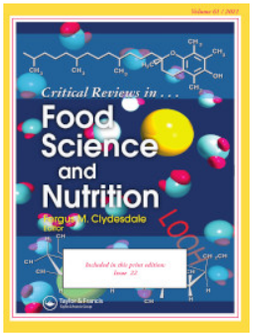

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REVIEW

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Recent developments in low-moisture foods: microbial validation studies of thermal pasteurization processes

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ABSTRACT

Outbreaks associated with low-moisture foods (e.g., wheat flour, nuts, and cereals) have urged the development of novel technologies and re-validation of legacy pasteurization process. For various thermal pasteurization processes, they share same scientific facts (e.g., bacterial heat resistance increased at reduced water activity) and guidelines. However, they also face specific challenges because of their different heat transfer mechanisms, processing conditions, or associated low-moisture foods' formulations. In this article, we first introduced the general structural for validating a thermal process and the shared basic information that would support our understanding of the key elements of each thermal process. Then, we reviewed the current progress of validation studies of 7 individual heating technologies (drying roasting, radiofrequency-assisted pasteurization, superheated steam, etc.) and the combined treatments (e.g., infrared and hot air). Last, we discussed knowledge gaps that require more scientific data in the future studies. We aimed to provide a process-centric view point of thermal pasteurization studies of low-moisture foods. The information could provide detailed protocol for process developers, operators, and managers to enhance low-moisture foods safety.

KEYWORDS

Thermal pasteurization; *Salmonella*; *Enterococcus faecium*; heating technology; lethality

1. Introduction

Low-moisture foods (LMFs) that display low water activity at 25 °C ($a_{w,25°C}$ ≤0.85) are less susceptible to microbial spoilage and foodborne pathogens, as they curtail the growth of microorganisms (Cordier 2014). However, LMFs still need to be decontaminated because pathogens and spore-formers may persist in desiccated conditions for a considerable period (Scott et al. 2009). Their impact can be substantial as many LMFs are ready-to-eat (e.g., nuts, chocolate, and dry fruits) and are commonly used as ingredients in food processing (Podolak et al. 2010). Outbreaks associated with *Salmonella*, *Listeria*, and pathogenic *Escherichia coli* (*E. coli*) include a wide range of LMFs (Centers for Disease Control and Prevention (CDC) 2016, 2018; Food and Drug Administration (FDA) 2016b). To minimize and prevent such hazards in LMFs, the Food Safety Modernization Act defined required science-based process controls for food production plants (Food and Drug Administration (FDA) 2016a). However, validation of these process controls can be challenging because thermal processing of LMFs is often implicated with different foods' properties and processing technologies (The Association of Food Beverage and Consumer Products Companies 2009; Verma 2021a).

Thermal pasteurization appears to be appropriate for LMFs to eliminate pathogens while remain shelf-life stable at room temperatures. Per National Advisory Committee on Microbiological Criteria for Foods' (2006) definition, pasteurization refers to "any process, treatment, or combination thereof, that is applied to food to reduce the most resistant microorganism(s) of public health significance to a level that is not likely to present a public health risk under normal conditions of distribution and storage." Therefore, the degree of decontamination of pasteurization can be lower than commercial sterility, but has to be validated its lethality prior to realistic process in food industry. To date, log reductions are typically set at a 5-log reduction (Food and Drug Administration (FDA) 2002). The suggested levels of pathogen control for different LMFs are listed in Table 1.

Validation refers to the evaluation of suitability of a process in controlling a potential hazard within tolerable limits (Codex 2008). In particular, microbial validation utilizes microorganisms to verify whether a designed process can achieve its target lethality (Guan et al. 2003). Under specific processing conditions, both thermal and process information are gathered to evaluate the inactivation level. Since pathogens are not allowed in industrial processing lines, non-pathogenic surrogates, yielding equal or higher thermal

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Table 1. Suggested levels of pathogen inactivation in processing LMFs.

Food products	Reduction	Reference
Almonds	≤ 4.0 log	7 CFR 981.442
Peanut-derived products	5.0 log	(Food and Drug Administration (FDA) 2009)
Pistachio-derived products	5.0 log	(Food and Drug Administration (FDA) 2011)
Meat jerky for humans	6.5 log	9 CFR 318.17
Poultry jerky for humans	7.0 log	9 CFR 381.150

resistance than pathogenic organisms, are used to access the thermal process controlling efficiency (Busta et al. 2003). Although the application of microorganisms in thermal processing validations are widely established, those in LMFs have only been partially addressed (Bianchini et al. 2012). Multiple organizations have published (incomplete) guidelines for studying the critical pathogens in LMFs, i.e., *Salmonella* spp., and for developing surrogate microorganisms to test the efficacy of the process (Almond Board of California (ABC) 2007c, 2007a, 2014; The Association of Food Beverage and Consumer Products Companies 2009). Additionally, a growing number of thermal inactivation studies on bacteria in LMFs are being conducted in different processes, including roasting (Beuchat and Mann 2011), moist-air heating (Jeong, Marks, and Ryser 2011), infrared heating (Bingol et al. 2011; Z. Yang et al. 2010), and radiofrequency-assisted pasteurization (Liu et al. 2018a; Zhang et al. 2021; Zhang, Zhao, et al. 2020). Numerous studies correlating microbial validation in LMF processes have also been reported over the past ten years (Anderson 2019; Sánchez-Maldonado, Lee, and Farber 2018; Wason, Verma, and Subbiah 2021).

Due to the greater heat resistance of microorganisms in LMFs and the low thermal conductivity of dry foods, thermal processing treatments are not as efficient in destroying pathogens when compared to moist foods (Doyle 2014). In LMFs, heat treatments require higher temperatures or prolonged treatments to ensure an equivalent level of lethality. The addition of moisture effectively inactivates microorganisms but may also alter product quality and shelf-life (Doyle 2014; Anderson 2019). Based on the different properties of raw materials and desired final products, emerging technologies were involved in thermal pasteurization. Researchers need to conduct well-designed laboratory-based study to assay both novel and existing technologies, either alone or in combination, to ensure microbial inactivation efficacy. Methods for controlling pathogens in LMFs were reviewed by Sánchez-Maldonado, Lee, and Farber (2018). Among these methods, thermal processes are widely used and validated using two primary groups of information: (a) the heat resistance of microorganisms under specific conditions, and (b) the temperature profiles of the treatment time of one product at the cold spot (Awuah, Ramaswamy, and Economides 2007). Furthermore, we review five thermal technologies (hot-air, hot-water, heat extrusion, radiofrequency heating, and infrared heating) and their (potential) advantages in thermal processing of LMFs, suitable food matrices, factors impacting the inactivation efficacy, and published studies on the microbial validation of LMFs' pasteurization.

2. Structures of validation studies in thermal pasteurizing LMFs

According to NACMCF's guideline (2016), six essential steps for pasteurization were often referred: determine the pertinent pathogen, the most resistant strain, the level of inactivation needed, the impact of the food formulation on pathogen survival, and validate the process applied. Guidance on validations of pathogen control in food industry were also published to provide details on conducting microbial validation studies of processing plants (Ceylan et al. 2021; Scott 2005). Those articles support the use of scientifically valid data, conduction of in-plant experiments, and the use of mathematical modeling to accomplish a successful challenge study for specific pasteurization processes.

