

### 1. Introduction

Microbiological challenge testing has been and continues to be a useful tool for determining the ability of a food to support the growth of spoilage organisms or pathogens. Microbiological challenge tests also play an important role in the validation of processes that are intended to deliver some degree of lethality against a target organism or group of target organisms. Quite often, with this latter purpose, there is an associated performance standard that the process must deliver (for example, a 5 log reduction of *Escherichia coli* O157:H7 for fermented meats). An appropriately designed microbiological challenge test will validate that a specific process is in compliance with the predetermined performance standard. The design, implementation, and assessment of microbiological challenge studies is a complex task that depends on factors related to how the product is formulated, manufactured, packaged, distributed, prepared, and consumed. An expert microbiologist must consider the relevant factors and design a study that best assesses the food safety of the product. Failure to account for specific product and environmental factors in the design of the test could result in flawed conclusions.

Microbiological challenge studies are also useful in determining the potential shelf life of certain refrigerated or ambient-stored foods. The determination of whether challenge studies are appropriate or useful must be made by considering such factors as the likelihood of the product to support growth of spoilage organisms or pathogens, or a knowledge of the previous history of the product. For example, it is not useful to conduct challenge studies on frozen foods that would not support growth under proper storage conditions; nor would it be especially useful to conduct challenge tests on commercially sterile retorted canned foods. However, in the canned food example, it may be appropriate to conduct inoculated pack studies as part of the protocol for process validation. Microbiological challenge testing is very useful for food products that may sustain the growth of pathogenic organisms and that are stored under refrigeration, elevated temperature, or at ambient temperature and vulnerable to the growth of microorganisms.

When conducting a microbiological challenge study, a number of factors must be considered (Vestergaard 2001). These include (1) the selection of appropriate pathogens or surrogates, (2) the level of challenge inoculum, (3) the inoculum preparation and method of inoculation, (4) the duration of the study, (5) formulation factors and storage conditions, and (6) sample analyses. The interpretation of the data and pass/fail criteria are critical in evaluating whether a food needs time/temperature control for safety.

While microbiological challenge testing is useful for determining the spoilage potential of a product formulation, the remainder of the discussion in this chapter will focus on pathogens relevant to foods that need time/temperature control for safety.

### 2. Selection of challenge organisms

Table 6-1 shows some pathogens that may be used in challenge studies for various types of foods (Vestergaard 2001). Knowledge of the food formulation and history of the food (for example, association with known illness outbreaks and/or evidence of potential growth) is essential when selecting the appropriate challenge pathogens. For example, *Clostridium botulinum* would be of concern with certain modified atmosphere packaged (MAP) products, and *Staphylococcus aureus* may be of concern in foods with little competitive microflora and in products with reduced  $a_w$ .

The ideal organisms for challenge testing are those that have been previously isolated from similar formulations. Additionally, pathogens from known foodborne outbreaks should be included to ensure the formulation is robust enough to inhibit those organisms as well.

Multiple specific strains of the target pathogens should be included in the challenge study. It is typical to challenge a food formulation with a “cocktail” or mixture of multiple strains in order to account for potential strain variation. It is not unusual to have a cocktail of 5 or more strains of each target pathogen in a challenge study. For example, botulinal challenge studies typically in-

**Table 6-1—Pathogens that may be considered for use in challenge studies for various food products**

Food Type	Type of Organism
Salad dressings	Salmonellae, <i>Staphylococcus aureus</i>
Modified atmosphere packaged products (that is, vegetables, meats, poultry, fish)	<i>Clostridium botulinum</i> (proteolytic and nonproteolytic strains) and other pathogens (for example, salmonellae, <i>Listeria monocytogenes</i> and enterohemorrhagic <i>Escherichia coli</i> ).
Bakery items (that is, fillings, icings, nonfruit pies)	Salmonellae, <i>S. aureus</i>
Sauces and salsas stored at ambient temperature	Salmonellae, <i>S. aureus</i>
Dairy products	Salmonellae, <i>S. aureus</i> , <i>C. botulinum</i> , enterohemorrhagic <i>E. coli</i> , <i>L. monocytogenes</i>
Confectionery products	Salmonellae
Formula with new preservatives	Salmonellae, <i>S. aureus</i> , <i>C. botulinum</i> , enterohemorrhagic <i>E. coli</i> , <i>L. monocytogenes</i>

Source: Adapted from Vestergaard 2001.

clude several strains of proteolytic types A and B as well as representative nonproteolytic strains (if appropriate). A single challenge strain with specific well defined characteristics may be used to screen products similar in nature to those formulations that have been extensively challenged with multiple strains in the past.

