Everything you need to know about microbiological challenge testing

WHITEPAPER

SGS

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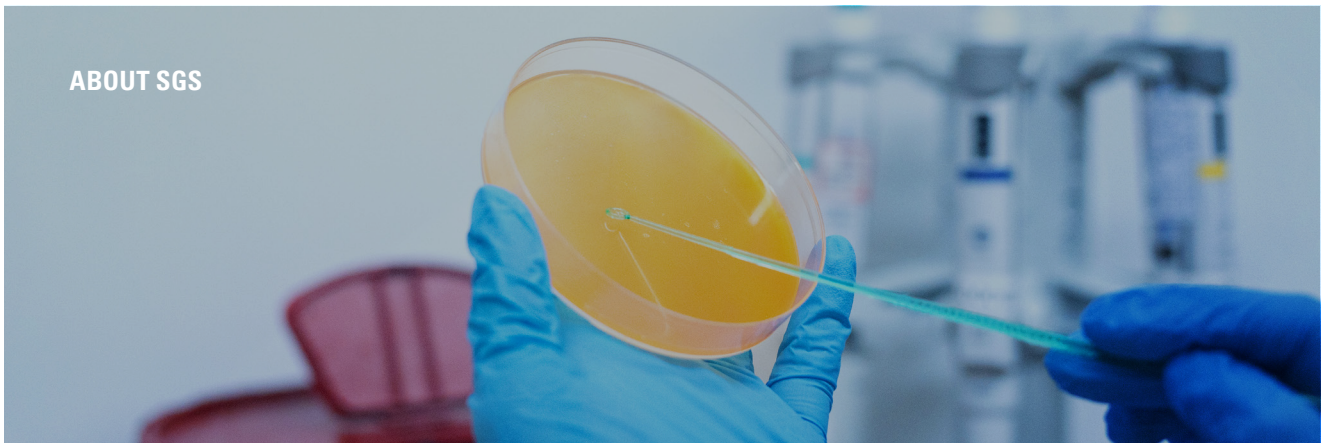


Introduction

Microbiological challenge testing is used for assessing growth, survival, or reduction of microorganisms when added to food products under well-controlled conditions. These challenge tests play an important part in validating processes intended to deliver some degree of lethality against a target organism or group of organisms.

However, while the design of these challenge studies is one of the most important steps to achieving an effective and scientifically sound investigation, there is no single standardized method to design or conduct them.

In this whitepaper, we will share some best practices for when to perform a challenge study, how to design and conduct your studies and how to interpret results to help prove food products are stable and safe.

We will also discuss how to carry out microbiological challenge studies while still meeting regulatory requirements and established finished product standards.

What is microbiological challenge testing?

Food processors use microbiological challenge testing to assess the **growth**, **survival**, or **reduction of microorganisms** within a food sample. It involves testing by adding these microorganisms to food products or food contact material, such as package material, under **well-controlled conditions**.

Microbiological challenge testing also plays an important role in validating processes intended to deliver some degree of **lethality** against a target organism, or groups of target organisms.

Challenge testing is also useful in determining the **potential shelf life** of certain refrigerated or ambient-stored foods.

When it comes to deciding whether challenge studies are appropriate or useful, food processors need to consider factors such as:

- The **likelihood** of the product to support the growth of spoilage organisms or pathogens. This involves **microbiological safety** to ensure no unwanted contamination of the food test and using the **susceptibility matrix** to determine the probability of infection for each organism, or group of organisms.
- A knowledge of the previous history of the food product, such as:
  - **Epidemiological data** — reviewing any past incidents of foodborne illnesses or outbreaks associated with the product, identifying pathogens involved, severity of illnesses and any patterns in contamination sources.
  - **Microbial testing results** — analyzing historical data on microbial testing, identifying which microorganisms were present, their levels and any trends over time.
  - **Storage and handling practices** — investigating how the product has been stored and handled at different stages, from production to retail, to reveal conditions that may contribute to microbial growth or contamination.
  - **Product recalls** — reviewing records of any past recalls involving the product, the reasons for those recalls and the measures taken to address the issues.
  - **Consumer complaints and feedback** — examining feedback from consumers to provide insights into quality issues, potential contamination and product safety.
  - **Regulatory compliance** — checking historical compliance with food safety regulations and standards, including any violations or warnings issued by regulatory bodies.
  - **Ingredient and formulation changes** — tracking any changes in the ingredients or formulation of the product, as these can impact its safety, stability and susceptibility to microbial growth.

