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Research Note

Bootstrapping for Estimating the Conservative Kill Ratio of the Surrogate to the Pathogen for Use in Thermal Process Validation at the Industrial Scale

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ABSTRACT

A surrogate is commonly used for process validations. The industry often uses the target log cycle reduction for the test (LCR_{Test}) microorganism (surrogate) to be equal to the desired log cycle reduction for the target (LCR_{Target}) microorganism (pathogen). When the surrogate is too conservative with far greater resistance than the pathogen, the food may be overprocessed with quality and cost consequences. In aseptic processing, the Institute for Thermal Processing Specialists recommends using relative resistance (D_{Target})/(D_{Test}) to calculate LCR_{Test} (product of LCR_{Target} and relative resistance). This method uses the mean values of D_{Target} and D_{Test} and does not consider the estimating variability. We defined kill ratio (KR) as the inverse of relative resistance. The industry uses an extremely conservative KR of 1 in the validation of food processes for low-moisture foods, which ensures an adequate reduction of LCR_{Test}, but can result in quality degradation. This study suggests an approach based on bootstrap sampling to determine conservative KR, leading to practical recommendations considering experimental and biological variability in food matrices. Previously collected thermal inactivation kinetics data of *Salmonella* spp. (target organism) and *Enterococcus faecium* (test organism) in Non-Fat Dried Milk (NFDM) and Whole Milk Powder (WMP) at 85, 90, and 95°C were used to calculate the mean KR. Bootstrapping was performed on mean inactivation rates to get a distribution of 1000 bootstrap KR values for each of the treatments. Based on minimum temperatures used in the industrial process and acceptable level of risk (e.g., 1, 5, or 10% of samples that would not achieve LCR_{Test}), a conservative KR value can be estimated. Consistently, KR increased with temperature and KR for WMP was higher than NFDM. Food industries may use this framework based on the minimum processing temperature and acceptable level of risk for process validations to minimize quality degradation.

Low-water activity foods (LWAFs) are defined as foods with a water activity (a_w) of less than 0.65 (Food and Agriculture Organization, 2003) and were historically regarded as low-risk commodities for microbial contamination (Beuchat et al., 2013). These include a variety of food items such as dried fruits and vegetables, grains, cereals, soups, confections, products made of protein powder such as dairy and egg powders, honey, herbs, spices, seeds, and nuts (Sharma et al., 2021; Wason et al., 2021). Despite their reduced a_w, pathogens such as *Salmonella enterica* can survive in the dry environments of LWAF for months to years (Podolak et al., 2010). Various serotypes of *Salmonella* were implicated in LWAF outbreaks between 2000 and 2019, which caused 3,880 illnesses, 659 required hospitalization, and 15 deaths (Dhowlaghar and Zhu, 2022).

One LWAF that is commonly utilized as an ingredient in various ready-to-eat foods such as infant formula, beverages, and protein shakes is milk powder (Wei, Lau, Chaves, et al., 2020). Powdered milk is usually made by spray drying nutrient-rich, pasteurized, liquid milk at 180–220°C. However, spray drying is not an effective kill step, and many heat-resistant bacteria such as *Salmonella enterica* and *Cronobacter sakazakki* can endure the high temperatures of spray drying (LiCari and Potter, 1970; Miller et al., 1972; Lin et al., 2020). Moreover, pasteurized milk powders may be cross-contaminated at various stages of processing if sanitation or quality control practices are violated, whether accidentally or negligently. Several foodborne illness outbreaks and recalls due to pathogenic contamination in milk and infant powder formula have been documented in recent years (Brouard et al.,

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2007; Cahill et al., 2008; U.S. Food and Drug Administration, 2022; Lin et al., 2020; Park et al., 2004; Rowe et al., 1987). Increasing numbers of outbreaks coupled with the high economic value of milk powders demand the need to ensure the microbial safety of milk powder for human consumption as a ready-to-eat product (Vashisht et al., 2022). One of the widely used techniques for pathogen inactivation in dairy powders is thermal processing. Various thermal inactivation studies conducted in the past on milk-related products (Dag et al., 2022; Lau et al., 2020; Wei, Lau, Chaves, et al., 2020) have pushed the dairy industry closer to the goal of having recognized and validated thermal processing steps following spray drying. To ensure that processes are adequate to control microbial hazards, the FDA requires that preventive controls be validated (CFR, 2015). Thus, process validation is conducted to obtain scientific and technical evidence and determine if a particular treatment technology can achieve the target microbial kill in the food products (NACMF, 2010). However, using pathogens (target microorganisms) in an industrial facility for process validation could pose a potential threat to the operator or contaminate the food products (Acuff et al., 2023). Thus, the suggested plan of action is to utilize a nonpathogenic surrogate (test microorganism) that has been proven to have thermal resistance comparable to or greater than that of the target food pathogen.