Prior to conducting the challenge study, selected strains should be screened for mutual antagonism. Antagonism between certain strains of *Listeria monocytogenes* has been reported, as well as between certain strains of *C. botulinum* and between bacteriocin-producing lactic acid bacteria. If these are not compatible when used as part of a challenge cocktail, then erroneous results may ensue.

It is also important to incubate and prepare the challenge suspension under standardized conditions and format. Shifts in the incubation temperature used to propagate the challenge organisms and the storage temperature of the product have been shown to change the length of the lag period of the challenge study itself (Curiale 1991). Consideration must also be given to adapting the challenge suspension to the environment of the food formulation prior to inoculation. For example, acid-adaptation of *E. coli* O157:H7 cells or salmonellae cells prior to inoculation can greatly influence their ability to survive when inoculated into an acidic food.

Finally, the use of genetic characterization tools may greatly aid the determination of which strains (if any) used in the challenge study are the most dominant over the length of the study. Tools such as ribotyping and pulsed-field gel electrophoresis can help distinguish among strains, whether inoculated or naturally occurring (Farber and others 2001). Challenge organisms may also be genetically modified to carry a marker that aids in distinguishing them from other strains and helps in their detection in the food matrix itself. Obviously, the genetically modified organism needs to have physiological characteristics comparable to the wild-type or parent strain.

For certain applications, surrogate microorganisms may be used in challenge studies in place of specific pathogens. For example, it usually is not possible to introduce pathogens into a processing facility; therefore, it is desirable to use surrogate microorganisms in those cases. An ideal surrogate is a strain of the target pathogen that retains all other characteristics except its virulence. In practice, however, many surrogates are closely related to but not necessarily the same species as the target pathogen. Traditional examples include the use of *Clostridium sporogenes* as a proxy for *Clostridium botulinum* in inoculated pack studies, *Listeria innocua* as a surrogate for *L. monocytogenes*, and generic strains of *Escherichia coli* as substitutes for *E. coli* O157:H7. Caution must be used with the latter, however, since generic strains of *E. coli* do not have the same level of acid resistance as *E. coli* O157:H7. Therefore, while it may be appropriate to use generic strains of *E. coli* in a challenge study to assess the impact of a heat process or preservative system in a high-pH food, it would be inappropriate to use these generic strains for evaluating acidic foods. Generally, surrogates are selected from a group of well characterized organisms and have the following desirable attributes (IFT 2000):

- Nonpathogenic.
- Inactivation characteristics and kinetics that can be used to predict those of the target pathogen.
- Behavior similar to the target pathogen when exposed to formulation and/or processing parameters (for example, pH stability, temperature sensitivity, and oxygen tolerance).
  - Stable and consistent growth characteristics.
  - Easily prepared to yield high-density populations.
  - Once prepared, population remains stable until utilized.
  - Easily enumerated using rapid, sensitive, and inexpensive detection systems.

- Easily differentiated from background microflora.
- Attachment characteristics that mimic those of the target pathogen.
- Genetically stable so that results can be replicated independently of laboratory or time of experiment.
  - Will not establish itself as a “spoilage” organism if used in a production area.
  - Susceptibility to injury similar to that of the target pathogen.

If a surrogate strain is to be used in a microbiological challenge study, preliminary work should be done to well characterize the strain before use in the study. Characteristics such as those discussed above should be determined and confirmed through preliminary laboratory work to assure that the surrogate strain is suitable for the intended purpose. The use of surrogates should be limited to only those cases where specific pathogens absolutely cannot be used for product or personnel safety reasons.

### 3. Inoculum level

The inoculum level used in the microbiological challenge study depends on whether the objective of the study is to determine product stability and shelf life or to validate a step in the process designed to reduce microbial numbers. Typically, an inoculum level of between  $10^2$  and  $10^3$  cells/g of product is used to ascertain the microbiological stability of a formulation. Higher inoculum levels may be appropriate for other products. Depending on the product formulation, some of the inoculum may die off initially before adapting to the environment. If too low of an inoculum level is used, the incorrect assumption could be made that the product is stable when it is not. Conversely, if the inoculum level is too high for this purpose, the preservation system or hurdles to growth may be overwhelmed by the inappropriate inoculum size, leading to the incorrect conclusion that the formulation is not stable. When validating a process lethality step such as heat processing, high pressure processing, or irradiation, however, it is usually necessary to use a high inoculum level (for example,  $10^6$  to  $10^7$  cells/g of product) to demonstrate the extent of reduction in challenge organisms. For example, in the United States, juice processors are now required to demonstrate a 5 log reduction of relevant hazardous microorganisms in their products (5 D performance standard). These log-reduction validation protocols usually require the use of plating methods. In order to measure this level of reduction within the statistical limits of the enumeration method, the inoculum level must be at least  $10^6$  CFU/g.

