Microbiological Testing of Fresh Produce

A White Paper on Considerations in Developing and Using Microbiological Sampling and Testing Procedures if Used as Part of a Food Safety Program for Fresh Fruit and Vegetable Products

OBJECTIVE

The purpose of this white paper is to briefly identify where a microbiological testing program may be useful and considerations to take for designing and implementing a program.

It is not the intention of this paper to establish specific microbiological testing recommendations or requirements for any fruit or vegetable product or commodity.

This paper was developed based on the best available current knowledge, and implications may change as more data are collected regarding the microbiology of fresh fruit and vegetable products.

INTRODUCTION

Food safety is an integral part of the production of all foods and the shared responsibility of all segments of the supply chain. In recent times there has been increased awareness for the need to evaluate the food safety practices in the production of agricultural products. Consumer demands for fresh and convenient forms of produce have led to the development of "Field to Fork" food safety practices in the fresh produce industry. The use of a microbiological testing program is one tool that may be used in the development and verification of a food safety program.

For purposes of this white paper, the term "produce" is synonymous with "fruits and vegetables".

BACKGROUND

Microbiological testing is not a guarantee of product safety. It is one component of an overall food safety system. Before microbiological testing is initiated, prerequisite programs must be in place. These should include programs that are appropriate to the specific operation, such as: Good Agricultural Practices (GAP), Good Manufacturing Practices (GMP), Sanitation Practices, Hazard Analysis Critical Control Point (HACCP), Traceability and Recall Management.

When sampling plans and methodology are properly designed and performed, microbiological testing can provide important information about an environment, a process, and even a specific product lot. However, when not properly designed and performed, testing can provide inaccurate information that can easily be taken out of context and create unwarranted concerns or false reassurances about the safety of the product.

Proper testing design depends on a number of pre-sample factors, including:

- o The intended purpose of the test
- o The intended target organism that is being tested for

- o   Where the sample is in the supply chain
- o   The commodity under consideration, knowledge of the growing, harvesting, and processing control strategies;
- o   The region where the product is grown;
- o   The intended use of the product.
- o   The level of "stringency" required and level of "confidence" required to demonstrate that level of stringency is being achieved.

The design of microbiological testing programs is a complex process and microbiological testing is not a stand alone program.

I.   ASSESSING THE NEED TO TEST

Risk can easily be defined: it is the possibility that an undesirable outcome will occur. However, the quantification of risk in order to develop sound risk management programs is a much more daunting task. Developing a thorough understanding of the probabilities of all alternative outcomes throughout the process is the essential first step in determining the need to test.   From a microbial food safety standpoint, this means identifying all the possible sources/points that pathogens may contribute to one of the two final, alternative outcomes: pathogen detected or not detected.

However, it is important to realize that microbiological testing can never determine whether a food is pathogen-free, unless 100% of the food is tested (and then there is nothing left to sell or eat). The most one can achieve with microbiological testing is "pathogen not detected" and understand the levels of sensitivity and confidence provided by the sampling plans and testing methodologies used.   International organizations recommend testing only when there is good evidence that there is a microbiological problem <u>and</u> that testing will help to control the problem (Codex and ICMSF). Any misunderstanding in what is achievable by microbiological testing, and the limitations of such testing, will tend to waste resources, product and potentially create a worse food safety situation than if no testing was performed.

A.   Why test?

Any testing program should be science-based and objective driven. Prior to implementation one should know why the testing is being performed, the basic assumptions underlying the test, the relative certainty of detecting an issue, and potential results. This will allow one to identify the type of samples to be collected, the sampling plan to be used, the specific test to be performed, and actions to be taken prior to and after the test results are obtained.

Typical reasons for testing in the fresh produce industry are:

1) Meeting product specifications(inputs and finished product)
2) Baseline development and identification of risk factors,
3) Process capability/validation,
4) Process verification,
5) Investigative testing and remedial activity verification, and
6) Verifying that regulatory guidelines have been met.

(1) Product specifications.

The most common reason for microbiological testing in the fresh produce industry today is to comply with a product specification. Inherent in any product specification

are assumptions that the sampling and test methods will provide a standard deviation and level of confidence in test results such that the user of the result will "know" that their specification was or was not met. In reality, specifications are rarely set by statisticians, and users wrongly assume that the number they've selected is an absolute limit. Consequently, test and method developers must take these expectations into consideration when establishing sampling plans and interpretations of the results.