General structure for conducting a validation study in thermal pasteurization process was plotted in Figure 1. The whole process involves seven important components (e.g., bacterial performance, process parameters, and surrogate microorganism), which requires data-collection and validations. Recently, our group has reviewed fundamentals of microbial studies in LMFs in laboratory (under review). Some widely-accepted microbial methods (such as lawn-harvest methods for preparing inoculum) have been outlined for LMFs, specifically. In this article, we focus on the microbial validation studies that have been recently conducted in support of a designated process.

2.1. Identification of specific foods, pertinent pathogen, and potential surrogates

Ranking of LMFs with microbial risks in descending order was: cereals and grains; dried protein products; spices and dried herbs; nuts and nut products; confections and snacks; dried fruits and vegetables, and seeds for consumption (Food and Agriculture Organization of the United Nations (FAO) 2014). These LMFs exhibit vast different physiochemical properties and may contaminate with various pathogenic bacteria. *Salmonella* has been recognized as the concerned pathogen in LMFs due to its high relevance to outbreaks in LMFs (Food and Drug Administration (FDA) 2020; Centers for Disease Control and Prevention (CDC) 2014, 2021), and high thermal resistance among foodborne pathogens (The Association of Food Beverage and Consumer Products Companies 2009; Villa-Rojas et al. 2013). Most of published articles on microbial safety of LMFs have utilized *Salmonella* as the target pathogen (Podolak, Lucore, and Harris 2017; Rachon, Peñaloza, and Gibbs 2016; Xie et al. 2021). However, rising numbers of articles have accessed the thermal resistance and survivability of *E. coli*, and *Listeria monocytogenes* (*L. monocytogenes*) at reduced $a_{w,25°C}$

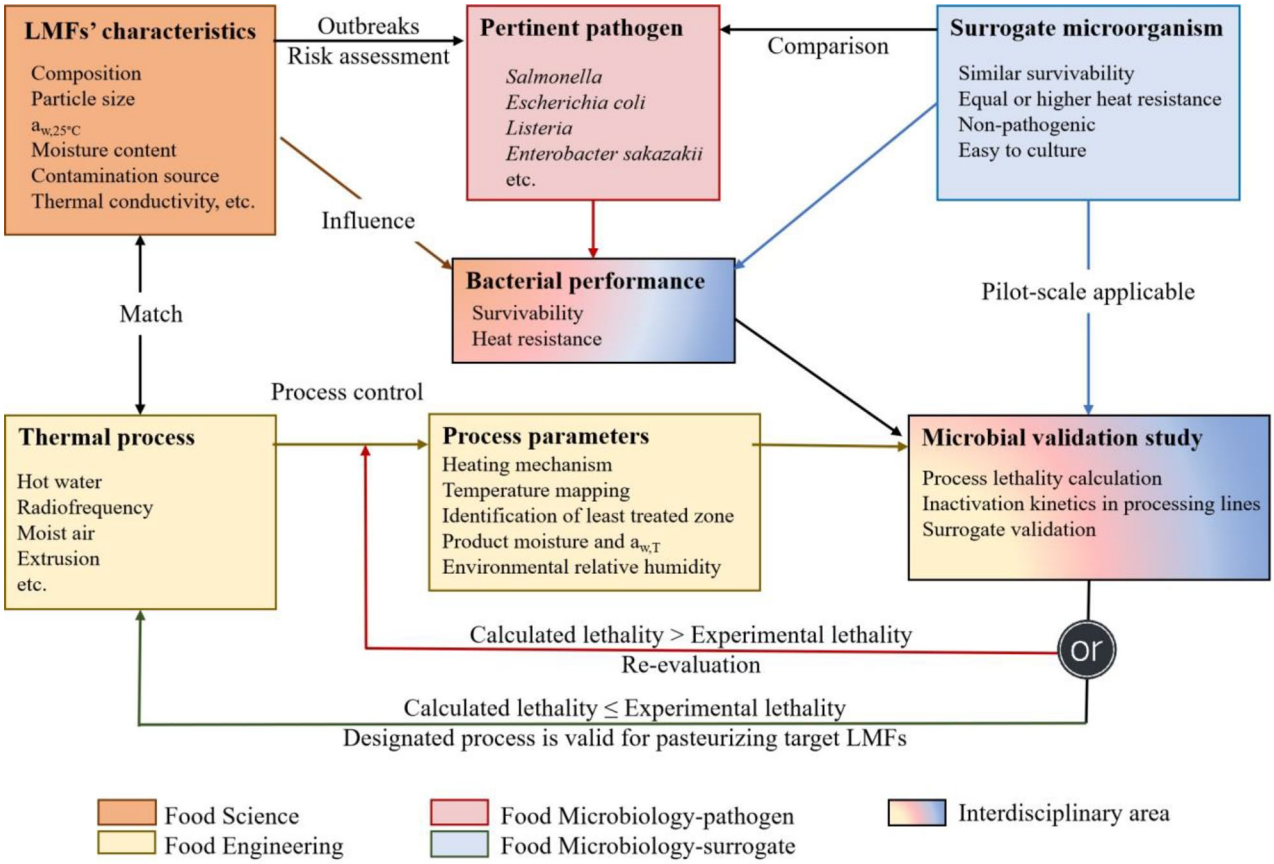

Figure 1. General structure for conducting a validation study of thermal processing LMFs.

(Daryaei et al. 2020; Quinn et al. 2021; Suehr, Anderson, and Keller 2019; Taylor et al. 2018) due to the outbreaks or recalls of these microorganisms in wheat flour and sunflower seeds (Food and Drug Administration (FDA) 2016b; Centers for Disease Control and Prevention (CDC) 2019). In addition, the presence of *Cronobacter sakazakii* (previously known as *Enterobacter sakazakii*) in powdered infant formula poses a severe risk due to its high mortality rate (27%) and serious social impact (Friedemann 2009; Koseki, Nakamura, and Shiina 2015).

Potential surrogates of pathogens in LMFs (e.g., *Enterococcus faecium* NRRL B-2354 (*E. faecium*) as *Salmonella* surrogate) were summarized by Theofel, Yada, and Harris (2019). Many studies have validated surrogate in specific LMFs (Arias-Rios et al. 2019; Brar and Danyluk 2019; Deen and Diez-Gonzalez 2019), and conducted the microbial validation studies using that surrogate (Channaiah et al. 2016; Liu et al. 2018a; Wei et al. 2020c). The use of surrogate *E. faecium* in validation studies were reviewed by Dhowlaghar and Zhu (2021).