According to various studies, *Enterococcus faecium* NRRL-2354 can be used as a potential nonpathogenic surrogate for in-plant validation of thermal inactivation of *Salmonella enterica* in various LWAF such as almonds (Almond Board of California, 2007), wheat flour (Villa-Rojas et al., 2017), oat flour (Verma et al., 2018), egg white powder (Wei, Lau, Reddy, et al., 2020), dried basil leaves (Verma et al., 2021a, b; Wason et al., 2022a), black pepper (Wei et al, 2018, 2021; Wason et al., 2022b), and cumin seeds (Chen et al., 2019, 2020). The industry often uses the target log cycle reduction for test (LCR_{Test}) microorganism (surrogate) to be equal to the desired log cycle reduction for target (LCR_{Target}) microorganism (pathogen). This is a worst-case conservative approach for heat-based processes that will ensure a minimum log inactivation (5-log reduction) for target pathogens tested in low-water activity food powders. However, to achieve this level of microbial inactivation, food products may have to be treated at extreme temperature conditions that may negatively impact product quality. Therefore, there is a need to provide a sound scientific basis that would warrant the use of less than 5 log CFU/g surrogate inactivation corresponding to a 5 log CFU/g inactivation of the target pathogen to minimize product quality deterioration.

In aseptic processing, the Institute for Thermal Processing Specialists recommends the use of relative resistance $(D_{Target})/(D_{Test})$ to reduce LCR_{Test} (IFTPS, 2011). However, this method does not consider the variability in determining D_{Target} and D_{Test}, and therefore, this approach is liberal. In this paper, we defined kill ratio (KR) as the inverse of relative resistance. Thus, the industry is either using an extremely conservative approach (KR = 1) or a liberal approach (mean KR = $(D_{Test})/(D_{Target})$). There is a need to develop a scientific framework to estimate a conservative KR (that falls between 1 and mean KR) so that the food product quality deterioration is minimized while ensuring food safety within acceptable levels of risk.

A statistical data-based simulation method called bootstrapping makes use of a resampling approach to account for the uncertainty of experimental data (Beasley and Rodgers, 2012). This method involves generating a large set of bootstrap samples by randomly selecting data points with replacement that leads to a sampling distribution of the statistic of interest. This offers valuable insights into the variability and uncertainty associated with the initial data (Hass et al., 1999). Bootstrapping offers a robust approach to empirically estimate sampling distributions without relying on predefined distributional assumptions (Efron & Tibshirani, 1991). Bootstrapping methods have been used to optimize food processing conditions and assess the food safety risks in several microbial research studies. Schaffner (1994) applied the bootstrapping method for calculating the variance in growth rate for three large datasets of *Listeria innocua*, *Listeria monocytogenes*, and *Yersinia enterocolitica*. In another study, the parameters of Baranyi and Roberts microbial growth models were estimated by generating resampled data sets using bootstrapping (Lee et al., (2007). Following this study, Lee and Park (2008) used the bootstrap method to estimate the experimental variability for modeling microbial growth on chilled food.

The objective of this study was to suggest an approach utilizing bootstrap sampling to estimate conservative KR that could lead to more practical recommendations considering experimental and biological variability in food matrices. The findings of this paper will guide the dairy industry in executing the thermal processing of milk powders to ensure the desired inactivation of *Salmonella* without compromising the product quality. The framework can be used to estimate conservative KR values in other food products.

Materials and methods

Thermal Inactivation of Salmonella and E. faecium in milk powders

The inactivation kinetics data of *Salmonella* and *E. faecium* were obtained from Wei et al. (2021). To summarize the methods used by Wei et al. (2021), Whole Milk Powder (WMP) and Non-Fat Dried Milk powder (NFDM) were inoculated with the 5-strain *Salmonella* and *E. faecium* cocktail. The homogenized inoculated sample was then equilibrated to an a_w of 0.10 using a specially built relative humidity chamber (Lau and Subbiah, 2020). After equilibration, inoculated samples were stored for 30 days and were then thermally treated in a thermal death time (TDT) sandwich at temperatures of 85, 90, and 95℃ for 6 uniformly spaced time points.

