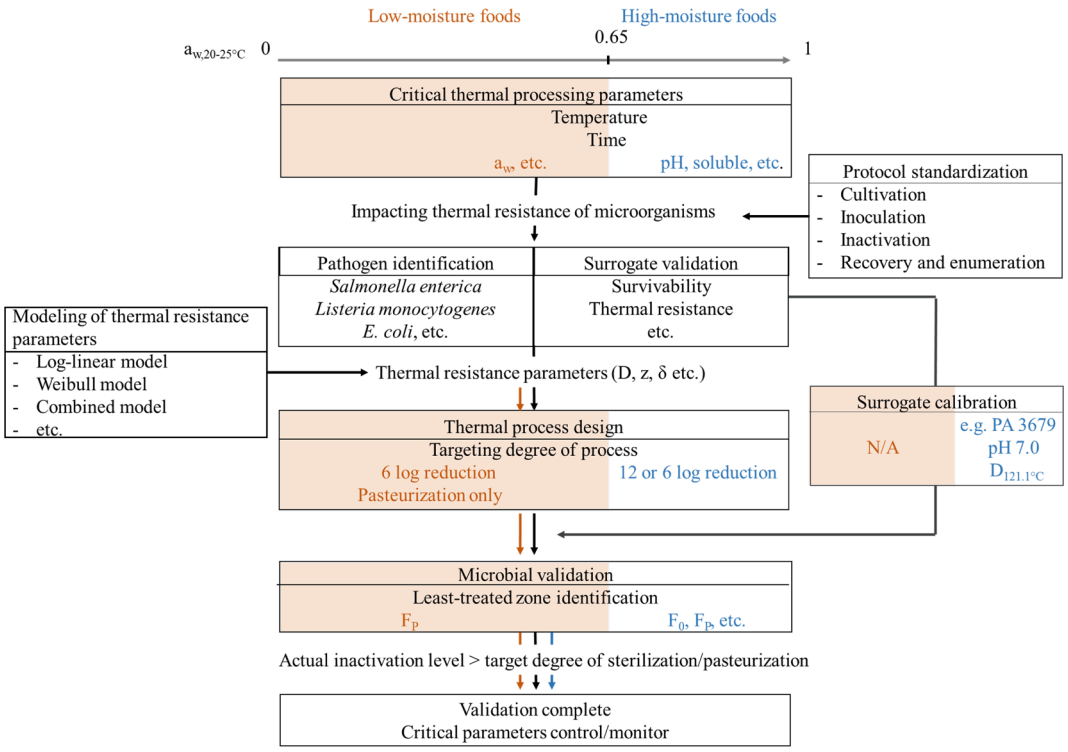

Fig. 1. Microbial validation of thermal processing of low-moisture foods (left) and high-moisture (right) foods.

desiccation stress in powdered infant formula. Therefore, *C. sakazakii* is another foodborne pathogen of concern in LMFs.

The pertinent pathogen is highly related to food systems. There is no "universal" pertinent pathogen for microbial studies of LMFs. Survival and resistance studies are necessary to qualify the bacterial performance in a given food system.

1.3. Surrogate selection and validation in LMFs

Surrogate is defined as the non-pathogenic bacteria with equal or higher thermal resistance as the target pathogen (Busta et al., 2003). Surrogates could be used to evaluate the inactivation efficacy of the target pathogen in specific thermal processes, given the fact that the target pathogen is prohibited in food processing lines. The non-pathogenic serotypes from the same family of pathogenic bacteria may be good surrogate candidates. For instance, *Listeria innocua* M1 serves as a *Listeria monocytogenes* surrogate in validating thermal processes (Friedly et al., 2008). Non-pathogenic bacteria (from environmental screening or existing surrogates for other pathogens) can also serve as surrogates. For instance, the non-pathogenic bacterium *Enterococcus faecium* NRRL B-2354 (Kopit et al., 2014) is a valid surrogate for *L. monocytogenes* in ground beef (Ma et al., 2007) and *E. coli* O157:H7 in apple cider (Piyasena, McKellar, & Bartlett, 2003). Desirable microbial characteristics of the potential surrogates are summarized by Busta et al. (2003). Quantitative information is needed to support the use of microbial surrogates in the development and validation of specific decontamination processes.

The selection and identification of a surrogate microorganism for a pathogen highly depend on their similarity in survival and heat resistance (Fig. 1). The general rules and methods in identifying possible surrogate bacteria under specific conditions were reviewed by Hu et al. (2017). In LMFs, specific serotypes of *Salmonella* (e.g., Enteritidis, Agona, Tennessee) (CDC, 1998, 2004, 2007) have shown robust survivability and thermal resistance. Since most *Salmonella* strains are considered human pathogens, it was difficult to find such highly heat-resistant surrogates in low-moisture environments. Validated surrogate organisms for LMFs were summarized by Theofel, Yada, and Harris (2019).

To date, all published data support the conditional usage of *E. faecium* B2354 as a *Salmonella* surrogate in the heat treatment of tested products (Wei, Agarwal, & Subbiah, 2020; Bianchini et al., 2014; Liu, Villa-Rojas, Gray, Zhu, & Tang, 2018a; Tsai et al., 2019). Hu and Gurtler (2017) compiled most of the bacterial pathogens that can be substituted by the surrogate *E. faecium* B2354 under certain conditions. Independent from food matrices, *E. faecium* B2354 also exhibited consistently higher $D_{80°C}$ values than *Salmonella* Enteritidis at $a_{w,\ 80°C}$ 0.13–0.72 in a desiccated environment i.e., silicon dioxide granules (Liu, Tang, Tadapaneni, Yang, & Zhu, 2018b).

Besides *E. faecium* B2354, an alternative generally recognized as safe (GRAS) bacterium, *Pediococcus acidilactici* ATCC 8042, was evaluated as a surrogate for *Salmonella* in toasted oats cereal and peanut butter (Deen & Diez-Gonzalez, 2019) and was found to exhibit similar heat resistance as that of *E. faecium* B2354 at test temperatures of 85–95 °C. Because of its GRAS status, *P. acidilactici* is easier to employ than *E. faecium* B2354 in LMFs.

As using *E. faecium* B2354 and *P. acidilactici* as *Salmonella* surrogates in thermal processes is food matrix-dependent, further validation studies are required before their applications as appropriate surrogates. Even in the same product undergoing the same thermal treatments, variation in data from heat resistance tests may still occur owing to inconsistencies in experimental protocols, strain variability, inaccurate recording of alternative parameters (e.g., $a_{w,25°C}$, moisture content, and food particle sizes), and the use of different modeling or analytical methods for describing inactivation kinetics. Given that pertinent pathogen and their heat resistance are highly associated with the food system, validation of surrogate microorganisms is also case-to-case. *E. faecium* appears to be a good choice because it was documented with higher heat resistance than most pathogens in various LMFs.

Table 1
Pathogens reported in different LMFs and correlated information (2007–2020).