2.2. Inactivation kinetics of microorganisms in LMFs

Accessing the lethality level of a thermal pasteurization process requires thermal resistance parameters of target pathogen or/and surrogate in selected LMFs (Awuah, Ramaswamy, and Economides 2007). These parameters were generated from mathematical modeling of thermal inactivation kinetics of specific microorganism under specific conditions (Peleg 2006). In addition, during most of thermal process, food matrix usually experiences dynamic changes of temperature and moisture content, such as increase in temperature while decrease in moisture content. Therefore, it is important to determine thermal inactivation kinetics at multiple temperature and water activity. Internal and external factors such as bacterial stress response, the $a_{w,25}$°C of LMFs, fat contents, and relative humidity (RH) were reported to impact bacterial thermal resistance in LMFs (Hildebrandt et al. 2016; Villa-Rojas, Zhu, Marks, et al. 2017; Yang et al. 2021). Therefore, microbial studies of LMFs involve a series of procedures (e.g., cultivation, inoculation, equilibration, inactivation study, enumeration, and mathematical modeling) that produce accurate and repeatable data. Methods to obtain the thermal inactivation data of microorganism in LMFs were reviewed by Cheng et al. (2021). Details in microbial studies of bacteria in LMFs were recently reviewed by our group (under review).

2.3. Temperature mapping and heat distribution

In thermal process, heat is the only factor that inactivates microorganisms. During the heating of LMFs, different locations in the same food matrix usually experience different temperatures history; therefore, measurement of temperatures is critical to evaluate a thermal process. Contacting (thermistor and thermocouples) and non-contacting

(infrared thermography) sensors could be used to measure the inactivation temperatures of microorganism in LMFs (Table 2) (Camuffo 2019; Vadivambal and Jayas 2011).

Temperature mapping identifies the lowest-temperature process condition in the tested equipment, and is critical for thermal processors (Anderson and Lucore 2012). Some LMFs processes have multiple zones with different temperature and RH: for instance, baking is a continuous process with heating and dehydration effects; while dielectric heating (e.g., microwave and radiofrequency) may heat up the LMFs non-uniformly (Luan et al. 2016).

Heat distribution measures the efficiency difference of a processor to deliver energy to the product, expressed as the temperature uniformity of products (Ozturk et al. 2017). Heating uniformity index (UI) is "the ratio of the rise in the standard deviation of sample temperatures to the rise in the average sample temperature during the heating" (Wang et al. 2005). The UI value is mainly utilized in temperature distribution studies of volumetric heating interventions such as radiofrequency and microwave heating (Hou, Ling, and Wang 2014; Jiao et al. 2012; Wang et al. 2005) because radiofrequency/microwave applies an uneven electromagnetic field to nonuniform food matrices, thus resulting in unsteady heating rates within tested foods.

2.4. Mathematical modeling and validation study

Lethality calculation of designated process is dependent on mathematical modeling of inactivation kinetic of bacteria in microbial studies (section 2.2) and temperature profiles of the least-treated zone (section 2.3) (Awuah, Ramaswamy, and Economides 2007). Isothermal inactivation kinetics of microorganism at multiple temperatures normally follow a first-order semi-logarithmic rate, generating the two key parameters (D- and z-values). The D-value represent a treatment time that results in 90% reduction of the existing microbial population at specific temperature (Eq. (1)), while the z-value represents the temperature needed to alter 10-fold in the D-values (Eq. (2)) (Gaillard, Leguerinel, and Mafart 1998; Peleg 2006).

$$\frac{N}{N_0} = 10^{\frac{-t}{D}} \quad (1)$$

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

where N_0 is the initial population of bacteria; N is the survivor populations (CFU/g) at given time t; D is the decimal reduction time at given temperature and t is the treatment time. The degree of pasteurization (P-value) was calculated by Eq. (3) based on the temperature histories of the least-treated zone (also namely cold-spot) (Lopez 1987):

$$P_T^z = \int_0^t 10^{(T-T_r)/z}\, dt \quad (3)$$

where P_T is the degree of pasteurization at reference temperature T for a certain z value; T is the actual temperature of the LMFs; T_r is the refence temperature. To estimate the ultimate microbial inactivation of thermal, it is necessary to consider the water activity change (moisture loss) during the process, since heat resistance of bacteria was in inversely proportion to water activity. Several secondary models were developed to estimate the D values of food pathogens at different temperatures and water activities (Pérez-Reyes et al. 2021; Wei, Agarwal, and Subbiah 2020b; Xie et al. 2021).

By embedding microorganism in the least-treated zone, we can make direct comparison between actual reductions of bacteria and calculated lethality (from Eqs. (1)–(3)). When the actual reduction is higher than the calculated lethality, a conservative validation is achieved for the designated process (Liu et al. 2018a). Notice that pathogens are rarely allowed in the food processing environment; non-pathogenic surrogate microorganisms are often used to implement the validation study in the real world (Busta et al. 2003; Almond Board of California (ABC) 2014).

3. Microbial validation studies of different thermal processes for LMFs

Based on the different properties of raw materials and desired final products, a few technologies (e.g., roasting, radiofrequency, moist-air, hot-room) were involved in the development of thermal pasteurization processes. Well-designed laboratory-based studies were conducted to assay both novel and existing technologies, either alone or in combination, to ensure microbial inactivation efficacy (Sánchez-Maldonado, Lee, and Farber 2018). Here, we review seven thermal technologies and their (potential) advantages in thermal processing of LMFs, suitable food matrices, factors impacting the inactivation efficacy, and published studies on the microbial validation of LMFs' pasteurization. These thermal technologies generally involve both heat and moisture in inactivation of microorganisms in LMFs (Figure 2); while the applicable treatment conditions of each technology were dependent on their heat-transfer mechanisms. Different technologies would involve specific temperature-moisture combinations and intensities (Figure 2). Therefore, we categorized these heating technologies into heat convection (moist and dry heat treatments), heat conduction (hot water, heat extrusion), and dielectric heating (radiofrequency and infrared heating).

3.1. Hot-air treatment (moist and dry heat)

Hot-air can efficiently transfer heat to the LMFs surfaces, and penetrate small cracks and crevices of LMFs that may contain numerous pathogens and molds (American Spice Trade Association (ASTA) 2017). To promote the decontamination efficacy, many advanced hot-air associated technologies were developed for pasteurizing LMFs: moist-heat (including vacuum-steam, superheated steam) and dry-heat (baking and roasting).

Table 2. Temperature sensors in thermal processing LMFs.

Mechanism	Appearance	Method	Suitable LMFs	Advantages	Disadvantages	References
Non-contacting sensors		Near-Infrared thermal imaging	Bulk powders Very thin cookies	Measure the samples' surface temperature	Noise in ambient may interact with the images	(Vadivambalan Jayas, 2011)
Contacting sensors		Thermistor sensor	Solids and liquids	Can insert in or attach on foods products	Sensor tips are fragile; Can only measure one spot.	(Camuffo 2019)
		Thermocouple sensor (K, J, T, E, N, S, R, and B types)	Bulk powders; Solids and liquids			