Why conduct microbiological challenge studies?

The overall goal of challenge testing is to **simulate what could happen to a food product if it is contaminated** during production, processing, distribution, or subsequent handling by consumers.

These tests are carried out by inoculating a relevant microorganism, or microorganisms, into a product before storing it in representative conditions that simulate every step of the process, from production to consumption.

In the past, food processors have relied on the many physical and chemical barriers in **food** — such as pH, salt content and water activity (aw) — to inhibit the growth of pathogens and extend a product's shelf life.

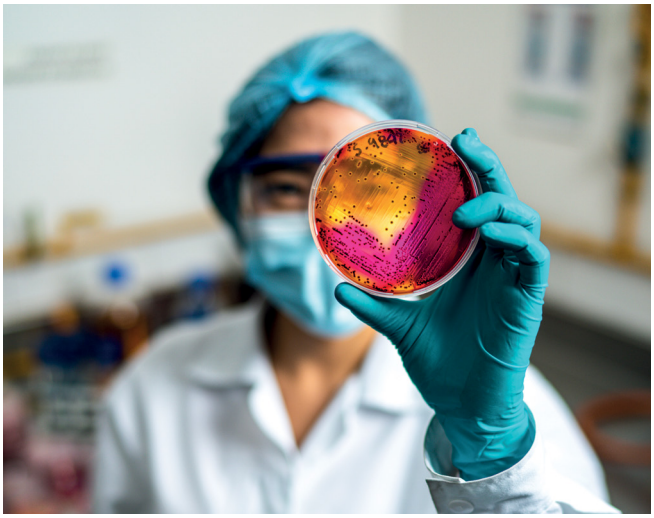
However, food safety professionals and regulators today have raised safety concerns over the reduction of these barriers in some products, demonstrating a need for Food Business Operators (FBOs) to carry out challenge testing.

As a result, the food industry conducts microbiological challenge testing more frequently than ever to help FBOs ensure the **safety of new, reformulated, or recaptured food products**. These products may have higher pH levels or avoid preservatives, making them susceptible to potentially hazardous microorganisms, even in low or incidental numbers.

In addition, changes in the product portfolio — like introducing new vegetarian product lines — also mean FBOs should undertake more frequent microbiological challenge studies.

What are 'recaptured' food products?

Recaptured food products are food items that have been retrieved or taken back during the manufacturing process for further inspection, reprocessing, or correction due to potential quality or safety issues. These products are typically identified through quality control checks or consumer feedback that indicates possible defects, contamination, or non-compliance with safety standards.



Microbiological challenge studies also help determine whether a food has the **ability to "kill off" any pathogens that may accidentally contaminate a product**. A key example here is in stress testing for the bacteriostatic effects of matrix formulations, looking for a reduction in preservatives like benzoate or sorbate.

Even if food processors do not expect the presence of any hazardous microorganisms, performing a challenge study will help them understand how an increased level of spoilage organisms through accidental contamination affects a product's shelf life.

Some of the key information food processors gain from challenge studies include **proper product code dating** and confirmation that changes in a product's formulation, processing, or packaging will inhibit microbial growth.

FBOs can then use this data to establish safety criteria at **critical control points** (CCPs) in food processing operations.

This data also allows FBOs to determine product testing specifications and decide on appropriate preservation techniques and packaging methods.

The difference between microbiological challenge testing and shelf-life analysis

The similarities between microbiological challenge testing and **shelf-life analysis** often mean food safety professionals are confused over which form of testing is needed.

However, there is one major difference — **the conditions around how each product is tested**.

In shelf-life analysis, the product is stored under normal conditions and analyzed over time to ensure it's safe and stable.

In challenge testing, pathogenic or spoilage microorganisms are added to the product to determine whether it would be safe and stable in case of accidental contamination.

Differences between microbiological challenge testing and shelf-life analysis

SHELF-LIFE ANALYSIS	MICROBIOLOGICAL CHALLENGE TESTING
Product is stored under normal conditions	Product is 'contaminated' with microorganisms under well-controlled conditions
Used to analyze normal background flora	Used to simulate contamination during processing, distribution, storage or preparation
Analysis targets naturally present spoilage microflora and pathogens growing during storage	Analysis targets pre-selected microorganisms with a relevant origin and prehistory for the purpose of the test
Performed on final food products	Performed on real food products or in model systems mimicking food compositions

When should you perform microbiological challenge studies?