Genus	Serotype	LMF	Consequence	Reasons of contamination	References
	Duisburg and Urbana	Cashew Brie	7 illnesses from 3 states	The cashews are likely the source	(FDA, 2021)
	NA	Dried fungus	43 individuals infected from 10 states	N/A	(FDA, 2020) (Administration, 2020)
Salmonella	Mbandaka	Cereal	100 individuals infected from 33 states	N/A	(CDC, 2018a)
	Typhimurium	Dried coconut	14 individuals infected from 8 states	N/A	(CDC, 2018b)
	Tennessee	Peanut butter	628 individuals infected from 47 states	Unknown. The products were from the same manufacturing plant.	(CDC, 2007)
	Typhimurium	Peanut butter	529 individuals infected from 43 states	A single facility at Blakely, Georgia, was associated with producing *Salmonella* contaminated peanut butter.	(Medus et al., 2009)
	Typhimurium	Commercial vegetable-coated snack food	69 individuals infected from 23 states; 93% were aged 10 months to 3 years old.	Contamination was suspected of having occurred after the final-lethal processing step, when powdered ingredients were added.	(Sotir et al., 2009)
	Agona	Puffed cereal	28 individuals infected from 15 states.	N/A	(CDC, 2008)
	Montevideo, Senftenberg	Pistachios	11 individuals infected from 9 states	Outbreak strains of *Salmonella* were found in the production facility.	(CDC, 2016a)
	Enteritidis	Turkish pine nuts	43 individuals infected from 5 states	N/A	(CDC, 2011)
	Bredeney	Peanut butter	42 individuals infected from 20 states	*Salmonella* was present in 28 environmental samples of the plant. Three of these samples showed the presence of the outbreak strain.	(CDC, 2012)
	Braenderup	Nut butter	6 cases from 5 states	Environmental isolates taken from the firm were related to the outbreak strain.	(CDC, 2014)
	N/A	Ground oregano	1,032 cases were recalled	The spice may have been contaminated while factory workers handled it.	(McCormick & Company, 2014)
	N/A	Products manufactured with organic garlic powder	Multiple cases were recalled in all 50 states	Raw material contained *Salmonella*.	(FDA, 2016a)
E. coli	O157: H7	Nut butter	32 individuals infected from 12 states	N/A	(CDC,2017)
	O121 or O26	Flour and flour products	63 individuals infected from 24 states	The recalled flours were produced in the same Kansas City facility, Missouri, and sold nationwide.	(CDC, 2016b)
Listeria	*monocytogenes*	Sunflower seeds	Various sunflower kernel products were recalled.	Originally from SunOpta's facility in Crookston, Minn	(FDA, 2016b)

1.4. Analytical protocols for detection and enumeration of microorganisms in LMFs

Standardized microbial methods must produce reliable, reproducible, and less variable results in heat resistance studies (Larry & James, 2001). For LMFs specifically, accurate measurement of water properties (water activity, moisture sorption isotherms, and moisture content) in LMFs and the preparation of microorganisms and their introduction to LMFs play essential roles (Fontana, 2007; Hildebrandt et al., 2016). Some procedures (such as the agar lawn-harvest method) have been widely accepted as standard operating procedures in different LMFs (ABC, 2014; Liu et al., 2018a; Wei et al., 2020). Based on these procedures, researchers can identify the real effects of experimental treatments and compare the results obtained across laboratories and food matrices. Standardization of all discussed aspects is necessary as otherwise published results will be incomparable. The suggested experimental procedures of microbial studies for LMFs are summarized in Table 2.

1.5. Culturing

The physiological state of a microorganism differs vastly depending on its growth in liquid (broth) or solid media (agar) (Keller et al., 2012): cells are in a planktonic state in liquid media. Still, they are in a sessile form when they are attached to a surface (Rivas, Fegan, & Dykes, 2008). Both liquid and lawn methods have been used to cultivate microorganisms in assessing their survival and thermal resistance in LMFs such as peanut butter (Komitopoulou & Peñaloza, 2009; Shachar & Yaron, 2006; Uesugi, Danyluk, Mandrell, & Harris, 2007). Agar lawn-based culture appears to be a more appropriate culturing method in laboratory studies of LMFs because lawn cultures were reported to be more desiccation resistant (Keller et al., 2012; Wiertzema et al., 2019). It is due to that the agar lawn-based method uses an agar plate to bacterial culture cells, and provides a completely different environment than the broth method (in liquids) for bacteria to grow. In addition, the lawn-based method enables researchers to produce less variation in the thermal resistance data of *Salmonella* (Hildebrandt et al., 2016). Only 2–3% differences in *Salmonella* $D_{80^{\circ}C}$ value (e.g., 250.9 min and 256.4 min) were observed from cross-laboratory comparisons in samples inoculated via lawn cultures harvested using peptone water.

1.6. Inoculation

Based on the proper culturing methods, most inoculated samples can reach an inoculated level of 10^5–10^8 CFU/g (piece) (in almonds, wheat flour, dates) for inactivation kinetics. This level is often associated with concentrated bacterial pellet or stock (Li, Huang, & Chen, 2014). The inoculation procedure can be challenging because it usually introduces a liquid (in bacterial pellet), which may alter the physical properties of LMFs and require a re-drying step after the inoculation. For instance, the ABC guidelines designed a "dipping" procedure for inoculating almonds and introduced an air-drying step using filter papers under ambient conditions. Inoculations that use a bacterial inoculum with water are often called wet or aqueous inoculation methods. Depending on the size of the bacterial inoculum droplets on the samples, inoculation methods can further be classified as spraying, dotting, dipping, or soaking.

Table 2
Current experimental procedures for different LMFs.

Procedure	Standardized method	Suitable for LMFs	References
Cultivation	Streak and transfer Lawn growth method Centrifuge and re-suspension (liquid inoculum)	All	(ABC, 2014)
Inoculation	Liquid inoculation (concentrated inoculum): spray, drop,or soak	Dry leaves, paste, butter, kernels.	(ABC, 2014)
	Dry inoculation (freeze-dried inoculum, carriers that provide desiccation stress)	Spices and powdered foods	(Xu et al., 2020)
Equilibration	Equilibrium chambers or saturated salt solution jars	All	(Syamaladevi et al., 2016b)
Thermal-death-time (TDT) test	Fill samples in closed/open containers Submerge filled containers in bath/ open systems Take containers out at given temperature/time treatment Recovery and enumeration	All LMFs that will be processed in sealed-package (closed system).	(Cheng et al., 2021)
	Fill samples in open containers Place the filled containers in specific conditions Take containers out at designated treatments Recovery and enumeration	All LMFs that will be processed in open packages (connect to the environment).	(OpX leadership network, 2016)
Mathematical modeling	Log-linear , Weibull, Bigelow, Surface response etc.	Depends on the inactivation kinetics	(Garces-Vega et al., 2016; Hildebrandt et al., 2015)
Surrogate validation	Access the characteristics (survivability, thermal resistance, safety etc.) of surrogates Compare with those of pertinent pathogen	All	(FDA, 2015)
Process validation	Identify the cold-spot Embed the inoculated-pack Track the inactivation kinetics Compare with modeled inactivation curves	All	(Liu et al., 2017)

Dry inoculation methods have been introduced and successfully applied for LMFs that cannot introduce water due to their physico-chemical properties (e.g., infant formula) or antimicrobial compounds such as those found in spices. The dry inoculum can be freeze-dried bacterial cells (10^{10}–10^{12} CFU/g) (Xu et al., 2018), inoculated dry carriers such as silica beads (Hildebrandt et al., 2017), talc powders (Enache et al., 2015), sand (Blessington, Theofel, & Harris, 2013), and a small portion of dried inoculated samples (Liu, Xu, Xie, Zhu, & Tang, 2019). Some of these carriers can be removed such that the cells are deposited onto the food, and the carrier no longer play a role in any inactivation kinetics (Hildebrandt et al., 2017); while in other cases, the carriers remain in the foods, possibly affecting survival curves (Enache et al., 2015; Liu et al., 2018b).