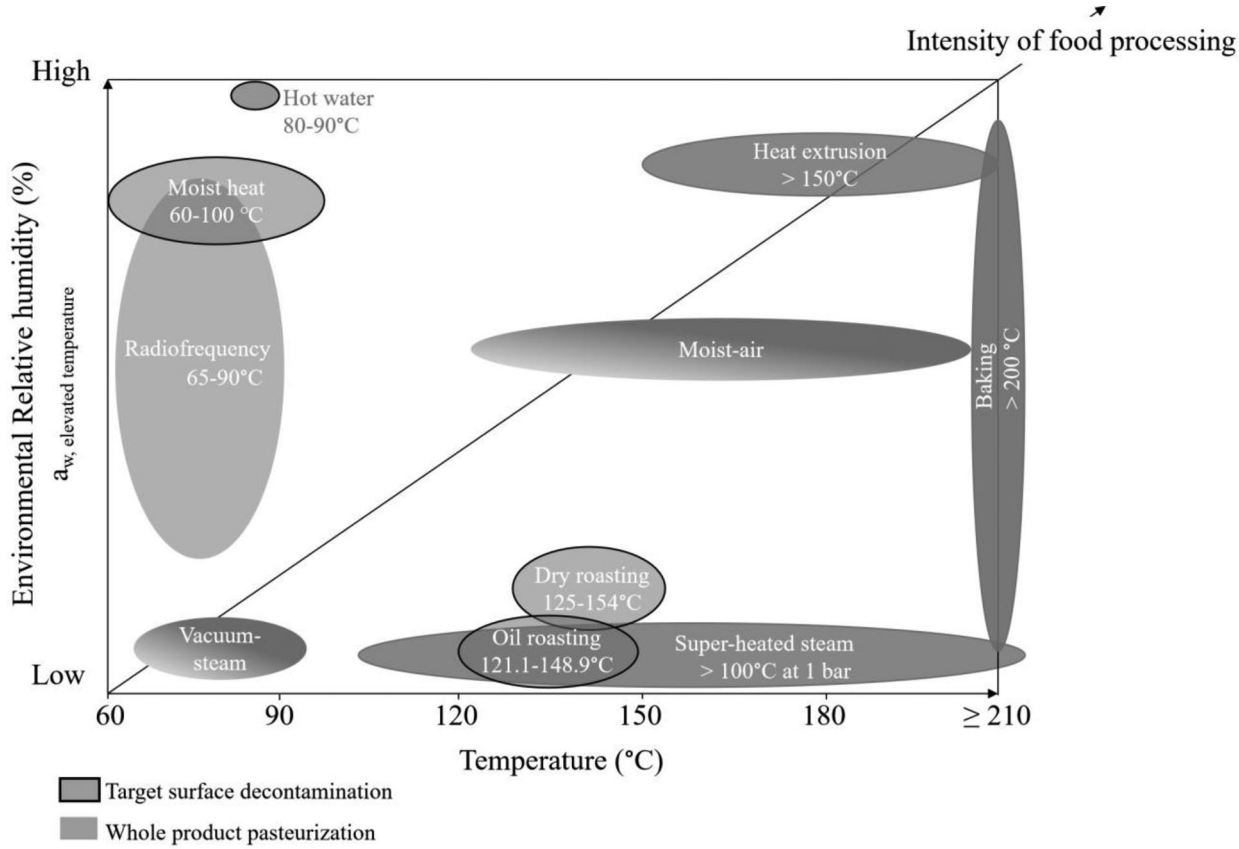

Figure 2. Potential thermal technologies for LMFs' pasteurization.

3.1.1. Moist-heat

The moist-heat process (i.e., moist-air or steam) introduces jets of moist-air mixture and increases the heat transfer rate by lowering the boundary layer thickness at the product surfaces (Moreira 2001). The steam first condenses on the surface of the samples during initial exposure, and water droplets begin to evaporate above the dew point temperature, thus generating high water vapor pressure inside the vessel. This dynamic process enables moist-heat to inactivate pathogenic microorganisms effectively on products' surfaces (Lilie et al. 2007) since the added moisture considerably lowers microbial thermal resistance (Doyle 2014).

Moist-heat exhibits as an open system because it involves hot-air flow and moisture transfer from the generators to treatment vessel. However, the treatment conditions may be fluctuant with the introduction of LMFs samples (Pan et al. 2012). LMFs also will generate specific water vapor pressure of food materials at elevated temperatures (Syamaladevi et al. 2016a). Ideally, iso-thermal and iso-humidity level could be achieved in inactivation of microorganism in LMFs by moist-heat oven (Anderson 2019). It appears to be a stable and predictable process that can eliminate microorganisms in LMFs. Jeong, Marks, and Ryser (2011) conducted thermal inactivation study of *E. faecium* B2354 and *S. enteritidis* on the surface of almonds in a convection oven. They obtained

D- and *z*-values of both strains, and concluded that *E. faecium* were 30% more thermal resistant than *S. enteritidis* PT 30 at four dry-bulb temperature levels (121–204 °C) and five RH levels (5–90%). Zhou et al. (2019) chose mild steaming treatments (<80 °C) at of peppercorns at $a_{w,25°C}$ values of 0.35, 0.57, and 0.69, and reported that steaming at 75 °C for 5 min ensured a ≥ 5-log reduction in the tested pathogens, (i.e., *Salmonella*, *L. monocytogenes*, and *E. coli* O157:H7). However, the increase of enthalpy of moist-air at elevated temperatures bring difficulty in setting iso-RH of convection ovens (Tadapaneni et al. 2017). Balancing both temperature and RH may be challenging in the pilot-scale ovens.

Apart from food matrices, inactivation studies of *S. enteritidis* and *E. faecium* in moist-heat treatment was conducted on silicon dioxide granules (0.2–0.7 mm) using a custom-designed thermal $a_{w,T}$ cell (Tadapaneni et al. 2017). Designed with the liquid-holding well at the geometric center, the thermal $a_{w,T}$ cell was able to control RH from 18% to 72% by adding lithium chloride solutions with different molality values, and bacterial cells were inactivated at different RHs. Results showed that *E. faecium* B2354 had higher $D_{80°C}$ values than *S. enteritidis* at RH values between 18% and 72% with equivalent $z_{aw,80°C}$ value (Liu et al. 2018b). This work supported the use of *E. faecium* as *S. enteritidis* surrogate in any low-moisture environment at 80 °C.

Use of vacuum in moist-air treatment, namely **vacuum steam pasteurization**, allows lower temperatures (70–100 °C) because the steam was saturated at reduced atmospheric pressure (Newkirk et al. 2018). It delivers efficient heat in a shorter period of time in a vacuum. By vacuum-steam process, Shah et al. (2017) achieved > 5 log reduction of pathogens in flaxseed and sunflower seeds at 75 °C for 1 min. Acuff et al. (2020) conducted inactivation studies of *Salmonella*, Shiga toxin-producing *E. coli*, *L. monocytogenes*, and surrogate *Pediococcus acidilactici* on raisins, apricot halves, and macadamia nuts in vacuum-steam treatments at 62, 72, and 82 °C. Reductions of the tested pathogens were comparable, which overall exceeded those of the surrogate. At same temperature and treatment time, the order of pathogen reductions on three products is: raisin, apricot halves, and macadamia nuts. This order might due to different increase of $a_{w,T}$ and moisture content of the treated samples. This study supported the use of surrogate in validating vacuum-assisted steam pasteurization, and the use of this technology as preventive control intervention of LMFs.