A challenge study should be carried out if a food product is susceptible to pathogenic growth and spoilage. For pathogens, any food with a pH ≥ 4.6 or aw ≥ 0.85 under the FDA Food Code is included.

The FDA Food Code added two interaction tables that use the values of pH and aw in a food to determine if the food is a non-PHF (Potentially Hazardous Food)/ non-TCS (Temperature/Time Controlled for Safety) Food, either because of its pH or water activity alone or due to interactions between the two factors.

PHF (Potentially Hazardous Food):

Food that has to be kept at certain temperatures to minimize the growth of any pathogenic microorganisms present in the food, or to prevent the formation of toxins in the food

TCS (Temperature/Time Controlled for Safety) Food:

Food that needs specific time and temperature controls to prevent the growth of harmful microorganisms that can cause foodborne illnesses

It is not easy to tell which foods are suitable for challenge testing and which are not, due to the ever-changing nature of foods (such as ingredients, manufacturing techniques, preservatives and preservation techniques, and packaging).

However, decisions can be made according to FDA reports like these:

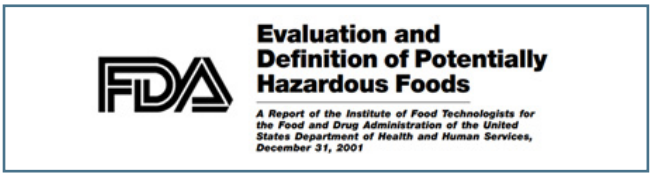


Table A—Control of spores:
Product treated to controlvegetative cells and protected from recontamination.

Critical a_w values	Critical pH values		
	4.6 OR LESS	> 4.6 TO 5.6	> 5.6
0.92 or less	Non-TCS	Non-TCS	Non-TCS
> 0.92 to .95	Non-TCS	Non-TCS	?
> 0.95	Non-TCS	?	?

Table B—Control of vegetative cells and spores:
Product not treated or treated but not protected from recontamination

Critical a_w values	Critical pH values			
	< 4.2	4.2 TO 4.6	> 4.6 TO 5.0	> 5.0
< 0.88	Non-TCS	Non-TCS	Non-TCS	Non-TCS
0.88 to 0.90	Non-TCS	Non-TCS	Non-TCS	?
> 0.90 to .92	Non-TCS	Non-TCS	?	?
> 0.92	Non-TCS	?	?	?

IFT/FDA Report: Evaluation and Definition of Potentially Hazardous Foods. Comprehensive Reviews in Food Science and Food Safety, 2001
https://www.fda.gov/files/food/published/Evaluation-and-Definition-of-Potentially-Hazardous-Foods.pdf

While challenge testing can be used for many different purposes, there are five key reasons why it may need to be performed:

1. The product does **not meet the shelf-life expectation** or goal set by the processor.
2. The FBO notices **spoilage in a product by the specified shelf-life date**, but due to changes in its formulation, packaging, distribution, storage, or preparation methods, the product now requires an extended shelf life.
3. There is a **risk associated with the product** that needs to be addressed as stated in legislation. This could be seen in requirements from the FDA's Food Modernization Safety Act (FMSA), the retailer's specifications, or standards such as those recognized by the Global Food Safety Initiative (GFSI).
4. The FBO needs **useful information about specific processing protocols**, particularly in facilities where raw materials are on the plant floor, or when a heat or lethal step — like pasteurization or sterilization — needs to be validated.
5. During the **formulation stage of a product**, where conducting a microbiological challenge study to get data on shelf stability, storage, preservatives and additives, and packaging helps processors establish sound finished product specifications.

The type of food product, processing methods and formulations also play a role in deciding whether to conduct a microbiological challenge study.

For example, products stored at extremely low temperatures are often unsuitable for challenge studies, since most microorganisms do not grow at these temperatures.

Canned food can also be excluded from challenge studies, as the retort process destroys harmful microorganisms, although inoculated pack studies might be appropriate to validate processes.

The **products best suited for challenge studies** are:

- Those stored at **refrigeration temperatures**, particularly those vulnerable to freeze-thaw cycles during the cool-chain, typically between 0°C and 4°C.
- Those stored at **room temperature**, also known as shelf stable and are vulnerable to spoilage organisms and/or pathogenic growth.