Dry inoculation methods can introduce an inoculation level as high as the liquid method. In some cases, the inactivation kinetics may differ due to alternative stresses (e.g., starvation, cold, heat) that may apply to bacterial cells during the dehydration process. One essential part of the dry inoculation method is that the drying of the inoculum is usually very rapid (much more rapid than when added wet to foods). Bacterial cell metabolism stops without many stress responses to gradient dehydration. Meanwhile, glass beads or silicon dioxide may provide extra starvation stress, and freeze-dried bacteria may encounter cold injuries (that may ultimately affect the bacterial thermal resistance in the suspending matrix). The merits and limitations of dry inoculation methods for LMFs have been recently reviewed (Xu, Song, Tan, Villa-rojas, & Tang, 2020).

1.7. Desiccation

The thermal resistance of bacteria increases in LMFs and depends on water (a_w and moisture content) (Farakos et al., 2013). Adjusting the $a_{w,25°C}$ of the inoculated sample also involves exposing the target bacteria to desiccation stress. In reality, bacteria can be desiccated in several critical control points in a processing line. Meanwhile, designing the desiccation step in the laboratory is associated with the thermal resistance and survivability of bacteria in the designated samples.

Air-drying is a controlled environment does not require complicated equipment and often dries out the samples rapidly. In non-food matrices, this process involves mounting a wet inoculum on/in silicon dioxide granules (Liu et al., 2018b), silica beads (Hildebrandt et al., 2017), or a 96-well polystyrene plate (Gruzdev, Pinto, & Sela, 2011) and air-drying inside a biosafety cabinet for 22–30 h at 25 °C. In the almond industry, a filter paper is used under wet-inoculated almond kernels, dried in ambient laboratory conditions for 24 h (ABC, 2014).

However, ambient air drying is dependent on the environment, especially the $a_{w,25°C}$ of air, and can vary across locations. Thus, an a_w-controlled environment is necessary to apply certain levels of desiccation stress to the target bacteria. Saturated salts that generate fixed levels of relative humidity, namely, LiCl (11.3%), CH_3COOK (Potassium acetate, 22.5%), $MgCl_2$ (32.8%), K_2CO_3 (43.2%), $MgNO_3$ (52.9%), $NaNO_2$ (65.8%), NaCl (75.3%), and KCl (84.3%), are often used in experiments to create desired relative humidity of the environment for desiccating bacteria to a certain level of $a_{w,25°C}$. Most of the generated water sorption isotherms have been obtained using the salt above solutions in hermetically sealed jars.

After dehydration of bacteria in LMFs in closed jars, it is critical to maintain its equilibrium status (at given $a_{w,25°C}$) before any survival and heat resistance tests. Environmental moisture can rapidly adjust the $a_{w,25°C}$ of food samples and bacterial cells (Syamaladevi, Tang, & Zhong, 2016a; Xie et al., 2020). Therefore, an equilibration chamber with controlled relative humidity was developed and applied in many laboratories (Hildebrandt, 2015). This chamber is a closed system with two in and out pipes that transport wet or dry air to adjust the internal relative humidity. Desiccating samples in the equilibration involves simultaneous equilibration between the pipes and the inner bacteria to the target relative humidity ($a_w \times 100\%$). A larger exposed surface and greater airflow increase the desiccation efficiency. However, the equilibration process can take 2–5 days depending on sample thickness and the sorption isotherms of food matrices (Hossain, Bala, Hossain, & Mondol, 2001).

To date, the effect of desiccation history on the thermal resistance of bacteria in LMFs remains unclear. In a study by Smith et al. (2015), *S.* PT 30 is rapidly desiccated (<4 min) wheat flour was as thermally resistant as that when slowly equilibrated (4–7 days) to the same $a_{w,25°C}$. Therefore, the desiccation speed of bacterial cells has a negligible impact

on performance in heat resistance studies. Another study (Liu et al., 2019) used dry inoculation methods, different desiccation histories of S. PT 30 and *E. faecium* B2354 in silicon dioxide and non-fat milk powder resulted in different inactivation kinetics in inoculated non-fat milk powders.

The ideal protocol should reflect the real-life scenario on how bacterial cells enter the LMFs and get desiccated. The liquid inoculation method introduces bacterial cells directly into LMFs, whereas the dry inoculation method applied desiccation stress prior to the food matrices. When a dry inoculation protocol reflects the natural routes of contamination, i.e., bacterial cells are dehydrated before food contamination, the use of liquid inoculation may produce deviated bacterial thermal resistance in LMFs (Liu et al., 2019). Further investigation on the contamination routes for risk assessment of LMFs is essential.

1.8. Recovery, detection, and enumeration

The general routines for the recovery and enumeration of bacteria in LMFs are related to the dilution and plating schemes in Bacteriological Analytical Manual (BAM) (Peter, Stephen, Michael, & William, 2002). Briefly, samples are suspended/dissolved in tryptic soy broth (TSB) (ABC, 2014) or Butterfield's phosphate buffer (Peter et al., 2002), serially diluted in the phosphate buffer, and spread-plated using the target dilution levels. The typical spread plating of *E. faecium* and pathogens (*Salmonella, E. coli,* and *L. monocytogenes*) uses tryptic soy agar (TSA) with no selective medium (Du, Abd, McCarthy, & Harris, 2010; Koseki & Nakamura, 2015; Margas et al., 2014; ABC, 2014) or TSA with yeast extract (TSAYE) (Keller et al., 2012).

Detection of foodborne pathogens in LMFs can have many obstacles such as low population levels of foodborne pathogens, low frequency of detection, and the presence of antimicrobials and inhibitors (Gurtler et al., 2019b). Pre-enrichments with added sodium pyruvate, catalase, and glycerol may help revive desiccated and heat-injured cells. This step can protect the desiccated cells from rapid exposure to a high a_w environment and reduce the chance of osmotic shock that renders cells unculturable (Kinsella, Rowe, Blair, McDowell, & Sheridan, 2006). Hasani, Wu, Hu, Farber, and Warriner (2019) applied a pre-incubation step in 1% w/v glycerol-TSB for 1 h prior to plating a cocktail of *Salmonella* or *L. monocytogenes* in raisins. They reported that the pre-incubation step increased the recovery of *Salmonella* but not of *L. monocytogenes*. Nevertheless, the effect of pre-enrichments on the recovery of different microorganisms in LMFs is not fully understood.