Superheated steam that raises the temperature above the saturation point at a given pressure (lower or higher than atmosphere) can effectively reduce pathogens in LMFs (Cenkowski et al. 2007; Deng et al. 2021). Due to the pressure difference, a temperature drop in the superheated steam will not produce condensation, unless the temperature falls below saturation. Several studies used superheated steam to obtain an adequate inactivation of pathogens and bacterial spores on grains (Cenkowski et al. 2007; Hu et al. 2016), nuts (Ban et al. 2018; Ban and Kang 2016; Bari et al. 2010), and black peppercorns (Ban et al. 2018). For instance, Ban and Kang (2016) reported more than 5-log reductions *of E. coli* O157: H7, *S. typhimurium*, and *S. enteritidis* on almonds and pistachios after the exposure to superheated steam at 200 °C for 15 s. The same group also obtained 5-log reductions of *Salmonella* in black peppercorns, almonds, and pecans in 180 °C superheated steam treatments within 3, 8, and 13 s, respectively (Ban and Kang 2016). Because superheated steam treatment is a high-temperature short-time process, it may not significantly affect the quality of LMFs. Bari et al. (2010) demonstrated that the overall quality of almonds was maintained well ($P > 0.05$) after superheated steam treatment at 115 °C for 70 s, and additional 70 s infrared heating. However, quality analysis on food samples after superheated steam treatments at very high temperatures (>150 °C) is lacking.

3.1.2. Dry heat

Dry heat treatment refers to any heating technique where heat is transferred to the food products without using any moisture. Roasting and baking are typical dry-heat processes that involve high heat (> 148.9 °C) and dehydration of food products.

Dry roasting is a typical process in nut and coffee industries and includes hot-air roasting and oil roasting (Beuchat and Mann 2011). In almond industry, its standard dry roasting process uses hot-air at 130–154 °C (Almond Board of California (ABC) 2007b), whereas oil roasting of almonds can provide ≥ 4 log reduction of *Salmonella* on almonds at 121.1–148.9 °C (Almond Board of California (ABC) 2007c). Beuchat and Mann (2011) also reported that the typical oil roasting process is sufficient to reduce *Salmonella* by 5 log CFU/g.

The designated temperature and time in the dry-roasting process depend on the roasting degrees (i.e., light, medium, or dark), the roasting conditions, and the variety, age, and moisture content of products (Mendes et al. 2001). Yang et al. (2010) achieved a 3.58-log reduction in the candidate *Salmonella* surrogate *Pediococcus spp.* by dry roasting almond kernels at 130 °C to the medium level. Du et al. (2010) reported that hot-oil treatment of almonds at 127 °C for only 1 min achieved a 5-log reduction for *S. enteritidis*. A recent study (Bingol et al. 2011) reported that promising technologies (e.g., infrared heating) and pre-wet treatment enhanced a dry-roaster's inactivation efficiency.

The Almond Board of California has published microbial validation guidelines for hot-air roasting and oil roasting almonds (Almond Board of California (ABC) 2007b, 2007c) with *S. enteritidis* as the target pathogen and *E. faecium* B2354 and *Pantoea agglomerans* as potential surrogates. For instance, 4.7 log destruction of *Pantoea agglomerans* at 121.1 °C is equal to a 4.0-log destruction of *S. enteritidis*. Further, a set of temperature and time combinations (e.g., 129.4 °C for 50 min) could also attain a 4.0 log reduction in *S. enteritidis*. The guidelines also outlined key elements ensuring the pasteurization of *Salmonella* on almonds' surfaces including the temperature profile of the center of almond kernels, treatment time, and thermal death data for *S. enteritidis* and potential surrogates (*D* and *z* values). The protocol applies to most nut kernels (e.g., pecans, walnuts, peanuts) and dry dates

(e.g., dry grapes). However, although specific details such as $a_{w,T}$, temperature, moisture content, and inoculation techniques reportedly impact bacterial thermal resistance in LMFs, these parameters were not mentioned (Hildebrandt et al. 2016; Laroche, Fine, and Gervais 2005; Liu et al. 2019). If researchers do not consider these parameters, they may get fluctuating microbial results. Full protocols on the challenge tests for *E. faecium* B2354-inoculated almonds and pistachios were available (Almond Board of California (ABC) 2014; Food and Drug Administration (FDA) 2011). They partially fulfilled the gaps in microbial validation of the dry roasting processes. However, this heat resistance study protocol is not suitable for water-absorbing kernels, LMFs with antimicrobial components on the surface, and water-soluble powders because it introduced water in the inoculation step.

Currently, the microbial validation protocol of dry roasting pasteurization is more developed than other thermal processes. Although its guidelines are not comprehensive, they have provided a well-developed standard for microbial validation study in LMFs.

Baking is a traditional heating, drying, and/or cooking process producing numerous types of LMFs. Most baked products were considered safe because they undergo a kill step, such as heating or cooking (Channaiah et al. 2016). However, outbreaks associated with *S.* Typhimurium PT 42 in a raw baking mixture including flour has been reported (McCallum et al. 2013). Validating baking process as a killing step is needed under the requirements of FSMA. A simulated industrial baking system for hamburger buns (Channaiah et al. 2016) was validated using both *Salmonella* and surrogate *E. faecium* B2354. Thermal resistance parameters of the strains in the dough were determined by sealing the inoculated samples in polyethylene Whirl-Pak filter bags, followed by submerging the sealed samples in a hot-water bath and enumerating survivors after heating and cooling at different time spots. The baking process was simulated in a baking oven at 218.3 °C. The authors presented a typical hamburger bun baking process by reporting the temperature profile of buns and the reduction in microorganisms in buns. They have verified that the baking process could achieve a > 6 log reduction in *Salmonella* at ≥218.3 °C for a minimum of 9 min. The same group (Channaiah et al. 2017) validated a typical commercial baking process of muffins using a *Salmonella* cocktail (Newport, Typhimurium, and Senftenberg). More than 5 log reductions of *Salmonella* cocktail were achieved by baking the muffin at 190.6 °C for ≥17 min. Therefore, baking process is proved to efficiently eliminate pathogens at high-temperature and long-term process conditions. However, one problem of the baking process is the nonuniform heating as the surface of the food product could be overheated while its inside remains undercooked. This replies on temperature tracking of both surface and core of foods during baking process.

3.2. Hot-water treatment

Immersing food products in hot-water can reduce pathogenic bacteria on their surface at mild temperatures (80–90 °C) and short times (<3 min) (Corry et al. 2007). For example, immersing raw almonds in water at 88 °C for 1.6–2.0 min could achieve a 4- to 5-log reduction in *S. enteritidis* (Harris et al. 2012). Bari et al. (2009) also documented that no *S. enteritidis* survivors were detected on almond surfaces after 20 s of hot-water treatment at 88 °C, followed by infrared drying for 70 s. However, they have detected *Salmonella* survivors 24 h after treatment. The authors claimed that the short and mild-temperature treatments using hot-water injure bacteria cells but do not eliminate nor inactivate them. Special attention is needed to monitor bacterial destruction after hot-water treatments following the re-drying process. In addition, hot-water treatment requires a re-drying step, which might result in significant quality loss of LMFs.

3.3. Heat extrusion

Heat extrusion is the process of mixing, compressing, and cooking dough-like ingredients in a barrel and forcing them through a specially designed die (Okelo et al. 2006). It is a continuous high-temperature (>100 °C) and short-time (in seconds) process (Doyle 2014), which could also include shear stress and high pressure. Standard products include low-density puffed cereals and snacks. Unlike hot-air and hot-water treatment, heat extrusion is too complex to be mimicked in a laboratory setting. Other validation methods include determining lethality based on the processing conditions (e.g., integrated time and temperature profile-based lethality) or choosing a reasonable surrogate to validate the processing time (The Association of Food Beverage and Consumer Products Companies 2009). The lethality determination of extrusion is rather difficult because thermal resistance parameters of *Salmonella* or other candidate surrogates are not available: extrusion is a continuous process, and samples cannot be taken out for measurements in the middle of any stage. The bacterial survival curves cannot be plotted, and only the endpoint survivors after the extrusion process can be counted.