### 4. Inoculum preparation and method of inoculation

The preparation of the inoculum to be used in microbiological challenge testing is an important component of the overall protocol. Typically, for vegetative cells, 18 to 24 h cultures revived from refrigerated broth cultures or slants or from cultures frozen in glycerol are used. The challenge cultures should be grown in media and under conditions suitable for optimal growth of the specific challenge culture. In some studies, specific challenge organisms may be adapted to certain conditions. Such adaptation will be tailored to the specific food. For example, *E. coli* O157:H7 may be acid adapted with the appropriate acidulant prior to use in the challenge studies on acidic products. Bacterial spore suspensions may be stored in water under refrigeration or frozen in glycerol. Spore suspensions should be diluted in sterile water and heat-shocked immediately prior to inoculation. Spores of *C. botulinum* should be washed thoroughly prior to use to ensure that no free botulinum toxin is carried over into the product undergoing challenge testing, and, if possible, the spores should be heat-shocked in the food to be studied. Quantitative counts on the challenge suspensions may be conducted to aid in calculating the

dilutions necessary to achieve the target inoculum in the challenge product. Appropriate procedures and containment facilities should be used when carrying out challenge tests with certain pathogens.

The method of inoculation is another extremely important consideration when conducting a microbiological challenge study. Every effort must be made not to change the critical parameters of the product formulation undergoing challenge. There are a variety of inoculation methods that can be used depending upon the type of product being challenged. In aqueous liquid matrices such as sauces and gravies with high  $a_w$  ( $> 0.96$ ), the challenge inoculum may be directly inoculated into the product with mixing, using a minimal amount of sterile water or buffer as a carrier. Use of a diluent adjusted to the approximate  $a_w$  of the product using the humectant present in the food minimizes the potential for erroneous results in intermediate  $a_w$  foods. In studies where moisture level is one of the experimental variables, the inoculum may be suspended in the water or liquid used to adjust the moisture level of the formulation. For batch type inoculations, the inoculum may be added directly to the product in a mixing bowl or container. For individual package or pouch type applications, the inoculum may be aseptically injected using a sterile syringe through the package wall containing a rubber septum. In solid matrices with  $a_w > 0.96$ , such as cooked pasta or meat surfaces, an alternative to the syringe method may be the use of an atomizer. An atomizer sprays the inoculum, which is suspended in sterile water or buffer, into the ground product or onto the surface of product. Spraying should be done in a containment hood or using other protective devices to avoid worker safety issues related to creation of pathogenic. In all these applications, the smallest amount of water or buffer practical for suspension of the inoculum should be used. Inoculum may also be transferred using a velvet pad, paint pad, or similar fibrous cloth provided the method is calibrated and reproducible levels of inoculum can be delivered with minimum moisture transfer. Preliminary analyses should be done to ensure that the  $a_w$  or moisture level of the formulation is not changed after inoculation.

Products or components with  $a_w < 0.92$  may be inoculated using the atomizer method with a minimal volume of carrier water or buffer. Again, the product should always be checked to ensure that the final product  $a_w$  or moisture level has not been changed. A short, post-inoculation drying period for some products may be needed prior to final packaging. Alternatively, they may be inoculated with challenge organisms that have been suspended in carrier water or buffer that has been added to sterile sand, flour, or a powdered form of the product (for example, dried pasta), and allowed to dry. Lyophilized culture may also be used for some applications. Inoculum viability and population levels should be determined in advance of the study. The dried inoculum preparation should be added aseptically to the test product and shaken or agitated thoroughly for even distribution of the inoculum.

Enough product should be inoculated so that a minimum of three replicates per sampling time is available throughout the challenge study. In some cases, such as in certain revalidation studies and for uninoculated control samples, fewer replicates may be used.

### 5. Duration of the study

It is prudent to conduct the microbiological challenge study over, at least, the desired shelf life of the product. It is even more desirable to challenge the product for its entire desired shelf life plus a margin beyond the desired shelf life because it is important to determine what would happen if users would hold and consume the product beyond its intended shelf life. Some regulatory agencies require a minimum of data on shelf life plus at least one-

third of the intended shelf life.