Differential media are also optional for the recovery and enumeration of microorganisms in LMFs. A modified TSAYE that contains TSA supplemented with 0.6% (w/v) yeast extract, 0.03% (w/v) sodium thiosulfate, and 0.05% (w/v) ferric ammonium citrate was used for *Salmonella* (Jeong, Marks, & Ryser, 2011). Sodium thiosulfate and ferric ammonium citrate serve as the differentiating ingredients because sodium thiosulfate can be decomposed into sulfate and H_2S gas by sulfate-reducing *Salmonella* strains resulting H_2S gas reacts with ferric ions to form ferric ammonium citrate and a brown-black precipitate. This precipitate can stain the *Salmonella* colonies in TSAYE plates, thus improving the visualization of *Salmonella enterica*. Selective media such as xylose lysine deoxycholate agar (XLD) and bismuth sulfite agar have also been used to recover and enumerate *Salmonella* (Harris, Uesugi, Abd, & McCarthy, 2012; Lambertini et al., 2016). Some of the supplements can reduce *Salmonella* recovery: *Salmonella* in XLD produces black colonies, but sodium deoxycholate can disrupt the integrity of the outer membranes of some injured cells and inhibit their recovery (Wang et al., 2015). The 0.1% (wt/vol) sodium pyruvate was used to enhance the resuscitation of injured *E. sakazakii* cells in infant formula, providing a 32% recovery rate of four *E. sakazakii* strains after 31 days of storage at 21 °C (Gurtler & Beuchat, 2005).

Inappropriate recovery media and methods would lead to a failure to count actual survivors in LMFs and give rise to inaccurate survival and resistance parameters. Gurtler and Beuchat (2005) found that a spiral plate produced more conclusive survivor counts of desiccated *E. sakazakii* than the econometric technique did.

After thermal treatments, a selective plating medium would inhibit the recovery of heat-injured bacterial cells (Ray, 1986). To facilitate recovery of heat-injured *L. monocytogens* cells while providing selectivity of isolation of *L. monocytogenes* from other bacteria in food samples, Kang & Fung (1999) developed a thin agar layer method that overlay 5 mL of TSA onto prepoured and solidified selective plate. The authors reported significantly higher numbers of *L. monocytogenes* were recovered by this method. It was also named "the agar overlay method" and was documented with the significantly higher recovery of heat-injured *Salmonella* (Kang & Fung, 2000), desiccated *E. sakazakii* (Osaili et al., 2010), and *Salmonella* survivors in spices (Caver Branden, 2016). In addition, the induction of viable but noncultural (VBNC) state in LMFs or during thermal treatments require additional resuscitation step prior to plate count (Morishige, Fujimori, Amano, 2013) or advanced methods for detection and quantification of non-culturable cells (e.g., bacteriophage, flow cytometry, PCR) (Fernandes et al., 2014; Malorny et al., 2004). For instance, Lv, Gu, Wang, He, & He, et al., (2021) developed a detection method based on propidium monoazide, combined with a single intact cell droplet digital PCR, for VBNC *C. sakazakii* in infant food. The detection limit was 23 CFU/ml, and the positive sample detection rate was 2.08% higher than that of the plate method. The significance of bacterial VBNC state in food safety and methods for recovery and quantification of VBNC cells were reviewed by Ayrapetyan and Oliver (2016) and Foddai and Grant (2020).

Rapid detection technologies were also tested to quantify culturable cells in LMFs. The BAM *Salmonella* culture method was able to detect 1 CFU/25 g sample with a minimum 4 days (FDA, 2014b); while rapid-detecting assays (e.g., qPCR assay) and commercial test kits (e.g., VIDAS Easy) were able to detect equal or lower mount of cells in 2 days (Elizaquível et al., 2009; Temellit et al., 2011; Crowley et al., 2011; Cheng et al., 2009). The qPCR detection has the detection limit of 10^3-10^4 CFU/ml, which requires a pre-enrichment step, that must support the growth of *Salmonella* (Wang, Gill, Cheng, Gonzalez-Escalona, Irvin, et al., 2015).

All these recovery methods and associated supplements are designated to facilitate survivors' recovery and increase the limit of detection of the analytical procedures. Some new technologies or rapid detection assays may not be available or affordable for all researchers. Therefore, "an appropriate recovery method" is a case-by-case decision for scholars and industries to make.

1.9. Containers for LMFs in TDT tests

The TDT test allows the quantification of microbial heat resistance (D- and z-values) in specific foods. High resistance is an essential factor in identifying target pathogens (section 3.2), and the pathogen's thermal resistance parameters can be used to validate its potential surrogates (section 3.3). The TDT test is usually conducted in a capillary glass tube at an isothermal temperature for microorganisms in high-moisture foods. The capillary glass tube provides high heating and cooling rates but is limited to liquid or pureed samples (Guan et al., 2003). For LMFs in kernel or powder forms, alternative containers that can seal in moisture during the treatment and be operated at higher temperatures (60–80 °C) are needed. These containers can provide either closed systems (properly sealed) or open systems (connecting to the environment) for the TDT tests, depending on the associated thermal processes. Both closed- and open-system test methods have been reviewed by Cheng et al. (2021). In the following content, three closed-container (glass tube, plastic bag, and metallic container) and the open container in general (the mesh-based tray/basket) were summarized.

2. Closed containers

2.1. Glass tubes

A glass container can be the capillary tube that has been used for high-moisture foods (1 mm inner diameter, 0.25–0.4 mm wall thickness). The capillary glass tube is suitable for liquid-like low a_w foods (e.g., oil and honey, in this case, honey is low a_w, but the moisture content is non-negligible) and enables a short come-up time (CUT) and minimum air space. Yang et al. (2020) pipetted 70 μl of *E. faecium*-inoculated peanut oil, adjusted the samples to the center position, and sealed both ends of the tubes using flames. They have conducted isothermal inactivation studies of *E. faecium* in peanut oil at 80 ℃ and four a_w levels. Chick et al. (2011) inserted the capillary tubes (1.5–1.8 × 90 mm borosilicate glass) through the Luer-lock tip of the syringes (loaded with *Salmonella*-inoculated flours) to fill the tubes properly. The filled tubes were then heat-sealed and immersed into an oil/water bath for thermal testing.

In addition, Daryaei et al. (2020) chose the regular glass tube (inner diameter 13 mm, wall thickness 1.15 mm) to hold 5-g inoculated wheat flour for TDT tests. The sample-filled tubes were closed with sanitized caps before any thermal treatments. The authors have completed TDT tests of pathogenic *E. coli* and their potential surrogates in wheat flour using these tubes.

2.2. Plastic containers

Vacuum plastic bags enable high heat transfer efficiency and minimize the container's heat loss. Low-moisture kernels (e.g., nuts, grains) and their meals (e.g., peanut butter) can be packed in plastic bags (vacuum-applied optional) for microbial studies in laboratories (Li et al., 2014; Limcharoenchat et al., 2018; Shachar & Yaron, 2006; Ceylan & Bautista, 2015). The CUT of plastic bags varied from 6 s (Li et al., 2014) to 2.7 min (Limcharoenchat et al., 2018), depending on the food sample sizes. The plastic container can also serve as stomacher bags for the first serial dilution in the recovery and enumeration step. However, extra attention is needed when immersing packed LMFs into water or oil bath in TDT tests because plastic bags tend to float and drag the samples out of the heating medium.