Fortunately, using a proper validation surrogate to test the processing line seems feasible. For instance, *E. faecium* B2354 was validated as a suitable *Salmonella* surrogate in the extrusion process of a carbohydrate-protein meal and oat flour (Bianchini et al. 2014; Verma et al. 2018a). Also, Okelo et al. (2008) reported more *E. faecium* B2354 survivors than *Salmonella* at designated treatments. However, product formulations and process parameters affected the inactivation kinetics of *E. faecium* B2354 in a different fashion than that of *Salmonella* (Verma et al. 2018a). The difference between two strains ultimately lead to a complex lethality determination of *Salmonella* in alternative LMFs or processing lines.

Previous studies that validated the extrusion as a killing step have used either *E. faecium* (Bianchini et al. 2012; Verma et al. 2018b) or *Salmonella* (Anderson et al. 2017; Okelo et al. 2008; Rokey and Baldwin 2013) in various LMFs. Processing parameters such as extruder barrel exit temperature, feed moisture content, screw speed, fat content,

and mean retention time, were deemed critical in these validation studies (Okelo et al. 2006). For instance, Bianchini et al. (2012) collected data of thermal destruction of *E. faecium* B2354 (targeting a final level of 5 log CFU/g) in a carbohydrate-protein meal as a function of treatment temperature (67.5–85.0 °C) and moisture content (24.9–31.1%) in a single-screw extruder. The highest reported reduction in *E. faecium* B2354 was approximately 5 logs, which should enable more than 5 log reductions in *Salmonella* in the same conditions. Another optimization study in extruding oat flour (artificially inoculated with *Salmonella enterica* serovar Agona) reported that processing settings above 82 °C and 0.89 $a_{w,25}$°C enabled a > 5-log reduction in *Salmonella* (Anderson et al. 2017).

Since extrusion can effectively eliminate *Salmonella* in carbohydrate-rich products such as cereals, the microbial contamination, if any, would likely occur during post-processing or via the addition of coatings and flavorings (Anderson et al. 2017). Microbial safety of flavoring and coating ingredients (e.g., spices, color additives, edible coating) need to be ensured before their addition to extruded products. Existing data of the processing conditions (targeting 5-log reduction in *Salmonella*) were limited to the single-screw extruder (representing the worst-case scenario) with a certain extrusion speed, specific feed formula, and minimum moisture content level and temperature. Extrusion can only be used for processing of certain category of foods, such as powders, pastes and grain products.

3.4. Radiofrequency heating

Radiofrequency is an electromagnetic wave with a range of frequencies between 3 kHz and 300 MHz. According to dielectric mechanisms, radiofrequency vibrates bound water into thermal energy and is therefore suitable for heating LMFs (Feng, Tang, and Cavalieri 2002). As a volumetric heating technology, the critical challenge in radiofrequency heating is temperature nonuniformity. Electromagnetic waves in the radiofrequency spectrum can penetrate deeper into products; thus, there is less overheated surfaces, as compared with microwave heating (Piyasena et al. 2003). Few studies have also reported strategies for improving the heating pattern of peanut butter (Jiao, Tang, and Wang 2014), corn flour (Ozturk et al. 2017), and dates (Tiwari et al. 2011). These data can support validation studies to ensure that the microbial results came from the least-treated zone, and that the lethality determination or microbial validation can use the temperature profiles of the least-treated zone. A laboratory study using TDT cells (in a closed system) can mimic the radiofrequency heating process when it heats food samples in a steady state. The reported thermal resistance of microorganisms in LMFs at isothermal treatments can support the lethality determination of the given temperature profiles.

The number of studies on radiofrequency -assisted pasteurizing LMFs boosted in the past few years. Validation studies have been successfully done in pasteurizing *C. sakazakii* in dry milk products (Michael et al. 2014; Y. Zhang, Zhao, et al. 2020), *Salmonella* in flours, spices, and nuts (Liu et al. 2018a, 2018b; Wei et al. 2019, 2020a; Zhang et al. 2020), *E. coli* in spices and nut-derived products (Cheng et al. 2020; Ha et al. 2013; Kim et al. 2012; Li et al. 2017), fungi in nuts (Hou et al. 2018; Zhang et al. 2021), and spores in peppers (Jiao et al. 2019). Recent developments and applications of radiofrequency on pasteurizing LMFs were reviewed (Dag, Singh, and Kong 2020; Jiao et al. 2018; Ling, Cheng, and Wang 2020).

Most of these articles involve both lethality calculation and microbial study. They followed the same protocol – inoculated pack study—to complete the microbial validation study (Ozturk et al. 2019; Xu et al. 2018; Zhang, Zhao, et al. 2020). Briefly, a small pack of inoculated samples (3–5 g) was positioned on the cold spot for process, at which the temperature profile was recorded by a fiber-optic sensor. The survivor curve of bacteria in the pack was traced and compared with the prediction trend (modeled from temperature profile and thermal resistance parameters of the target strain). Slightly higher reduction of the bacterial survivors in experiments (than predicted lethality) indicated the completion of a conservative validation. This inoculated-pack procedure enables researchers to use a few inoculated samples as the representative of cold spot without contaminating the rest of the samples. However, this procedure utilized inoculated samples in a closed bag and is only applicable in closed-system pasteurization (Liu et al. 2018a, 2018b). In addition, the lethality calculation of RF-assisted pasteurization, using lab-based heat resistance parameters, did not consider the sublethal effect of RF heating. Zhang, Zhao, et al. (2020) recently reported that sublethal injured cells of *S.* Typhimurium existed in RF-pasteurized red pepper powders when the food samples have initial $a_{w,25}$°C ≥ 0.53. Although these sublethal injured cells (induced by RF heating stress) did not show direct and cross protection effects (Jiao et al. 2021), we still need to be cautious on the lethality prediction and microbial validations of RF-treated LMFs. Further investigation on stress response of pathogens in LMFs would be helpful to understand the sublethal effect of RF heating.

Without lethality calculation, studies applying pathogens and their surrogates (if available) serve as direct validation approaches (Gao et al. 2011; Guo et al. 2010; Wang et al. 2012). Most of them provided sets of parameters (e.g., temperature, treatment time, moisture content) that can ensure a certain level of reduction. For instance, Villa-Rojas, Zhu, Marks, et al. (2017) applied radiofrequency -assisted heat treatment to reduce *S. enteritidis* and *S. tennessee* K4643 in wheat flour, achieving 4-log decrease in *Salmonella* after 6 min at 80 °C. This procedure is more effective than conventional heating due to the short come-up-time of the wheat flour sample. Similar studies were documented on radiofrequency heating of almonds (Gao et al. 2011), peanut butter cracker sandwiches (Ha et al. 2013), black and red pepper spices (Wei et al. 2019; Tong et al. 2022), cumin seeds (Chen, Peng, et al. 2019), egg white powder (Wei et al. 2020a), and basil leaves (Verma et al. 2021b). These studies confirmed that radiofrequency heating can

potentially eliminate pathogens in LMFs, and documented processing conditions regarding specific products.