Estimation of mean KR

Wei et al. (2021) used the non-linear regression method in the Python SciPy library to estimate D-values using equation (1).

$$\log_{10}\left(\frac{N}{N0}\right) = -\left(\frac{t}{D}\right) \tag{1}$$

Instead of fitting nonlinear regression, we used an equivalent simple linear regression. Therefore, the mean D-values calculated using this method are slightly different in some of the cases from what was reported by Wei (2021). The D-values calculated as the negative inverse of the slope value (inactivation rate, k) were then used to calculate the mean KR using equation (2):

$$\text{Mean KR} = \frac{k_{Sal}}{k_{EF}} = \frac{D_{EF}}{D_{Sal}} \tag{2}$$

where k is the inactivation rate, D = D-value (time (mins) required for 1-log reduction), EF = *E. faecium* and Sal = *Salmonella*. Using the above formula, the mean KR of *E. faecium* to *Salmonella* in WMP and NFDM at 85, 90, and 95℃ were calculated.

Bootstrapping

A bivariate fit of the response (Log CFU/g adjusted for Time = 0 min) vs Time (mins) was conducted in JMP Pro 17, using a no intercept model. Bootstrapping generated 1000 bootstrap values of slopes (inactivation rates, k) for each of the 12 groups (2 milk powders x 3 temperatures x 2 bacteria). D-value was calculated by the negative inverse of the inactivation rate. The 1000 bootstrap values for *Salmonella* and *E. faecium* were combined to generate bootstrap KR's as shown in equation (3).

$$\text{Bootstrap KR} = \frac{Bootstrapk_{Sal}}{Bootstrapk_{EF}} = \frac{BootstrapD_{EF}}{BootstrapD_{Sal}} \tag{3}$$

Selected percentiles of the sampling distribution of KR are displayed in Table 2.

The distribution of bootstrap KR was calculated for each of the temperature-product combinations. The bootstrap KR values were then sorted in ascending order. From the sorted distribution of KR values, the conservative $KR_{x\%}$ is determined such that x% of bootstrapped KR values are less than $KR_{X\%}$, where $0 < x < 50$. The value of x should be selected by the industry or regulators based on the risk tolerances. For example, the conservative $KR_{1\%}$ is the 10th value in the sorted 1000 bootstrap KR values. In practical terms, this means that if the experiments were repeated 100 times, there is a 1% chance that the conservative KR may be less than the value of $KR_{1\%}$. In those 1% of the cases, the desired LCR_{Target} may not be achieved. Similarly, the $KR_{5\%}$ and $KR_{10\%}$ values correspond to the proportion of samples $< KR_{5\%}$ and the proportion of samples $< KR_{10\%}$. They are the 50th and 100th values in the sorted 1000 bootstrap kill ratio values. The $KR_{50\%}$ is the median value of distribution of KR, which should be close to the mean KR. Note that the conservative $KR_{X\%}$ ranges between 1 (conservative value used by the industry) and mean KR or $KR_{50\%}$ (a liberal estimate, as recommended by IFTPS for aseptic packages).

Results and discussion

Thermal Inactivation of Salmonella and E. faecium in WMP and NFDM

In brief, the survival values of *Salmonella* and *E. faecium* provided by Wei et al. (2021) were used to calculate the inactivation rate (Table 1). The negative inverse of inactivation rate was then calculated as D-value. As shown in Table 1, the inactivation rate of *E. faecium* ranged from −0.052 to −0.202 for NFDM and from −0.059 to −0.241 for WMP. Similarly, the inactivation rate of *Salmonella* ranged from −0.087 to −0.555 for NFDM and from −0.127 to −0.601 for WMP. This shows that *E. faecium* had a significantly lower inactivation rate (or higher D-values) than *Salmonella* at each treatment condition, indicating that *E. faecium* was more resistant to thermal inactivation relative to *Salmonella*. Therefore, *E. faecium* is an acceptable non-pathogenic *Salmonella* surrogate for thermal processing of milk powders at process temperatures between 85 and 95℃. Similarly, a study conducted by Ahmad, Hildebrandt, et al., (2022) found that *E. faecium* exhibited three times higher thermal resistance than *Salmonella* at 85, 90, and 95℃ in NFDM. Another study found that $D_{90℃}$ values were two times higher for *E. faecium* than *Salmonella* in skim milk powder, lactose powder, and 90% milk protein isolate but statistically similar in lactose-free skim milk powder (Ahmad, Marks, et al., 2022). While *E. faecium* has been demonstrated to be a good surrogate in various products, it is extremely more resistant than *Salmonella* in some cases. The current industry standard uses a KR = 1, meaning that the log reduction of *E. faecium* required for process validation is the same as the desired log reduction for *Salmonella*. While this assures food safety, the product may be over-processed resulting in quality deterioration. In such instances, the industry may desire a conservative KR (>1). This study has proposed a bootstrapping sampling approach.