Forghani et al. (2018) used 0.5 mL thin-wall PCR tubes with flat caps as the TDT containers to perform the thermal treatments of a five-strain serogroup cocktail *E. coli* in wheat flour. Each tube can hold 0.33 ± 0.02 g wheat flour samples. The heat resistance tests were conducted via a digital dry bath at 55 ℃ to 70 ℃. The authors also emphasized the necessity of keeping the tubes closed during the thermal treatment procedure to prevent any changes in a_w during the process.

2.3. Metallic containers

Compared with plastic bags or capillary tubes, metallic containers' main advantage is their reusability and heat transfer rate: they provide a steady sample space and can be manually opened and closed (Chung et al., 2008). When the metallic container is closed for TDT tests, no moisture transfer occurs of the tested samples. Most of the metallic containers' heating and cooling rates are markedly high despite the small sample size (0.5–0.7 g); minor inconsistencies in handling may lead to vast differences in thermal resistance results. Therefore, careful attention is required to maintain the uniformity of inoculation, the heating rate of cells in a water or oil bath, and the recovery of survivors in treated samples.

With the merit of high heat rate, the metallic containers were optimized by adding the control system of heating rate (1–10 °C/min) (Kou, Li, Hou, Huang, Ling, 2016), separating the metallic container with heating plates via a sandwich heating block (Wei et al., 2020; Lau et al., 2020), and adding multiple wells with a shared headspace inside aluminum cells (Tadapaneni et al., 2017). These metallic containers are suitable for many LMFs, such as food powders, nut particles, raisins, and date paste. Details on different sizes and types of metallic containers used in TDT tests for LMFs were recently reviewed by Cheng et al. (2021).

2.4. Open containers

Without sealing the food samples, mass transfer between food samples and the environment may occur at elevated temperatures. The open container can take many forms, but a mesh-based container is most commonly used. Bacteria-inoculated almonds are spread on a metal mesh tray. They have been placed in hot air, steam, infrared heating, or using a combined process for testing the heat resistance of *Salmonella* (Beuchat & Mann, 2011; Brandl, Pan, Huynh, Zhu, & McHugh, 2008; ABC, 2014; FDA, 2013; Jeong, Marks, & James, 2017; Lee et al., 2006). For instance, infrared can rapidly remove surface moisture of almonds compared with hot air (Bingol et al., 2011); however, in the steam process, the moisture content was found to increase during the treatment, which could cause economic loss to the company (Lee et al., 2006). Enclosed wire mesh baskets can also hold the almond kernels in hot-oil/water treatments, allowing the almond kernels to be submerged in oil throughout the entire treatment (Bari et al., 2009; Du et al., 2010). These lab-scale containers are akin to pilot-scale trays in food processing conditions that allow effective heating of food products. For such thermal processes, designated microbial tests using open containers serve well to acquire data pertaining to microbial thermal resistance and quality analysis of food samples.

Besides the containers, temperature control of the isothermal inactivation system is also critical in TDT tests. Accurate measurement and/or control of temperatures of LMFs, using thermocouples or heating units, is essential to determine the actual bacteria's heat resistances. Thermocouples were often inserted on the geometric center of the container (Kou e al., 2016) or attached to food kernels (Du et al., 2010) to ensure that the temperature in the containers reached the experimental temperature. The time interval from admitting the heating medium until the actual processing addition is established was named CUT (Ramaswamy, 1993). Low heating rates would result in pre-adaptation of bacterial cells to heat and enhanced thermal resistance (Chung et al., 2007; Kou et al., 2018).

Last but not least, one should also consider the degree of convenience and previous experience in experimental operations. For instance, the heating block system requires rather specialized equipment, which is more complicated than a simple heating cell or plastic bags and not available for all researchers. In addition, most microbiologists may already have more experience in isothermal inactivation studies by capillary tubes than metallic cells. Capillary tubes appear to be the better choice if the LMFs can fit in them. On the other hand, liquid baths (water or oil) have disadvantages, such as liquid spillage and liquid infiltration into samples (Lau et al., 2020). The liquid circulation and temperature control also need to be calibrated frequently prior to any thermal inactivation tests.

3. Mathematical modeling of thermal inactivation kinetics in LMFs

Describing microbial inactivation kinetics involves model fitting and the generation of parameters that can be used to describe bacterial behavior in foods. Appropriate mathematical models can provide detailed information on the trend of inactivation kinetics, bacterial resistance, and external factors, thereby allowing the accurate and robust calculation of process lethality. To date, the log-linear, Weibull, response surface, Bigelow, Arrhenius, Bigelow-type, Geeraerd, and combined models have been used by many researchers to fit the inactivation data generated under numerous situations. Based on these models, secondary models that estimate the influence of temperature and a_w have also been proposed by a few studies (Garces-Vega, Jeong,

Dolan, & Marks, 2016; Smith et al., 2016; Taylor et al., 2018).

When the target bacteria's survivor curve (treated at specific temperature T) approaches a log-linear trend, its thermal resistance-related parameters can be computed, namely, the decimal reduction time (D) and temperature sensitivity (z_T), as follows:

$$\log(N_t/N_0) = -t/D$$

where N_t and N_0 are the populations (CFU/g) at times t and 0, respectively; t is the time of the isothermal treatment (min) after CUT; and D is the time (min) required to reduce the microbial population by 10-fold at the specified temperature (°C), and

$$d(\log D)/d(T) = -1/z$$

where z is the temperature change necessary to change the TDT by one log-cycle (Gaillard, Leguerinel, & Mafart, 1998). A complete survivor curve requires a sufficient inactivation range (generally, 3–5 log reduction) and enough data points (≥5 time points), whereas an accurate z_T value requires a minimum of three D values over an adequate temperature scale (Gaillard et al., 1998).

Mattick et al. (2001) reported that a Weibull distribution with a polynomial secondary model best fitted the inactivation data generated in intermediate a_w broth solutions ($a_{w,25°C}$ = 0.65–0.90). In non-isothermal inactivation of *Staphylococcus aureus* in walnut shell powders at different heating rates (0.2–10 °C/min) (Zhang, Kou, Zhang, Cheng, & Wang, 2018), the Weibull model fitted better (R^2 = 0.97–0.99) than the first-order kinetics (R^2 = 0.88–0.98).

Several secondary models could describe D- or β-values as a function of a_w or food nutrients (e.g., fat and protein). Besides log-linear secondary model, modified Bigelow-type model that involves temperature and product a_w was widely used in thermal inactivation studies of LMFs (Gaillard et al., 1998):

$$D_{T,a_w}(t) = D_{ref} \times 10^{\frac{T_{ref}-T(t)}{z_T}+\frac{a_{w,ref}-a_w(t)}{z_{a_w}}}$$

Where $a_{w,ref}$ is the reference water activity, $a_w(t)$ is the water activity at t, and z_{a_w} is the water activity change required for a 10-fold change in D-value. The modified Bigelow-type model could always change the factor a_w into moisture content, food composition, and others (Casulli et al., 2021; Verma et al., 2021; Zhang et al., 2022, under review). Both modified-Bigelow and secondary log-linear models have a degree of phenomenological meaning, which benefits the readers to understand, and the model parameters (D- and z-values) can be directly applied in industrial situations.