Some latest studies have provided quality analyses of LMFs samples (spices, dates, and flours) after radiofrequency treatment (Cui et al. 2021; Tong et al. 2022). For example, Wei et al. (2020a) achieved 5.98 log CFU/g reduction in *Salmonella* spp. in ground black pepper at an average temperature of 80.1 °C for 130 s. After radiofrequency treatment, the major volatile compounds of ground black pepper only changed slightly. A similar study was performed by Saka et al. (2021) in white and whole wheat flour. The overall impact of radiofrequency pasteurization on physical, chemical, rheological and bread-baking properties was minimal. Both studies support the use of RF in LMFs pasteurization. Radiofrequency heating was found to enhance the gelling and foaming properties of egg white powder (Boreddy et al. 2016; Kar et al. 2020). However, Liu et al, 2021 did report severe quality deterioration of spice Sichuan pepper (*Zanthoxylum bungeanum*) after radiofrequency heating step. The oil droplets on Sichuan pepper surface broke rapidly because the dielectric properties of oils were far different than other tissues of Sichuan pepper. This study warns the application of radiofrequency on LMFs that have uneven oil distribution.

3.5. Infrared heating

Infrared radiation is an electromagnetic radiation that is transmitted as a wave and gets converted into heat when it impinges on the food surface (Navin 2012). Infrared heating provides rapid and uniform heating for foods' surfaces, and is normally utilized as one key step in combined thermal pasteurization of LMFs. Bari et al. (2009) assessed a series of approaches (sanitizers, dry heat, hot-water, and gas catalytic infrared heat) to inactivate *Salmonella* on raw almonds, and found that although a single method could not ensure *Salmonella* target lethality, the combined methods could. Venkitasamy et al. (2017) conducted a validation study on pistachios and found that tandem infrared drying, tempering, and hot-air drying can reduce *E. faecium* by 6.1 log CFU/g on pistachio kernels.

Shirkole et al. (2021) applied short time intensive microwave-infrared (MW-IR) radiation to paprika (*Capsicum annuum* L., a pulverized product of dried red pepper), and obtained the inactivation kinetics of *S.* Typhimurium and *A. falvus* on paprika's surface. MW-IR heating eliminated 7.3 and 6.2 log CFU/g of *S. Typhimurium* and *A. falvus*, respectively, at various treatment conditions (e.g., 10 W/g microwave powder density, 150 °C IR temperature, and 8 cm IR distance, 20 s heating time). However, the authors did not conduct temperature mapping of the heated paprika. It brings difficult to estimate the actual level of lethality because the end-points fell out of detection limit of bacteria in all treated samples.

3.6. Evaluation of microbial validation studies

In the validation studies mentioned above, the tested food matrices were mostly nuts, flour, and powders. For the same product, experimental factors such as methodology, mathematical models describing the thermal behavior of microorganisms, and process technology were all critical to select and implement protocols. Ongoing regulatory changes require predictable process validation protocols for pathogen reduction aimed for LMFs; therefore, the reproducibility of different validation methods is critical.

However, systematic comparisons of these validation studies are limited. Jeong, Marks, and Ryser (2011) inoculated almonds using *S. enteritidis* and *E. faecium*, followed by equilibration to four $a_{w,25°C}$ levels (0.24, 0.45, 0.58, and 0.78) and heat treatments in a moist-air impingement oven. They built four validation models (two biological and two time-temperature models) and reported a vast difference in repeatability and accuracy. They highlighted the significance of a particular methodology, $a_{w,25°C}$, and process humidity in the thermal pasteurization processes of LMFs. Furthermore, Hildebrandt et al. (2016) noted that the inoculation method would affect the performance of the pathogen under heat treatment, thus ultimately affecting validation efficacy. The acclimation of *Salmonella* inoculated LMFs has been observed in several microbial challenge studies (Lambertini et al. 2016; Lang et al. 2017; Wei et al. 2020a), which could allow the bacteria adapt to the extreme environment and enhance the resistance of *Salmonella* in the subsequent inactivation treatment. Therefore, acclimation prior to the experiment is critical for improving the external validity of the process validation (Allison and Fouladkhah 2018).

Lambertini et al. (2012) emphasized that enhanced awareness of the process deviation and uncertainty of pathogen decline would likewise enhance risk assessments. Therefore, strictly controlling these key factors should be the focus of any processors to implement and interpret validation results and ensure food safety.

Researchers are expected to build mathematical models in microbial validation studies and apply them in real-world thermal processes. However, their application has multiple challenges such as enlarged sample size, reduced heating rate, scale-up of equipment, temperature nonuniformity, moisture transfer and evaporation of heated samples, unstable bacterial performance, and other uncertainties. These variables lead to non-isothermal and non-iso-moisture treatments, which are very different from isothermal and iso-moisture studies conducted in laboratories. Therefore, three key challenges in microbial validation of LMFs' pasteurization are (Jeong, Marks, and James 2017): (a) monitoring the dynamic process parameters, and estimating their roles in lethality calculations: (b) quantifying uncertainties through the implementations of microbial validation studies; and (c) accounting for inconsistencies in validating the target process and ensuring its efficacy.

4. Scientific data and knowledge gaps in microbial validation of thermal processes of LMFs

Despite the identification of pathogen *Salmonella* and selected surrogate *E. faecium* B2354, there are still knowledge gaps in the microbial validation of the thermal

processing of LMFs. Microbial procedures (such as cultivation and inoculation) used in thermal inactivation studies are not standardized for most LMFs. To date, almond industry has published the most standardized validation guidelines and required validation studies to be reviewed by a panel of thermal processing experts (Almond Board of California (ABC) 2014). Scholars have kept generating reduction curves and thermal resistance data in the thermal processing of LMFs and subsequently applied them to food safety analyses without fully understanding the impacts of external and internal factors on bacterial thermal inactivation (Hildebrandt 2015; Syamaladevi et al. 2016b). Because the thermal resistance of most microorganisms is dependent on food matrices, a full-factorial comparison of *Salmonella* and *E. faecium* B2354 independently from food systems may provide clearer insight. More information on critical process parameters is desirable to understand real-time inactivation kinetics (Villa-Rojas, Zhu, Marks, et al. 2017). Some of the gaps are explained in detail in the next sections.

4.1. Standard operating procedures (SOP)

Many guidelines are available for controlling *Salmonella* in LMFs (Anderson and Lucore 2012; Almond Board of California (ABC) 2014; Food and Drug Administration (FDA) 2009, 2011; OpX Leadership Network 2016; The Association of Food Beverage and Consumer Products Companies 2009). In particular, the ABC guidelines were specifically developed and have been extensively tested to validate almond pasteurization technologies (ABC 2007b, 2007c, 2014). Each guideline has provided specific protocols for one certain process of limited LMFs (e.g., hot-air treatments for nut kernels). Knowledge gaps in SOPs include, but are not limited to, process variability, uncertain lethality outcomes for a given process, the influence of measurement approaches, and replication required to achieve a target food safety outcome.