So, in a practical sense, specifications should identify:

- The product to be tested
- The frequency of testing (e.g., every fifth lot shipped to Customer)
- The sample size and how the sample is to be collected (e.g., a 125 g composite of five 25 g samples collected from the beginning, middle and end of the production run)
- The target organism
- Test method
- Acceptance criteria (examples: "not to exceed 10^6 cfu/g aerobic plate count", "not to exceed 1000 cfu/g yeast and mold", or "no detectable *Salmonella* or *E. coli* O157:H7 in 25 g")
- Actions to be taken in the event that the acceptance criteria are exceeded.

Best practices dictate that any lots tested for pathogens are maintained in the supplier's control until cleared by the test results.

(2) Baseline determination and identification of risk factors.

Prior to using microbiological testing to assess quality, safety or process verification, it is important to understand what's statistically "normal". Microbiological testing can be useful to understand the range of microbial populations that can be observed and how they may change by specific type of produce, growing and handling practices, season, weather, geography, environmental controls and other effectors that may not be as obvious. Baseline assessment should take place over a timeframe sufficient to capture the variability of interest, e.g., hourly, daily or seasonally.

Key elements of a baseline assessment are:

- Standardization of test methodology to enable comparing and compiling of data;
- Establishing the frequency, number of tests and/or period of time required to have confidence in the accuracy of the baseline;
- Managing such data through "control charting" (a graphic representation of the data) and/or in a database; and
- Analyzing for trends and patterns

(3) Process capability/validation.

Microbiological testing can be used to "validate" the process' capability to reduce a particular or overall microbial population, or at least to ensure that the process does not allow microorganisms to grow or spread throughout a lot. Validations most often begin with whatever background microflora that comes with the test lot. It is important to have an accurate assessment of the variability (levels and type) of this target microflora in the starting material. Samples are collected at points in the process, to assess the impact of individual steps. Properly performed, a validation study may conclude that "under the conditions of this study, this process is

consistently capable of producing product with an acceptable level of microbial quality."

The benefits of a validated process are:

1) The operator understands the factors that are critical to control to produce reliable results
2) The operator understands the limits at which those factors must be maintained, and
3) Routine monitoring of the microbiological quality of individual lots can be greatly reduced.

(4) Process verification.

Process verification utilizes microbiological testing to "verify" (i.e., confirm) that the "process" performed as anticipated. Process verification differs from validation in that validation utilizes an initial, fixed, predetermined number of repetitions and tests, while verification involves periodic, ongoing testing. Process verification is intended to demonstrate your validated process is functioning as designed, i.e., one is not getting statistically significantly different results than those observed during the validation trials.

(5) Investigative testing and remedial action verification.
Microbiological testing can be a very effective tool to investigate sources and causes of an unexpected microbiological result. For example, if a process verification test indicates a much higher aerobic plate count than expected, or if an undesirable and unexpected microorganism is detected in a finished product, targeted microbiological testing can be used to:
   a. investigate the source of the unexpected microorganisms
   b. verify that remedial action was successful in eliminating the source.

(6) Verifying that regulatory guidelines have been met.

Microbiological testing can be used to demonstrate compliance to published regulatory guidelines or requirements.

B. Why Not to Test

1) Used as a substitute for sound process controls
2) Repeat testing to negate an unwanted or undesired result
3) "Prove" that a contaminated product is "safe"

(1) Process Control

Microbiological testing is not a substitute for a sound process. Process control, if achievable, will always be more effective and reliable than microbiological testing in assuring microbiological quality and safety.

(2) Unwanted/Undesired Results

An unacceptable microbiological result is always valid unless there is a sound reason (i.e. lab error) that the result may be false. Produce grown outdoors is subject to random environmental factors. This results in the microorganisms present to be non-uniform in distribution. Multiple testing of the same lot can provide very different results. Retesting and getting a "negative" result after getting a "positive" result does not negate the positive result.

(3) Contaminated Product

If a produce product becomes contaminated with a pathogen of public health significance, it is considered adulterated. Unless an acceptable, effective reprocessing method can be employed to eliminate the contaminant, the product cannot be "tested" into safety. The FDA does not currently recognize any reprocessing method, other than diversion to a cooked or otherwise pasteurized product, as an acceptable method to "clear" an adulterated fresh produce product.

C. What to test

In selecting what should be sampled and tested, first understand the objective of the test as noted in section A. Second, select samples or sampling points most likely to achieve that objective.