4. Factors influencing the thermal resistance of microorganisms in LMFs

4.1. Food components

Food components and their ratios are the major factors that need to be considered in validating potential *Salmonella* surrogates, estimating thermal resistance of bacteria, and designing thermal pasteurization processes (Bianchini et al., 2014; Jin et al., 2018). Bacteria exhibit vast differences in thermal resistance parameters (D- and z-values) in carbohydrate-, protein-, or fat-rich products. For instance, at $a_{w,20–25°C}$ = 0.45, the $D_{80°C}$ of *S.* PT 30 in wheat flour, almond flour, and whey protein powder (prepared and treated using similar microbial methods) were 4.9 ± 0.5 min, 21.2 ± 09 min, and 10.6 ± 0.2 min, respectively (Xu et al., 2019). Jin et al. (2018) also reported that the z_T of *Salmonella* Agona varied according to food composition (high-protein and high-fat model food matrices) at the same $a_{w,25°C,}$ and treatment temperature.

Food components in LMFs influence bacterial resistance by serving as shields from decontaminants, structural components that interact with water (Quirijns, Boxtel, Loon, & Straten, 2005; Dhaliwal, Gänzle, & Syamaladevi, 2021), and potential stress inducers, all of which may enable cross-protection that subsequently increases resistance (Yang, Xu, Lombardo, Ganjyal, & Tang, 2020). In particular, special components such as oil and sugar could have different impacts at various physical states (liquid, solid, or powder) and structures (i.e., saturated or unsaturated fat, glucose, or sucrose) (Alshammari, Dhowlaghar, et al., 2020a; Alshammari, Xu, Tang, Sablani, & Zhu, 2020b). Because of the complexity of food components, to date, there is no reported relationship or available mathematical model that accounts for the effect of one specific food component (e.g., fat, sugar, and protein) on bacterial thermal resistance in LMFs.

4.2. Moisture content

Moisture content describes the amount of water in a food system and is not temperature-dependent (Fontana, 2007). Due to the hysteresis in foods' moisture sorption isotherms, the specific moisture content of a food system can be linked with different a_w levels. Garces-Vega, Ryser, & Marks (2019) reported no significant preferences ($P > 0.05$) of $a_{w,25°C}$ or moisture content on its effect on S. PT 30 inactivation kinetics on almonds.

Increased moisture content reduces bacterial thermal resistance. Verma et al. (2018) adjusted oat flour to different moisture contents (14–26% wet basis), inoculated the samples with a five-strain cocktail of *Salmonella* and extruded the inoculated samples at different temperatures and screw speeds. Moisture content showed significant linear (P = 0.0014) and quadratic (P = 0.0005) positive effects on microbial reduction. The same trend was also reported in the extrusions of animal feed mash (Okelo et al., 2006), balanced carbohydrate-protein meal (Bianchini et al., 2012), and oat flour model foods (Anderson et al., 2017). Besides extrusion, Xie et al. (2020) conducted an isothermal inactivation study of freeze-dried *S.* PT 30 at 80 °C with different moisture content levels (7.7–15.7 g water/100 g dry solids). They built a relationship between the moisture content of bacterial cells and corresponding $D_{80°C}$ values of *S.* PT 30 using freeze-dried bacteria data and available data from five low-moisture matrices (wheat flour, almond flour, whey protein powder, honey powder, and silicon dioxide). The authors suggested bacterial cell moisture content is the intrinsic parameter determining *Salmonella* thermal resistance in LMFs.

During dielectric heating of LMFs, increased moisture content alters the dielectric properties of foods, accelerates the heating rate (e.g., radiofrequency heating) (Jeong & Kang, 2014), and therefore partially reduces bacterial thermal resistance (section 3.5 and 3.7).

4.3. Water activity

Water activity (a_w) is the ratio between the water vapor pressure in a food system (Pv) and the saturation water vapor pressure (Pvs) at the same temperature (Labuza, 1975). A food system and water with different temperatures would also have different Pv/Pvs ($a_{w,\ T}$) because food composition generates various water sorption isotherms according to composition, structure, and water sorption histories. Therefore, a_w can be further expressed as $a_{w,\ T}$, indicating a temperature-dependent parameter (Syamaladevi et al., 2016b). Because water activity at 25 °C ($a_{w,25°C}$) categorizes foods into LMFs ($a_{w,25°C} \leq 0.85$) and high-moisture foods ($a_{w,25°C} > 0.85$), a_w can dramatically influence microbial resistance (Liu et al., 2019; Farakos et al., 2013; Smith et al., 2016).

At a sp,ecific temperature T, a_w plays a key role in the D values and z_T (temperature sensitivity) of bacteria (*Salmonella*, *E. coli*, *Listeria*, and *E. faecium*) in various LMFs. Jin et al. (2018) studied the influence of a_w and quantified this as z_{a_w}, which has the exact definition as z_T, to describe a_w bacterial sensitivities in LMFs. However, the a_w and z_T values of microorganisms in LMFs are not well studied. Given the fact that thermal resistance of *S.* PT 30 and its surrogate *E. faecium* increased exponentially with decreasing $a_{w,T}$ (Liu et al., 2019), and the general increasing $a_{w,T}$ at elevated temperature (Syamaladevi et al., 2016c), the possible synergistic effects of temperature and a_w on the D_T values of microorganisms in LMFs are presented in Fig. 2. This figure is based on