Mean KR calculation

The mean KR values were calculated as ratio of mean D-values (negative inverse of inactivation rate, k) of *E. faecium* to that of *Salmonella* for six treatment conditions (3 temperatures × 2 milk powders). As shown in Table 1, with increasing temperature from 85 to 95°C, the mean KR increased from 1.67 to 2.71 in NFDM and from 2.13 to 2.37 in WMP. Similarly, when ground black pepper was exposed to thermal treatment at 75, 80, and 85℃, KR increased from 1.57 to 2.03 with increasing temperature (Wason et al., 2022). Another study reported that with an increase in temperature from 70 to 80℃, KR increased from 1.71 to 2.74 in dried basil leaves at 0.7 a_w (Verma et al., 2021). On the contrary, there are studies that have reported an inverse impact of temperature on KR in various LMFs such as wheat flour (Liu et al., 2018), almond meal (Ahmad et al., 2019), and cocoa powder (Tsai et al., 2019). In this study, a slightly higher KR was observed in WMP as compared to NFDM at all treatment conditions. This variation may be attributed to the different impact of fat in WMP and NFDM on the D-values of *E. faecium* than *Salmonella* (Aviles et al., 2013). Ahmad, Marks, et al. (2022) reported a higher KR of 2.5 in lactose powder as compared to 1.6 in 90% milk protein isolate at 90℃, and this was attributed to the presence of lactose. The higher fat content in WMP can also be attributed to subsequent quality loss poststorage due to the development of rancid, oxidized, and stale flavors (Bryce and Pearce, 1946; Christensen et al.,1951). Therefore, KR is unique to temperature and product matrix.

Framework for selection of KR based on bootstrapping

Figure 1 shows the distribution of KR from bootstrapping for each temperature and product combination. As the temperature increased, the distribution shifted to the right indicating that KR increases with an increase in temperature. This shift was more pronounced in NFDM as compared to WMP. Table 2 discusses different bootstrap KR parameters such as minimum, maximum, percentiles, and standard deviation values for each of the six treatment combinations. The bootstrap KR values for 1000 iterations ranged from 1.25 to 2.10 at 85°C, 1.75 to 2.81 at 90°C, and 2.41 to 3.48 at 95°C for NFDM. Similarly, these values ranged from 1.86 to 2.80 at 85°C, 2.18 to 2.96 at 90°C, and 2.22 to 2.90 at 95°C for WMP. The median KR values (IFTPS recommended, $KR_{50\%}$) calculated from the distribution of bootstrap D-values (Table 2) were approximately equal to mean KR values (Table 1). IFTPS recommended the use of mean KR (IFTPS, 2011) and that means there is a 50% chance that the actual KR in each situation can be lower than the mean KR, which would result in achieving less than desired log reduction of pathogen in 50% of times. Based on the acceptable level of risk determined by the food industry and regulatory agency, a conservative KR can be estimated from the distribution. If a 5% level of risk is considered as acceptable (because zero risk is impossible, when considering biological variability), then the conservative $KR_{5\%}$ value can be determined such that only 5% of iterations are below that value and 95% of the time the selected KR value will be higher than the

Table 1
Thermal inactivation kinetics parameters (k - inactivation rate along with standard deviation (SD) of three replicates) of Salmonella (Sal) and E. faecium (EF) and mean Kill ratio (KR) values in Whole Milk Powder (WMP) and Non-Fat Dried Milk powder (NFDM) at 85, 90, and 95°C calculated using the data provided by Wei et al. (2021)

Kinetic Parameters	NFDM			WMP		
	85℃	90℃	95℃	85℃	90℃	95℃
Mean (k_{EF})	−0.052	−0.102	−0.202	−0.059	−0.126	−0.241
SD (k_{EF})	0.002	0.004	0.007	0.002	0.005	0.005
Mean (k_{Sal})	−0.087	−0.209	−0.555	−0.127	−0.307	−0.601
SD (k_{Sal})	0.004	0.007	0.020	0.005	0.005	0.020
Mean KR	1.67	2.03	2.71	2.13	2.42	2.49