Commonly included food products in challenge studies			
Bakery items stored at room temperature like icings and non-fruit pies	Refrigerated ready-to-eat (RTE) products like pasta entrees and deli salads	Dairy products and juice drinks	Modified atmosphere packaged (MAP) products like vegetables, meats, poultry and fish
Shelf-stable salad dressings and condiments	Confectionery	Foods formulated with new preservatives	

How to design your microbiological challenge study

Microbiological challenge testing mainly determines the **effect of the external environment** on a food's shelf life.

During challenge testing, products are intentionally contaminated with microorganisms to assess the food's ability to inhibit or control the growth of these microbes under expected storage and handling conditions. These microorganisms are chosen because they **share similar characteristics to those that might naturally occur in or contaminate** the food.

The contaminated product is then stored at the recommended storage temperature and packaging conditions — such as the appropriate gas atmosphere — for a specific time period that corresponds with the product's current or intended shelf life.

NSF Standard 75 for Non-Potentially Hazardous Foods

In 2000, NSF International published ANSI/NSF Standard 75 for Non-Potentially Hazardous Foods, which is a guideline for challenge study design and performance in baked foods.

This standard applies to baked goods held without temperature controls and considered non-potentially hazardous by formulation or manufacturing process.

Examples of food categories specified in NSF Standard 75 include:

- Specialty breads or pastries containing vegetables or soft cheeses added before baking.
- Bakery products filled or topped with cream, crème, custard, or cheese after baking.
- Products filled before baking, such as pumpkin, sweet potato, custard, or meringue pies.
- Components like toppings, glazes, icings, or fillings stored without temperature control prior to use in other products.

EURL Technical Guidance Document

There are also other legislative requirements to follow in your microbiological challenge studies, such as food safety criteria in the EURL Technical Guidance Document for Listeria monocytogenes in ready-to-eat (RTE) food. In this case, FBOs need to perform microbiological challenge tests to trace the behavior of *L. monocytogenes* in their products and demonstrate their adherence to the legislation to the relevant authorities.

The EURL document describes laboratory studies, challenge tests and durability studies mostly related to *L. monocytogenes* in packaged products. However, for unpackaged products, additional factors like hygrometry need to be considered for storage under foreseeable conditions.

The shelf life is then determined for the product as marketed by the manufacturer and a new shelf life must be assessed once the product is opened and stored by a retailer or restaurant.

The EURL document provides the information required before implementing a challenge test, as well as recommendations for performing challenge tests and durability studies.

What to consider in your microbiological challenge study design

When an FBO determines a challenge study is needed for a particular food, you should consider the following factors in your study design:

- The challenge microorganisms
- The right number of control and test samples
- The inoculum preparation and level
- The storage temperature
- The duration of the study

Choosing your challenge microorganisms

The expected storage temperature and the product's history of past microbial contamination are used to assess how likely it is for microorganisms to grow in it.

Generally speaking, challenge organisms are selected from those previously isolated in similar foods.

Surrogate strains, such as *Clostridium sporogenes* for *Clostridium botulinum, or Listeria innocua* for *Listeria monocytogenes*, are commonly used when pathogenic strains cannot be used in processing plants.

Table-1: Typical pathogenic microorganisms for diferent matrices which are also used in challenge testing of various foods (adapted from IFT/FDA 2003b and NACMCF 2009).

Food category	Challenge microorganism(s)
Salad dressings	*Salmonella* spp., Coagulase (+) Staphylococci
Dairy products	*Salmonella* spp., C. *botulinum, E. coli* O157:H7, Coagulase (+) Staphylococci, *Listeria monocytogenes*
Confectionary products	*Salmonella* spp.
Sauces and salsas stored at ambient temperature	*Salmonella* spp., Coagulase (+) Staphylococci
Cooked or dried meat and poultry	*C. botulinum, C. perfringens, L. monocytogenes, Salmonella spp., S. aureus, E. coli* O157:H7
Fish and seafood	*B. cereus, C. botulinum, L. monocytogenes, Salmonella* spp., *Shigella* spp., *Vibrio* spp., Coagulase (+) Staphylococci
Fruits and vegetables	*B. cereus, C. botulinum, E. coli* O157:H7, *Listeria monocytogenes, Salmonella* spp. *Shigella* spp., *Yersinia enterocolitica*
Cereal grains and related products (e.g. fresh pasta, cooked rice)	*B. cereus, C. botulinum, Salmonella* spp., Coagulase (+) Staphylococci

There are many factors to consider when choosing surrogate strains of challenge microorganisms. Typically, they must be:

- Non-harmful to humans (non-pathogenic).
- Able to grow and survive in a stable and consistent manner, similar to the target pathogen when exposed to the same conditions (like pH, temperature sensitivity and oxygen levels).
- Similar in characteristics for being killed off and the rate at which this happens, just like the target pathogen.
- Easy to produce, remain stable over time and grow in large numbers that are easy to count and identify in a mix of different organisms.
- Genetically stable to ensure consistent results in different tests.
- Similar in susceptibility to stress and injury as the target pathogen.