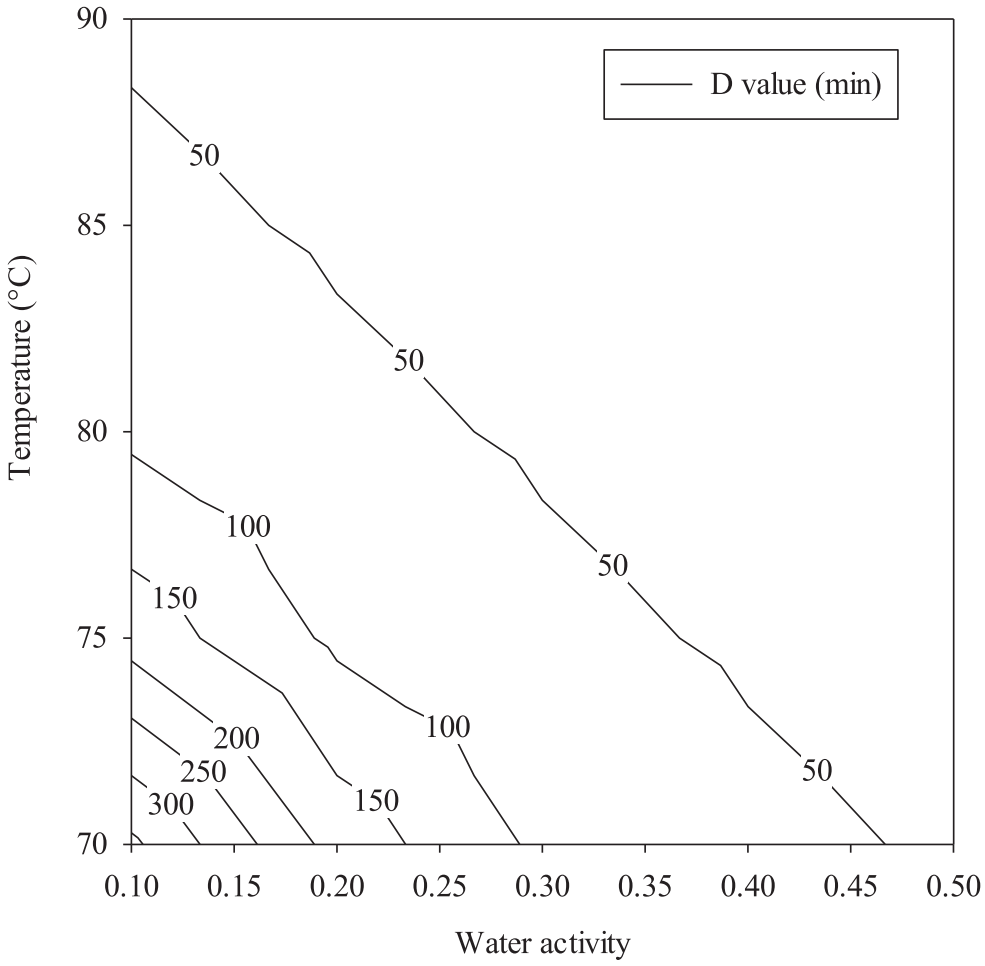

Fig. 2. Combined effect of water activity ($a_{w,T}$) and temperature (°C) on D values (min) of microorganisms in LMFs (based on the assumption that D value changes exponentially with $a_{w,T}$ and temperature).

the assumption that bacteria's heat resistance (D_T value) altered exponentially with a_w at temperature T. This assumption could be risky when the inactivation kinetics do not follow a log-linear trend (upward concavity or tailing) (Peleg, 2006).

As a temperature-dependent factor, the a_w of a food system appears highly influenced by moisture content and food composition (Syamaladevi et al., 2016a). The influences of temperature and moisture content can be expressed as, where T is the measurement temperature, and X_m is the moisture content of food samples in a closed system. Food components can also affect the $a_{w,\ T}$ of samples (Jin, Tang, & Sablani, 2019; Syamaladevi et al., 2016c). However, more information is needed to understand how $a_{w,\ T}$ changes at elevated temperatures and how these changes influence bacterial thermal resistance. To date, $a_{w,\ T}$ highly correlates with the heat resistance of microorganisms in LMFs (Liu et al., 2018a; Xu et al., 2019). Therefore, in this review, the $a_{w,\ T}$ represents the a_w value and its measurement temperature.

4.4. Desiccation history

Most of the reported *Salmonella*'s thermal resistance data were associated with an equilibrium step of inoculated samples and bacteria inside (section 3.4). The equilibrium process can be the dehydration or rehydration of bacterial cells depending on the target and the original $a_{w,25°C}$ value of the food samples. Here, bacterial cells are exposed to desiccation stress either way because the final aw,25 °C values of bacterial cells in LMFs are consistently lower than those in the inoculum.

The desiccation history of bacteria refers to the series of steps that occurred to reduce the bacterial $a_{w,25°C}$ to<0.85 (within a low-moisture zone). Bacterial responses to a low-moisture environment were reviewed by Finn et al. (2013) and Spector & Kenyon (2012). Multiple cellular mechanisms might be involved in maintaining microorganisms' osmolarity balance with that of a low-a_w environment, such as the production and collection of osmoprotectants like trehalose, glycine-betaine, and proline (Jiang, Liu, Chi, Hu, & Chi, 2018); loss of water and transition into a VBNC state (Abdelhamid & Yousef, 2020; Gruzdev, Pinto, & Sela, 2012); and production of glycocalyx layers outside bacterial cells as extracellular defenses (Tamaru, Takani, Yoshida, & Sakamoto, 2005). These responses can lead to increased thermal resistance in pathogens such as *Salmonella* (Gruzdev et al., 2011), *Listeria* (Zhu et al., 2020), and *C. sakazakii* (Shaker, Osaili, Abu Al-Hasan, Ayyash, & Forsythe, 2008). In contrast, the induction of VBNC pathogens in LMFs after heat injury brings difficulties in plotting survival curves by the regular protocol (using the plate-count method) (Oliver, 2005).

The degree of bacterial persistence changes is linked to factors during the desiccation process, including drying temperature, drying speed, nutritional state, and previous equilibrium history (desorption and absorption). A slow dehydration rate could enhance bacterial survival through the de novo synthesis of critical metabolites required to adapt to desiccation stress (Gruzdev et al., 2012). In contrast, an accelerated drying process (drying inoculated almonds at 37 °C for 12 h) led to significantly smaller thermal reductions of *S.* PT 30 on almonds in both blanching and roasting processes ($P < 0.05$) (Mohammad, Murano, Moreira, & Castillo, 2020). Because of bacterial cell exposure to various

desiccation histories, quantifying their corresponding resistances can be very complex (to date, virtually impossible). This complicates understanding the mechanisms involved in increased thermal resistance of bacteria in LMFs and the application of laboratory data to industrial pasteurization validations.

Considering that microorganisms in LMFs are in a dormant state (Wen et al., 2011), moisture transition between bacterial cells and the environment may be critical for bacterial persistence. Smith et al. (2015) equilibrated two batches of S. PT 30-inoculated wheat flour to $a_{w,25^{\circ}C}$ of 0.30 and 0.60. They applied rapid dehydration and hydration treatment ($a_{w,25^{\circ}C}$ 0.60–0.30 or reversed) over 4 min to investigate the effect of moisture fluctuation on the thermal resistance of S. PT 30 at a reduced a_w. The results showed that $D_{80^{\circ}C}$ values of S. PT 30 have a negligible correlation to the response period. Still, a strong correlation to the $a_{w,25^{\circ}C}$ before the heat resistance study, e.g., the $D_{80^{\circ}C}$ in $a_{w,25^{\circ}C}$ 0.60 flour and rapid hydrated flour (from 0.30 to 0.60) are statistically similar (1.33 ± 0.15 min and 1.15 ± 0.09 min, respectively). Therefore, Smith et al. (2015) claimed that desiccation and hydration history might have little impact on bacterial thermal resistance once the cells reach $a_{w,25^{\circ}C} < 0.85$. The heat resistance of foodborne pathogens in LMFs and its factors have been reviewed by Podolak et al. (2017).

5. Factors influencing the microbial validation of thermal processing of LMFs

Validation refers to the evaluation of the suitability of a process in controlling a potential hazard within tolerable limits (Codex, 2008). Microbial validation is carried out in food matrices to verify the lethality of the process using microorganisms (Guan et al., 2003). Under specific processing conditions, both thermal and process information are gathered to evaluate the inactivation level.