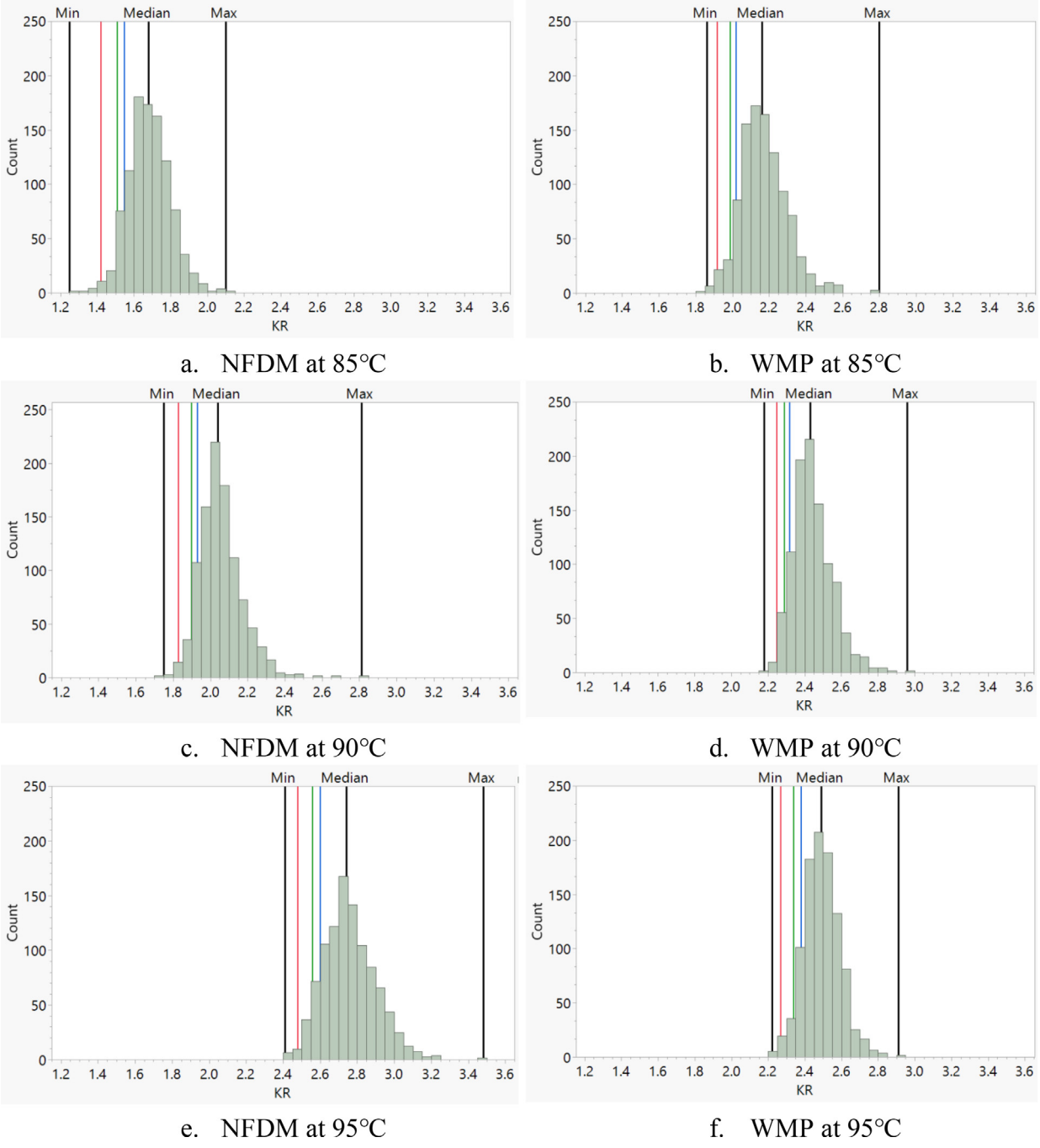

Figure 1. . Distribution of 1000 bootstrap kill ratio (KR) in Non-Fat Dry Milk powder (NFDM) and Whole Milk Powder (WMP) at 85, 90, and 95℃. The min, median, and max refer to the minimum ($KR_{0\%}$), median ($KR_{50\%}$), and maximum ($KR_{100\%}$) values of the KR distribution. The red, green, and blue vertical lines correspond to the conservative $KR_{1\%}$, $KR_{5\%}$, and $KR_{10\%}$.