Another consideration impacting the duration of the challenge study is the temperature of product storage. Refrigerated products may be challenged for their entire shelf life under the target storage temperature, but under abuse temperatures they are typically held for shorter time.

In certain foodservice venues, it may be convenient for the food establishment to hold specific refrigerated products at room temperature for short periods of time. For example, some fast food operations may find it convenient to hold processed cheese slices at room temperature for up to 8 h. This allows the cheese to temper and melt faster when preparing food items such as hot sandwiches. However, pathogens may be present on the cheese slices due to cross-contamination through handling in the restaurant, and therefore challenge testing will be needed to provide evidence that this practice is safe. If the restaurant would like to hold the cheese slices at room temperature for an 8 h shift, the duration of the challenge study should be at least 12 h. This challenge study is performed to ensure that the rapid growth of pathogens does not occur if the cheese slices are cross-contaminated in the restaurant through handling.

It is also desirable to test the product over and significantly beyond its entire shelf life because sublethal injury may occur in some products. This can lead to a long lag period, where it may not be possible to culture the inoculum, but over time, a small number of the injured cells recover and grow in the product. This rebound, or "Phoenix" phenomenon, has been observed in a number of products (Jay 1996). If the product is not tested for at least its entire shelf life, it is possible to miss the recovery and subsequent growth of the challenge organism late in its shelf life.

The frequency of testing is governed by the duration of the microbiological challenge study. It is desirable to have a minimum of 5 to 7 data points over the shelf life in order to have a good indication of the inoculum behavior. Typically, if the shelf life is measured in days, the frequency of testing should be at least daily, if not multiple times per day. If the shelf life is measured in weeks or months, the test frequency is typically no less than once per week. All studies should start with "zero time" testing, that is, analysis of the product right after inoculation. For some types of products, it may be desirable to also allow an equilibration period for the inoculum to adapt to the product before testing. It may be desirable to test more frequently (for example, daily or multiple times per day) early in the challenge study (that is, for the first few days or week), and then reduce the frequency of testing to longer intervals.

### 6. Formulation factors and storage conditions

When evaluating a formulation, it is important to understand the range of key factors that control its microbiological stability. Intrinsic factors such as pH,  $a_w$ , or preservative level may be key to preventing the growth of pathogens or to preventing spoilage that would influence the safety of the product during its intended shelf life. It is, therefore, important to test each key variable singly and/or in combination in the formulation under worst-case conditions. For example, if the target pH is  $4.8 \pm 0.2$  and the process capability is within that tolerance range, it is important to challenge the product on the high side of that range (that is, pH 5.0). Similarly, if sorbic acid is used at a level of  $0.15 \pm 0.05\%$ , the product should be challenged at the low concentration of 0.10%. This is recommended to ensure that the challenge study covers the process capability range for each critical factor in the formulation. Relevant intrinsic properties such as pH,  $a_w$ , and salt level should be documented for each study for future comparison and reference.

Test samples should ideally be stored in the same packaging as

intended for the commercial marketplace. If the commercial product is vacuum- or MAP-packaged, then the samples used in the microbiological challenge study should be packaged under the same conditions using the same packaging film.

The storage temperature used in the microbiological challenge study should include the typical temperature range at which the product is to be held and distributed. A refrigerated product that may be subject to temperature abuse should be challenged under representative abuse temperatures. Products that may encounter high humidity environments should also be challenged under those conditions (Notermans and others 1993). Some challenge studies may incorporate temperature cycling into their protocol. For example, the manufacturer may distribute a refrigerated product under well controlled conditions for a portion of its shelf life, after which the product may be subjected to elevated temperatures immediately prior to and during use.

### 7. Sample analysis

Typically, in a microbiological challenge study, the levels of live challenge microorganisms are enumerated at each sampling point. Usually, it is desirable to have at least duplicate and, preferably, triplicate samples for analysis at each time point. In cases where higher levels of certainty are needed, a larger number of replicates should be used or the study should be replicated. The selection of enumeration media and method (for example, direct plating versus Most Probable Number) is dependent on the type of pathogens or surrogates used in the study. If the product does not have a substantial background microflora, nonselective media for direct enumeration may be used. In cases where toxin-producing organisms are used (for example, *Staphylococcus aureus* or *C. botulinum*), appropriate toxin testing should be performed at each time point using the most current validated method. Toxin levels may not always be tested at each time point in the study, but should be done at frequent enough intervals throughout the desired shelf life of the product to determine if that shelf life is acceptable. Where appropriate, resuscitation methods may be used to avoid erroneous results.