It's also important to know the minimum, maximum and optimum microbial growth conditions for effective risk assessments before conducting microbiological challenge studies.

Whether the food product — or food test matrix — can support or inhibit microbial growth and survival (and in turn affect shelf life) also involves a combination of important intrinsic and extrinsic factors.

Intrinsic factors	
Water activity (a_w)	pH
Nutrient availability	Oxidation-reduction (redox) potential
Presence of naturally occurring antimicrobial compounds	Background (competitive) microflora
Extrinsic factors	
Packaging	Processing
Storage time	Temperature

Table-2: Most common pathogenic and spoilage organisms and their minimum pH and a_w requirements (adapted from NSF/ANSI 75, 2000 and Microorganisms in Food 5, 1996).

	Challange organism	Minimum pH of product components	Minimum aW of product components
TOP PATHOGENIC CHALLENGE ORGANISMS	*Staphylococcus aureus*	>4,6	>0,85
	Bacillus cereus	>4,4	>0,91
	Salmonella spp.	>4,6	>0,94
	E. coli O157:H7	>4,6	>0,95
	Listeria monocytogenes	>4,6	>0,92
	Clostridium perfringens	>5,5	>0,93
OTHER BACTERIA	*Pseudomonas sp.*	5,5	0,97
	Most spoilage	variable	0,90
	Lactic acid bacteria	3,8	0,94
YEAST	Refrigeration	4,0-8,5	0,80
	Most spoilage		0,88
	Osmophilic		0,61
MOLD	Refrigeration	2,0-8,5	0,60
	Most spoilage		0,80
	Xerophilic		0,61

Table-3: Typical pathogenic pH and aW requirements and spoilage organisms by food groups (adapted from NSF/ANSI 75, 2000 and Microorganisms in Food 5, 1996).

Food group	pH	aW	Predominant spoilage organisms
Fresh meats Fish Poultry	>4,5	>0,95	**Aerobic:** Gram-negative rods such as *Pseudomonas* **Anaerobic:** Lactic acid bacteria
Vegetables	>4,5	>0,95	**Aerobic:** Gram-negative rods such as *Erwinia, Pseudomonas* **Anaerobic:** Lactic acid bacteria
Fruits	>4,5	>0,95	Molds, Lactic acid bacteria, Yeast
Breads, Rolls, Cakes	>4,5	0,95	Yeast, Mold (high starch and baking inhibits bacteria)
Salad dressings	>4,5	>0,95	Lactic acid bacteria, Yeast, Mold
Confectionary	>4,5	<0,90	Yeast, Mold
Dried vegetables, Cereals, Cocoa	>4,5	<0,90	Mold
Juices	>4,5	>0,95	Lactic acid bacteria, Yeast, *Alicyclobacillus, Acetobacter, Gluconobacter*
Pasteurized milk, Ice Cream	>4,5	>0,95	*Bacillus*

It's also vital to ensure that your selected microorganisms for challenge testing are appropriate and reflective of the real-world conditions the product is likely to encounter:

- **Food formulation and product components** — consider the packaging and storage temperature of the product.
- **Storage temperature** — pay special attention to products that might allow the growth of psychrotrophic pathogens, such as *Listeria monocytogenes, Yersinia enterocolitica* and *Bacillus cereus*.
- **Type of packaging** — this can include:
  - **Modified atmosphere or vacuum packaging** — these may be susceptible to anaerobic organisms like *Clostridium botulinum* or microaerophilic spoilage organisms like lactic acid bacteria.
  - **Aerobic environment packaging** — items like bakery goods, fresh meats and produce may be more susceptible to aerobic organisms such as *Pseudomonas* and molds.
  - **Facultative anaerobes** — organisms such as *Salmonella* and *E. coli O157:H7*, which can grow with or without oxygen, should be considered for all types of packaging.
- **Foodborne illness data** — reviewing data on the incidence, mortality and product recalls associated with foodborne illnesses helps in selecting appropriate pathogens for challenge studies.
- **Use of specific strains** — although individual strains provide some information, it is recommended to use at least five strains of an organism to increase the confidence that the challenge study accurately reflects real-life susceptibility. For spoilage organisms, using samples taken directly from the specific product being tested will give the most accurate results.