Thermal inactivation kinetics and resistance parameters of target bacteria, generated from laboratory-based thermal inactivation kinetics, would ultimately be applied to validate real processing plants. For instance, Du et al. (2010) and Beuchat and Mann (2011) have completed the inactivation studies of *Salmonella* in almonds and pecan nutmeats by hot oil and hot air in the laboratory. Their data supported that the typical oil roast nutmeats would be sufficient to reduce *Salmonella* to a safe level. Liu et al. (2017) obtained the D- and z-values of *S.* Enteritidis and its surrogate *E. faecium* in organic wheat flour in the laboratory (using metallic cells) and correlated them with the temperature profiles of the cold spot within 3 kg wheat flour (heated by pilot-scale radio-frequency oven). Based on the mathematical modeling, they have completed a conservative validation study and verified the given processing parameters to achieve 5-log reduction of *Salmonella* in wheat flour. However, microbial validation of thermal processing of LMFs may encounter different critical control points because of the various physiochemical properties of LMFs. Here a couple of factors that may play important roles were listed:

5.1. Particle sizes

Foods with various particle sizes provide a different degree of contamination of microorganisms (surface or the whole product) and degree of exposure of bacterial cells to thermal treatments. In surface-contaminated foods such as shelled nuts, relative humidity is a critical element in thermal processing (Garces-Vega et al., 2016) because heat is not required to penetrate food matrices for the inactivation of bacterial cells attached on the surface. The inactivation temperature of the target microorganisms is considered as the foods' surface temperature, which rapidly increases to the designated temperature. Higher relative humidity allows the acceleration of the inactivation of a surface-inoculated microorganism because of the overall higher thermal conduction and lower bacterial thermal resistance (Liu et al., 2018b). Therefore, adding water to increase the relative humidity in a processing oven/chamber is commonly used to pasteurize nuts (ABC, 2007a; Venkitasamy et al., 2017; Villa-Rojas et al., 2013). In the nut industry, several reports have been generated over the past ten years on the survival kinetics and thermal resistance of *E. faecium* B2354 (Brar & Danyluk, 2019; Kataoka et al., 2014). From there, the FDA has developed two industrial guidelines addressing the presence of *Salmonella* species in peanut- (FDA, 2009) and pistachio-derived products (FDA, 2011). These two guidelines require the industries to evaluate the effectiveness of specific Salmonella control measures and recommend manufacturers be cautious on purchasing peanut- and pistachio-derived ingredients (by verifying the suppliers' decontamination controls and relying on microbial tests of incoming ingredients).

In LMFs with small particle sizes, such as powdered foods and ground nuts and spices, studies have focused on traditional steam processing, as well as the application of pasteurization technologies, including radio-frequency, extrusion, and moist air. Challenges include the following: (a) sticking, caking, and agglomeration that may occur with the introduction of water (while inoculating the products with an aqueous inoculum) or chemical degradation at high temperatures (onion powder cake at 71 °C at $a_{w,25^{\circ}C} = 0.50 \pm 0.2$); (b) difficulty in implementing laboratory-scale experiments using dusty powders in pilot validation studies; (c) alteration of the survivability and thermal resistance of microorganisms upon exposure to particular components in LMFs (e.g., lipids in ground nuts enhance thermal resistance, whereas antimicrobial compounds in spices inactivate bacterial cells); and (d) reduced heat transfer efficacy owing to the lower thermal conductivity of LMFs. Regarding these challenges above, dry inoculation methods that avoid introducing water droplets have been developed and reviewed in section 3.4. Emerging technologies to pasteurize LMFs have also been developed and include infrared heating (Bingol et al., 2011), radio frequency (Liu et al., 2017; Sánchez-Maldonado, Lee, & Farber, 2018; Wei et al., 2019), superheated steam (Ban et al., 2018), and combined hurdle technologies (Bari et al., 2009, 2010; Venkitasamy et al., 2018).

5.2. Packaging

Packaged products are less likely to be re-contaminated by pathogens (the risk remains if the product is re-packed). In addition, thermal pasteurization of packaged LMFs generally results in naturally-increased $a_{w,\,T}$ at elevated temperature T (Syamaladevi et al., 2016c) and therefore in higher decontamination efficacy because the bacterial heat resistance decreases as the $a_{w,\,T}$ increases. An exception can be oil-rich products with a low $a_{w,\,T}$ (Yang et al., 2020). Without packaging, the thermal pasteurization of LMFs is exposed to open systems (such as hot air), which can be significantly affected by the $a_{w,\,T}$/relative humidity of the environment. Rapidly increasing $a_{w,\,T}$/relative humidity might condense water on the products' surface, leading to broken surfaces and severe quality deterioration (Liu et al., 2020). More importantly, the relative environmental humidity ($a_{w,\,T}$) during the process can be challenging to control and lead to challenges in predicting inactivation efficacy and microbial validation.

5.3. Other external factors

In addition to the factors in microbiological protocols that induce stress responses in bacterial cells in LMFs, external factors influencing thermal resistance parameters are not fully understood. In particular, how factors interact and how thermal pasteurization efficacy can be maximized without affecting other parameters remain unknown. For instance, most studies support the idea that higher $a_{w,25^{\circ}C}$ /$a_{w,\,\text{elevated temperature}}$/relative humidity could reduce bacterial thermal resistance; however, adding moisture could vastly affect food properties. Thus, balancing the heating technologies, processing parameters, and LMF properties is critical in controlling pathogens in the LMF industry.

5.4. Processing technology

Although food properties are the primary factors in designing and validating pasteurization processes, processing technologies and their corresponding factors can also be alternative aspects to review progress in LMF pasteurization. For that, a couple of review articles have summarized the recent advances in the pasteurization of LMFs in terms of legacy technologies (e.g., baking, roasting, and drying) and alternative technologies (e.g., radiofrequency, non-thermal plasma, and irradiation) (Anderson, 2019; Deng et al., 2020, 2021; Liu et al., 2021). In real-life scenarios, predicting and validating the processes is challenging because of non-isothermal and non-*iso*-moisture processes.

6. Conclusions

Ensuring LMFs microbial safety requires comprehensive understanding and effective control of foodborne pathogens. This process can be very complicated since LMFs represent a large variety of food products, involving many technologies and processing lines. Standardizing experimental procedures and monitoring the critical factors during the process would significantly reduce the data errors. To provide this information to the LMFs industry, this review covers the fundamentals of microbial validation studies and the developments in preventive controls and process validations.

As the last step before industrial application, a validation study involves the design, conduction, evaluation, and implementation of the thermal processing of LMFs. Three validation approaches are commonly used: obtaining scientifically valid data from regulatory agencies, challenge studies, and mathematical modeling and monitoring factors involved in the models. Here, relevant studies that have contributed to these three aspects were summarized. These studies have provided scientific information on the behavior of the pathogen *Salmonella* and its surrogates (product component-related, moisture content, a_w, and temperature), and the lethality of existing processes and novel thermal processes based on experiments and modeling.

However, despite the progress in microbial validation studies of different thermal technologies, many challenges to conducting a successful validation study using microorganisms remain.

Remarkably, the preparation and calibration of microorganisms are as crucial as identifying cold spots and the design of processing conditions. More studies are needed to develop a systematic procedure applicable to different kinds of LMFs and various types of processing technologies. Close collaboration among microbiologists, process engineers, modeling statisticians, food quality scientists, and other related professionals will help ensure positive outcomes to achieve successful microbial validation studies on the thermal processing of LMFs.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contributions

Shuxiang Liu compiled data from the literature review and drafted the manuscript. M. S. Roopesh revised the manuscript and contributed to sections 3, 4. Professor Juming Tang revised the manuscript and contributed to section 5, Professor Wu revised the manuscript and contributed to section 4. Professor Wen Qin supervised the work, revised the manuscript, and contributed to section 2. All authors reviewed the manuscript before submission.

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