It is prudent to analyze the product, including uninoculated control samples, at each or selected sampling points in the study to see how the background microflora is behaving over product shelf life. For example, if a product has a high background microflora, it may suppress the growth of the challenge inoculum. In some cases, this is useful and desirable because the product spoils before pathogens can grow. In other situations, the background microorganisms may not be universally present, leading to a potentially false sense of security. Also, under some circumstances, the background microorganisms can change the formulation parameters in the product to favor or inhibit growth of the inoculum over time (for example, molds can raise product pH; lactobacilli can decrease product pH).

It is also important to track pertinent physicochemical parameters of the product over shelf life to see how they might change and influence the behavior of the pathogen. Understanding how factors such as  $a_w$ , moisture, salt level, pH, MAP gas concentrations, preservative levels, and other variables behave over product shelf life is key to understanding the microbiological stability of the product.

### 8. Data interpretation

Once the microbiological challenge study is completed, the data should be analyzed to see how the pathogens behaved over time. Trend analysis and appropriate graphical plotting (that is, semi-log plots) of the data will show whether the challenge organisms died, remained stable, or increased in numbers over time. In

the case of toxin-producing pathogens, no toxin should be detected over the designated challenge period. Combining the quantitative inoculum data for each time point with data on the background microflora and the relevant physicochemical parameters gives a powerful and broad representation of the microbiological stability of the formulation under evaluation. Based on these data, a reasonable shelf life can be established or adjustments can be made to the formulation so that it is less susceptible to pathogen growth.

When using microbiological challenge testing, as part of a process validation protocol, analysis of the data will show whether the process is capable of delivering the required level of lethality (that is, conforms with the predetermined performance standard). Based on this information, adjustments can be made to the process, if necessary, in order to meet the lethality requirements.

The data from microbiological challenge testing can be used in developing predictive microbiological models or in validating existing ones. Predictive models are computer-based programs that simulate or predict how specific microorganisms will behave in a formulation under specific conditions (for example, pH,  $a_w$ , moisture, salt, and preservatives). Microbiological challenge tests are used both to generate these types of empirical models and to validate their applicability.

Overall, well designed challenge studies can provide critical information on the microbiological safety and stability of a food formulation. They are also invaluable in validating key lethality or microbiological control points in a process. Challenge studies can be an invaluable aid in determining if a food product requires temperature control throughout its shelf life or if it can tolerate storage at room temperature for a portion or all of its shelf life.

### 9. Pass/fail criteria

Selection of microorganisms to use in challenge testing and/or modeling depends on the knowledge gained through commercial experience and/or on epidemiological data that indicate that the food under consideration or similar foods may be hazardous due to pathogen growth. In addition, the intrinsic properties (for example, pH, water activity, and preservatives) and extrinsic properties (for example, atmosphere, temperature, and processing) should be considered. The significance of a population increase varies with the hazard characterization of each microorganism. For example, the growth of infectious pathogens should always be controlled, whereas most toxin production requires substantial growth before a hazard exists. In this case, growth of the toxigenic organism alone does not result in a health hazard, but toxin production will.

The following list identifies microorganisms that can be used in a microbiological challenge study along with the panel's recommendations and rationale for selection and assessment of tolerable growth.

**Toxigenic molds such as *Aspergillus*, *Penicillium*, and *Fusarium* spp.** Challenge studies related to the need for time/temperature control for safety are not recommended because mold provides a visual clue to prevent consumption of the spoiled product.

***Bacillus cereus*.** The absence of toxin formation is the preferred criterion. However, since toxin measurement is difficult, a 3 log increase over inoculum levels would indicate the need for time/temperature control. This growth limit determination is based on the following:

- Typical initial levels of *B. cereus* are low; therefore, 1000 CFU/g would be a conservative initial level based on the literature.
- Populations of  $> 10^6$  CFU/g are needed to produce toxin at levels hazardous to health (FDA 2001).
- The emetic toxin of *B. cereus* is heat stable; therefore, no reduction is likely.

Other considerations:

- *Bacillus cereus* spores are relatively heat sensitive. Baking is likely to destroy low levels found in flour used in bread products (Kaur 1986); however, unusual high heat resistance has been reported in pumpkin pie (Wyatt and Guy 1981). The potential for survival of *B. cereus* should be evaluated for specific products.