Setting the optimal number of control and test samples

For microbiological challenge testing, you should prepare at least two control samples to assess changes in pH, water activity, aerobic plate count and yeast and mold count during the test period.

Control samples for most food categories should be sliced to evaluate the presence of naturally occurring challenge organisms and assess parameters at the beginning (Day 0) and end of the test period. These sample slices may be repackaged, if necessary.

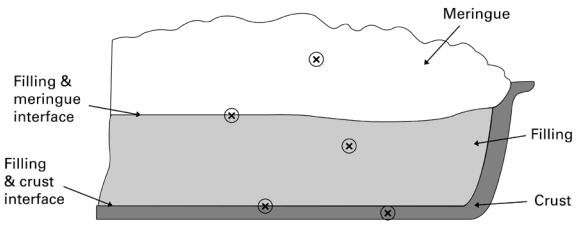
Test samples can then be prepared to assess the product's ability to support rapid and progressive growth of challenge organisms inoculated into the product.

Separate test samples are prepared for each composite of challenge organisms, with five samples prepared at t_0 (time zero), using the following formula:

$$\left(\begin{matrix}5\\ t_0 \text{ samples}\end{matrix}\right) \times \left(\begin{matrix}3\\ \text{different lots}\end{matrix}\right) = \left(\begin{matrix}15 \text{ samples}\\ \text{per product, } t_0\end{matrix}\right)$$

Here are some common examples of how food processors might choose different control and test samples:

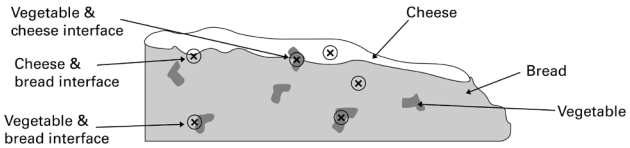
CROSS-SECTION OF A CHOCOLATE MERINGUE PIE



⊗ POINT OF INOCULATION

Points of inoculation
1. Meringue
2. Filling & meringue interface
3. Filling
4. Filling & crust interface
5. Crust

CROSS-SECTION OF VEGETABLE AND CHEESE BREAD



⊗ POINT OF INOCULATION

Points of inoculation
1. Bread
2. Bread & ceringue interface
3. Cheese
4. Cheese & vegetable interface
5. Vegetable
6. Bread & vegetable interface

Figure-1: Cross-section of two example multi-component food products and recommended points of inoculation (from NSF/ANSI 75 – 2000).

To figure out the total number of products needed per composite of challenge organisms, you first determine the number of time points, then use the formula below:

$$\left(\begin{matrix}2\\ \text{test samples}\end{matrix}\right) \times \left(\begin{matrix}3\\ \text{different lots}\end{matrix}\right) \times \left(\begin{matrix}\text{number of}\\ \text{time points}\end{matrix}\right) = \left(\begin{matrix}\text{number of}\\ \text{samples needed}\end{matrix}\right)$$

For products large enough, you might require two samples for each time point — each slice of a cake, for example.

In cases where products are too small to provide two samples, you can use multiple products.

Timing your study right

A challenge study should cover the entire expected shelf-life of a product. The number and timing of analyses are determined based on studies conducted with similar products. The product should be evaluated at intervals, with a minimum of five to seven data points included in the study to capture significant changes in counts.

For products with shelf life measured in days, tests should be conducted daily. For products with shelf life measured in weeks or months, tests should be conducted at least once per week.

Organism counts at t_0 (time zero) should be determined two hours after inoculation.

In most cases, a challenge study is conducted for the length of a product's expected shelf- life. However, an additional margin of safety is added when specifying the duration of the study:

- 50% for products with shelf life of 7 to 10 days
- 25% for products with shelf life of 3 to 6 months.

Preparing your inoculum

There are no specific guidelines for inoculum preparation. Instead, it varies based on the type of inoculum and the nature of the food matrix.

A basic rule in inoculation trials is to standardize protocols for culture preparation. This includes:

- Clear instructions on culture maintenance (examples include refrigerated cultures; slants; cultures frozen in glycerol; and freeze-dried cultures).
- Isolate sub-culturing and recovery.