actual KR. The conservative KR values at 1, 5, and 10% in addition to 50% (median KR), 0% (minimum value), and 100% (maximum value) are shown in both Figure 1 and Table 2. Conservative $KR_{x\%}$, where $0 < x < 50$, ranges between 1 (extremely conservative) and the mean kill ratio (most liberal). As 'x' increases, the value of $KR_{x\%}$ increases and the factor of safety decreases.

For WMP at 90℃, the conservative KR values decreased slightly with decreasing levels of 'x', when compared to other product-temperature combinations. This is because the KR distribution is tight with a smaller standard deviation as the standard deviation of inactivation rates of both bacteria were lower. Therefore, if the industry would like to have a conservative $KR_{x\%}$ closer to the mean KR, then they must control the experimental error in the determination of inactivation rates which should result in a lower standard deviation.

When the bootstrapping was repeated to get 10,000 iterations, the KRs estimated were approximately the same (results not shown). Based on this framework, the dairy industry can pick the temperature for pasteurizing milk powders and based on the level of safety (x%), the corresponding $KR_{x\%}$ value can be selected. For instance, if milk powders are pasteurized at 90℃, the corresponding conservative KR for WMP and NFDM are 2.29 and 1.90, respectively, with a 5% acceptable level of risk. The selected conservative KR values fall between 1 (extremely conservative) and mean KR (2.42 and 2.03 for WMP and NFDM – liberal estimate). The lower the experimental and biological

Table 2
Bootstrap kill ratio (KR) parameters in Non-Fat Dry Milk powder (NFDM) and Whole Milk Powder (WMP) at 85, 90, and 95°C for selected percentiles of the bootstrap distribution

KR Parameters	NFDM			WMP		
	85℃	**90**℃	**95**℃	**85**℃	**90**℃	**95**℃
Industry Standard (extremely conservative)	1	1	1	1	1	1
Minimum ($KR_{0\%}$)	1.25	1.75	2.41	1.86	2.18	2.22
Conservative $KR_{1\%}$	1.42	1.83	2.48	1.92	2.25	2.27
Conservative $KR_{5\%}$	1.51	1.90	2.55	1.99	2.29	2.34
Conservative $KR_{10\%}$	1.55	1.93	2.59	2.02	2.32	2.38
IFTPS recommendation ($KR_{50\%,}$ liberal estimate)	1.68	2.04	2.74	2.16	2.43	2.49
Maximum ($KR_{100\%}$)	2.10	2.81	3.48	2.80	2.96	2.90
Standard deviation	0.11	0.11	0.14	0.13	0.11	0.10

variability, the closer the selected $KR_{x\%}$ will be to the mean KR. The higher the experimental and biological variability, the closer the selected KR will be to 1. In this case study for conducting thermal validation studies at 90℃, processors would need to achieve a minimum 2.18 and 2.63 log reduction of *E. faecium* to ensure >5 log reduction of *Salmonella* in WMP and NFDM, respectively, at 5% levels of risks. This would require a shorter treatment time than would be required for a minimum of 5 log reduction of *E. faecium*, which would positively impact milk powder quality.

This framework can be used by the food industry to determine the conservative KR to be used for validation at the industrial scale. The use of such KR values will minimize the impact on quality, while ensuring the food safety within the acceptable levels of risk. This will minimize over-processing and enhance sustainability (due to lower energy usage).

Conclusion

This study introduced an innovative approach to calculate a conservative KR between a surrogate and pathogen to minimize over-processing while ensuring food safety with an acceptable level of risk. As a case study, the proposed approach was used to estimate conservative KR at various acceptable levels of risk for two milk powders. Results show that conservative KR values are unique for product-temperature combination. Currently, the food industry uses either a worst-case conservative approach (KR = 1) or a liberal approach (mean KR). This proposed method using bootstrapping provides a balanced approach for estimating a conservative KR. This method also allows the food industry and regulatory agency to customize the levels of risk depending on the product, such that the estimate falls closer to extremely conservative value (close to KR value of 1) or liberal value (mean KR). If the industry wants to use a higher KR without compromising the factor of food safety, then inactivation rates must be estimated with high precision (low standard deviation).

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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