- Rice and potatoes have a history of association with *B. cereus* foodborne illness and therefore products containing rice or potatoes should be evaluated for time/temperature control requirements.

**Campylobacter spp.** No challenge testing is recommended because other organisms such as *Salmonella* have similar routes of contamination, are less fastidious, and are easier to culture. Furthermore, its minimal growth temperature and water activity of 32 °C (90 °F) and 0.98, respectively, make *Campylobacter* spp. an unlikely candidate for challenge studies.

**Clostridium botulinum.** The absence of toxin formation based on current methodology is the recommended requirement. Other considerations: *C. botulinum* is appropriate to consider for certain cooked products, particularly those packaged under anaerobic and micro-aerophilic conditions such as MAP products; and those with a history of associated illness, such as products under oil and baked potatoes.

**Clostridium perfringens.** A 3-log increase is recommended based on the following facts:

- Although other products may contain surviving spores, *Clostridium perfringens* is relevant mainly to meat and poultry products, including sauces and gravies. Most products subject to the Food Code requirements will be either raw or freshly cooked.

- Vegetative cells of *C. perfringens* are easily destroyed by cooking meat and poultry products, and spore levels are typically low due to demanding sporulation requirements. An initial population of 100 CFU/g was considered to be a conservative worst case by the panel. A population of >10<sup>5</sup> CFU/g is needed to result in illness; therefore, a 3-log increase would control the hazard.

**Enterohemorrhagic E. coli.** If modeling programs are used to predict the growth of the pathogen, time/temperature holding conditions should maintain enterohemorrhagic *E. coli* in lag phase due to the infectious nature of the microorganism. However, if laboratory challenge studies are used, the inherent variability in quantitative methods necessitates the use of a progressive increase of < 1 log as indicative that growth is controlled.

**Listeria monocytogenes.** Recent risk assessments (FDA/USDA 2001) indicate that low numbers of *L. monocytogenes* present a low risk to public health. In recognition of this, some countries such as Canada and Germany have established a tolerance for low levels of this organism in certain ready-to-eat foods that will not support growth to high levels. However, a tolerance for *L. monocytogenes* has not been established in the United States for these types of foods. It is also recognized that products that support the growth of the microorganism present an increased risk. A *L. monocytogenes* level of 100 CFU/g at the time of consumption may provide an acceptable level of consumer protection (Ross and others 2000). However, data are insufficient to determine general worst-case initial levels. Overall, the panel concluded that a 1 log increase was an appropriate level of control for *L. monocytogenes*. This level accounts for variability in enumeration techniques and represents a view that growth of this organism to high levels represents a risk to public health that must be controlled.

**Salmonella spp.** Appropriately validated pathogen modeling programs for growth can be used to verify that *Salmonella* spp. is

maintained in the lag phase. Otherwise, population growth should be limited to < 1 log, following the same rationale as for enterohemorrhagic *E. coli*.

**Shigella spp.** No challenge studies are recommended for *Shigella* spp. because it has the same potential source as *Salmonella* spp. and has more fastidious growth and survival requirements.

**Staphylococcus aureus.** No detectable toxin should be formed under the time/temperature studies evaluated. As with *C. botulinum*, current methodology should be used for toxin detection and specific toxin levels should be determined. In lieu of testing for toxin, limiting growth to < 3 logs may be used. This limiting growth level is based on an initial population of 1000 CFU/g, and a minimum of 10<sup>6</sup> CFU/g to produce toxin.

Other considerations: *Staphylococcus aureus* is appropriate to study in foods that receive extensive handling because of the human source of the microorganism. *S. aureus* does not compete well with other microorganisms; therefore, it is not appropriate to consider in foods with high levels of other organisms, such as raw vegetables or properly fermented products.

**Vibrio spp.** Appropriately validated pathogen modeling programs for growth can be used to verify that *Vibrio* spp. are maintained in the lag phase. Otherwise, population growth should be limited to < 1 log, following the same rationale as for *E. coli*.

**Vibrio parahaemolyticus** can be used as a surrogate for other *Vibrio* spp. *V. parahaemolyticus* studies are only appropriate for marine foods. It should also be noted that most fish are highly perishable and therefore will be temperature controlled for spoilage reasons.

**Yersinia enterocolitica.** Challenge studies are not recommended as *Salmonella* spp. and *Y. enterocolitica* have similar sources and salmonellae are easier to culture.

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