It's also crucial to maintain cultures properly during challenge studies. For instance:

- Ensure strains previously isolated from extreme environments (for example, in low pH or high sugar matrices) retain specific characteristics developed from previous exposure.
- Store strains in a medium similar to their original isolation environment (such as a yeast strain from a low-pH beverage should be stored in a similar pH medium).
- Avoid prolonged exposure to different conditions to maintain the strain's ability to grow under original conditions.

Cultivating and preparing cells and spores

Cell cultivation should happen under optimal growth conditions for each strain. Typically, this involves growth for 18 to 24 hours under optimal temperature and atmospheric conditions, although certain procedures may require 48 to 72 hours, especially for certain yeasts.

Culture enumeration is crucial at this stage to determine the required dilutions to achieve the target inoculum in the challenge product.

Spores may need special preparation, such as:

1. Washing and storing in distilled water or alcoholic suspension to prevent germination.
2. Heat-shocking immediately prior to inoculation for prompt germination and growth.
3. Thorough washing to minimize the transfer of free toxins during inoculation — for example, botulinal toxin from *C. botulinum*.

Centrifuging cultures and cells

Most protocols involve centrifuging cultures and thoroughly washing cell pellets to avoid transferring compounds from laboratory media to the food matrix.

For liquid products, washed pellets should be resuspended in the same food product, and subsequent dilutions should be made with the same product.

For solid products, cell pellets should be resuspended in appropriate diluents, using minimal inoculum volume to avoid altering the product's intrinsic characteristics, such as pH and water activity.

Microbial cell recovery

Alternatively, microbial cell recovery from lawn plates prepared from 24–48-hour cultures is another method of inoculum preparation. Lawn plates, prepared using grown cultures, are incubated under optimal growth conditions.

Cell recovery is carried out using an appropriate carrier and the resuspended cells or spores are diluted and used in challenge trials.

Lawn-collected cells have enhanced survival potential compared to broth-collected cells, representing a worst-case scenario in challenge experiments.

Selecting the right inoculum level

Inoculum levels per unit weight or volume of a product should be realistic and directly correlated to the purpose of the challenge testing.

For evaluating the safety and stability of a product during a specified period, initial inoculum levels should be between 100 and 1000 cfu per gram or mL of the product.

Levels lower than 100 cfu per gram or mL may fall below detection limits in many sampling methodologies, potentially leading to incorrect assumptions about product safety and stability.

On the other hand, levels higher than 1000 cfu per gram or mL may overwhelm the food's intrinsic preservation properties, potentially leading to false assumptions about product safety and stability.

For trials aiming to determine microbial log reductions following the application of specific stress, such as heating or irradiation, inoculum levels can range from 10^6 to 10^8 cfu per gram or mL of the product, depending on the required log reduction.

For example, to confirm that a specific heating process can achieve a five-log reduction in *Listeria monocytogenes* in cheese, the product should be inoculated with a minimum of 10^6 cells per gram and then subjected to the heating process.

Inoculating your test sample

A successful challenge test ensures that inoculation does not affect any intrinsic or extrinsic properties of the product. The methodology used must be reproducible, properly validated and include pre- and post-inoculation analysis of critical characteristics like moisture content, water activity and pH.

The choice and volume of the liquid inoculum carrier are crucial parameters affecting the trial's success. The aim should be to use the minimum possible volume of carriers to minimize changes in product characteristics.

In liquid products, the inoculum can be suspended in a sample of the product matrix itself, creating a stock for inoculating different product samples. In cases where maintaining moisture levels is vital, the inoculum carrier can be the same diluent used to adjust the moisture content of the product formulation.

The inoculation methodology should ensure even distribution of the inoculum within the product matrix to minimize sampling and enumeration errors.

Mixing of inoculum within the product matrix can be done in products with water activities higher than a_w 0.96, such as sauces, using minimal carrier volume.

Spraying or surface dot contamination can be employed for inoculating product surfaces, with care taken to minimize exposure to pathogenic aerosols.

For multi-component foods, each component should be inoculated at the product slice by micropipette with a fraction of the total inoculum volume.

Following inoculation, slices of the product should be reassembled into their original shape and repackaged according to the manufacturer's recommendation. The product is stored in the repackaged state during storage until evaluation, with potential need for additional packaging materials from manufacturers.