Several publications have addressed the importance of microbial protocols in validation studies, such as inoculation procedures (Hildebrandt et al. 2016), a_w of tested samples (Smith et al. 2016; Tadapaneni and Foods 2018), moisture level of bacterial cells (Xie et al. 2020), biofilm/non-biofilm forming strains (Villa-Rojas, Zhu, Marks, et al. 2017), bacterial strains (Acuff et al. 2020), recovery and enumeration methods (Hasani et al. 2020), and food components (Syamaladevi et al. 2016a). To date, some critical steps (e.g., lawn-harvest method, equilibration of a_w, and inoculated pack study) of the published protocols are widely accepted as SOPs (Almond Board of California (ABC) 2014; Liu et al. 2018a, 2018b; Wiertzema et al. 2019).

4.2. Calibration of surrogate strain

In the calibration of bacterial spores in high-moisture foods, *C. sporogenes* (PA 3679) spores are suspended in phosphate buffer (pH 7.0) and injected into capillary glass tubes for TDT tests at 121.1 °C (Norusiuen et al. 1978; Odlaug and Pflug 1977). The generated $D_{121.1\,°C}$ and z_T values can validate the combination of microwave and circulated water heating technology (Guan et al. 2003). The guideline published by ABC includes a "heat resistance test" section characterizing surrogate resistance. Briefly, the inoculated and dried almond kernels were placed onto a metal mesh and transferred into a convection/forced-air oven for heat treatment at 137.8 °C for 15 min. The acceptable heat resistance of *E. faecium* B2354 was that of ≤ 2.5-log reduction. Nonetheless, the equipment (i.e., metal mesh) is not entirely relevant to alternative LMFs, such as powdered foods, date paste, and nut meals. The ABC guidelines also neglect the need for monitoring RH or the temperature of the food matrix, which later proved as crucial points (Syamaladevi et al. 2016b). The inadequate control of these factors might cause a broad variance. Last but not least, the acceptable heat resistance range (137.8 °C/15 min≤ 2.5-log reduction) on almonds may not apply to other LMFs.

The different thermal resistance amounts of *Salmonella* and *E. faecium* B2354 are associated with a_w, food components, physical structures, and water isotherms. Syamaladevi, Tang, and Zhong (2016c) reported that bacterial cells rapidly adapt to the environment (within seconds). The $a_{w,\text{treatment temperature}}$ is the real-time a_w that affects microbial thermal resistance and determines isothermal treatment efficiency (Syamaladevi, Tang, and Zhong 2016c). This concept has been validated for *S. enteritidis* and *E. faecium* B2354 in wheat flour at 80 °C (Liu et al. 2018b), in silicon dioxide at 80 °C (Liu et al. 2018b), and for *S. enteritidis* only in three types of flours at 80 °C (Xu et al. 2019). These studies were conducted in different TDT cells (Cheng et al. 2021). By using these aluminum cells, one can generate more information on the behavior of other microorganisms in alternative food matrices and in a wider temperature range to verify the relationship before applying it in surrogate calibration.

When using surrogate for process validation, many food manufacturers prefer to targeting a 5-log reduction of the surrogate, which works well when the thermal resistance of surrogate is similar or slightly higher than the target food pathogen. However, when the thermal resistance of surrogate is much higher (two or three times) than the target food pathogen, the food products are expected to be overprocessed (Ma et al. 2007). Using kill ratio between surrogate and the target food pathogen would be a way to avoid over-processing (Grocery Manufactures Association 2010; Perry, Peña-Melendez, and Yousef 2019). Since the bacterial thermal resistance in LMFs are highly dependent on the a_w and food components, identify a general way to use the kill ratio for process validation could increase the efficient of thermal process, save energy and improve overall food quality.

4.3. Monitoring systems

The reliability and consistency of a designated process validation need to be performed under a precise monitoring system. The monitoring of the operating system includes performing a defined sequence of observations and assessments of control parameters to determine if a

measure is completely controlled (Codex 2008). During the production of target foods, temperature readings of the process equipment, processing time, and product moisture/a_w readings are recorded to ensure minimum required levels.

When receiving food ingredients from different suppliers, inconsistent food quality could be expected even between batch. For example, moisture content of milk powder required to be less than 4.5%, therefore the moisture content of milk powder usually could be varied from 2 to 4% (Vieira, Freire, and Freire 2015). The thermal process required to pasteurize milk powder is highly dependent on its moisture content or water activity: because the enhance heat resistance at lower a_w, more serve thermal would be necessary for milk powder with 2% than 4% moisture content (Wei, Agarwal, and Subbiah 2020b). Therefore, the thermal process should be developed in a way that could response to the monitoring system, so it could be adjusted to overcome this challenge by manipulating its operation temperature or treatment time.

4.4. More challenges of thermal processing LMFs

LMFs have various physical and chemical properties. Most products have poor heat transfer rate because of their low thermal conductivity, whereas some cannot bear high temperatures. Onion powder with $a_{w,25\,°C} < 0.30$ is very heat sensitive with a glass-transition temperature of 71.1 °C. Even though a 4–5-log reduction of the target pathogen (*Salmonella*) is a suggested requirement, food companies may demand greater inactivation values than the requirement for further insurance. For instance, spices require pasteurization of 5 log reduction in *Salmonella* to be pasteurized before the flavoring and coloring steps in LMFs (section 4.4). When used as ingredients by other food manufacturers, the spice also needs a standard plate count of <1,000 CFU/g to eliminate microorganisms from the sources for most high-moisture foods. However, this requirement imposes difficulties on spice producers to balance quality and standard plate count.

In general, these are some of the knowledge gaps in the microbial validation of thermal processing for LMFs: (a) lack of information on the effects of various extrinsic and intrinsic factors on the thermal resistance of microorganisms; (b) lack of protocols that can calibrate surrogate *E. faecium* B2354 before its application in validation studies; and (c) lack of information on critical control points in different thermal processes. Reliable scientific data are needed to fill the gaps to ensure LMF safety by thermal treatments with confidence.

5. Conclusions

This review covers the fundamentals of the microbial validation study and the developments on preventive controls and process validations. As the last step before industrial application, a validation study involves people that design, conduct, evaluate, and implement the thermal processing of LMFs. Three validation approaches are commonly used including scientifically valid data provided by authoritative guides, microbial experiments by measuring systems and enumerating survivors at the endpoint, and mathematical modeling and monitoring of factors involved in models. Here, we summarized studies that have contributed to all three aspects. Besides, these studies have provided scientific information on the behaviors of the pathogen *Salmonella* and surrogates (product components-related, moisture content, a_w, and temperature) and the lethality of existing technologies and novel thermal processes based on experiments and modeling.

On the other hand, despite the progress on microbial validation studies of different thermal technologies, many challenges remain in conducting a successful validation study using microorganisms. The preparation and calibration of microorganisms are as crucial as the identification of the cold spot and the design of processing conditions. Research needs to be done to develop a systematic procedure that can apply to different kinds of LMFs and types of processing technologies. Close collaboration among microbiologists, process engineers, modeling statisticians, and other related professionals will ensure the positive outcomes needed to accomplish microbial validation studies of the thermal processing of LMFs.

Author contributions

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

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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