Storing your samples correctly

Challenge study samples should be stored at the same temperatures as the expected storage conditions of the product. If the product is to be refrigerated, then the challenge study samples should also be refrigerated. The same applies to products stored at room temperature.

Products prone to temperature abuse, such as those stored in hot warehouses or under substandard refrigeration, should be challenged at those temperatures.

Test samples should be placed in the same packaging as the final product delivered to retailers to reflect real-world conditions as accurately as possible.

If the tested formulation is expected to be shelf-stable, using sterile containers may be advisable. Although this approach does not simulate typical storage conditions, it eliminates sample variation.

Interpreting data: what can you learn from your challenge test?

In most cases, the average count of major pathogenic challenge organisms such as *Salmonella* spp., *Listeria monocytogenes, Bacillus cereus, Clostridium perfringens, Staphylococcus aureus,* or *E. coli O157*:H7 should not increase more than 1 log for two consecutive time points, or more than 1 log by the last time point compared to the average count on Day 0.

For toxins and toxin producers, the criteria include the following:

- Pathogens → shall not increase more than 1 log
- Toxin producers (e.g. *S. aureus*) → shall not increase more than 3 log
- *C. botulinum* → no toxin production is allowed

The average count should be the geometric mean value of two samples per lot for each time point, calculated by reducing each value to its logarithm, adding these values and dividing by the number of determinations to obtain the log average.

Interpreting data from the challenge study is based on trend evaluation. An increase of one log is generally considered significant, if noted at two or more intervals.

Replicating samples is vital to determine the validity of the challenge test, ideally with duplicate or triplicate samples for each sampling point, with the trial repeated two or three times independently for a high degree of certainty.

Selecting the right media and reagent for sampling and enumeration depends on the type of microorganisms and the challenge matrix, using selective media with care to avoid additional stress on microorganisms.

Monitoring parameters like water activity (a_w) and pH throughout the challenge tests also helps validate results.

The most common way to analyze trends and present results is by graphical plotting of mean log counts against time, or mean survivor curves.

These challenge trial outcomes are crucial for determining product shelf life, indicating potential changes in formulation affecting safety and stability and developing predictive models for current and future product formulations.

Conclusion

Designing a challenge testing study requires a combination of skills and knowledge, starting with a risk assessment to identify potential hazards. This involves selecting appropriate challenge microorganisms, determining inoculation methods, setting up controls and interpreting results based on guidelines and past experiences.

Since challenge trials often involve pathogenic microorganisms, they need to be conducted by qualified microbiologists familiar with handling these pathogens and their toxins. This expertise ensures safety but also makes challenge testing expensive.

The demand for natural ingredients in food products is rising due to consumers preferring healthier options — a trend expected to continue long into the future.

In turn, this has led to the reformulation of existing products to include natural ingredients and to meet "clean label" standards. This makes it crucial to verify the safety and stability of these new formulations.

Reformulating products also involves replacing chemical preservatives with natural alternatives, accommodating diverse product lines like vegetarian options and meeting retailer demands for larger production lots.

With these changes increasing the pressure on internal quality assurance processes within the industry, it is more important than ever to conduct effective microbiological challenge studies and keep up with the rapid pace of the evolving food industry.

About SGS

We are SGS – a culture built on trust, with a passion for excellence.

We are SGS – the world's leading testing, inspection and certification company. We are recognized as the global benchmark for sustainability, quality and integrity. Our 99,600 employees operate a network of 2,600 offices and laboratories around the world.

Food safety services with SGS

We perform microbiological safety testing in our global network of food laboratories to detect foodborne pathogens and spoilage organisms, to assist food safety along the supply chain, during production and in the final product. Our services range from enumeration of indicator organisms to identification of foodborne pathogens, including *Listeria spp., E. coli, C.perfringens, Campylobacter spp., S.aureus, Salmonella spp.* and others.

Amongst our many services, we offer challenge testing. Challenge testing is an effective method for gaining an understanding of how robust your product is against spoilage or pathogens' behavior during storage. Our challenge test protocols are designed using established principles and guidance (EURL Lm, ISO 20976-1:2019) as well as our extensive experience with a broad range of products, packaging, organisms and inoculation methods.

In addition to routine testing and environmental monitoring, we offer microbiological shelf-life determination for a wide range of foodstuffs. We have extensive storage capacity in both ambient and chilled conditions.

Our microbiology laboratories operate seven days a week.

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