Items to consider when determining what to test are:

- What is the target microorganism of interest and where may it be observed?
- The expected prevalence of the microorganism in the product, process or environment: Is it commonly found or rarely found?
- The expected distribution of the microorganism in the product, process or environment: Is it uniformly distributed or a sporadic event?
- Are there practices (or failure to follow them), conditions, or events with a history of leading to contamination events? For example: Product flow fails to follow a raw to process pattern, which causes a mingling of raw product and processed product and potential cross contamination of the finished product

Where answers to these questions may indicate a need for testing, an evaluation must be done to determine the appropriate step in the process where testing may provide information that is most useful.

The following are examples of what could be subject to testing and the rationale for testing:

- Water: Generic E. coli, which may be present in irrigation canal water if fecal contamination has occurred, in levels and distributions depending on the source of the E. coli and, if present in high numbers, may indicate a fecal contamination which indicates the potential presence of human pathogens.
- Compost: Thermotolerant coliforms, which are expected to be present in raw manure in a generally uniform distribution and, if present in composted manure, may indicate incomplete composting.
- Environmental Testing: *Listeria spp.* are not expected to be present in the produce processing area. If present, *Listeria spp.* are expected to be distributed sporadically and, if detected, may indicate harborage of *Listeria monocytogenes.*

D. When to test

As with all aspects of microbiological testing, when to sample, the frequency of sampling/testing and the size/number of samples to analyze, should be objective driven. The timing or frequency of sampling and testing affect the likelihood of achieving the objectives of the testing.

QUESTIONS TO ANSWER TO DETERMINE WHEN TO SAMPLE AND TEST

1. What information do we want the test to give us?
2. Where are those test organisms most likely to be found?
3. Do we have any information or evidence of contamination or potential contamination?
4. When is contamination most likely to occur?
5. What is the expected prevalence of the target organism?
6. What is the expected distribution of the test organism?
7. What are the expected levels of the organism, if present?
8. Do we have sampling plans and testing methodologies available that can reliably detect the test organism at the expected distribution and levels, if present?
9. Will the test results be available in time to take action, if needed?

When testing finished product, the best time to sample the product would be after the last potential source of contamination, as defined by a hazard assessment. In the absence of a hazard assessment, then sampling might be performed as soon after completion of the process as feasible; e.g., after packaging or from shipping containers.

E. Limitations of testing.

Just as one should know why the testing is being performed, it is important to know that the reasons for testing are valid and that testing is an effective tool towards achieving the objective.

- Microbiological testing is not a substitute for a reliable and validated process. Ongoing, validated process control, if achievable, will be more effective and reliable than microbiological testing in assuring microbiological safety.

    - Example – real time monitoring and verification of antimicrobial levels in flume water provides actionable information for immediate process control, as opposed to microbiological testing which provides information after the fact and too late to take effective action.

- Testing cannot assure the absence of pathogens. There is a natural tendency to believe that a negative test result means the product is safe, even if a process goes out of control and there is reason to believe that the product may be contaminated. Before relying solely on a negative test result to affirm the safety of a material, remember there is truth to the adage, "absence of evidence is not evidence of absence." The effectiveness of microbiological testing to detect lots that are contaminated decreases when the defect rate (e.g, the percentage of contamination in a single produce item or lot of items) falls below approximately 5%

- Product reconditioning. FDA does not recognize any process (other than diversion to a product that is cooked or will otherwise receive pasteurizing treatment) for reconditioning fresh produce that may have been adulterated with pathogens.

II. MICROBIOLOGY

A. Which microorganisms to test for

Many different kinds of microorganisms can be found on fresh produce, and most have little to no effect on humans, even if consumed in large numbers. Only a relative few have the ability to cause human illness. Fresh and fresh-cut produce are not sterile products. The microorganisms present fluctuate greatly depending on the type and variety of produce, the season and weather, the growing conditions and locations, as well as the health and condition of the produce.

Aerobic Plate Count

- Aerobic plate count (APC), also known as Total Plate Count (TPC) is used as an indicator of the number of bacteria in a food product. APC only measures those microorganisms capable of growing at 30-37°C in the presence of oxygen.
- Aerobic plate counts are typically incubated at 35±1°C for 48±3 hours, but other temperatures (e.g. 25°C) may be used.
- It is not unusual for Aerobic Plate Counts on produce to range from thousands to millions (10^3 to 10^7/g) depending on the commodity. Many of these organisms cannot grow at the low temperatures used for storing fresh and fresh-cut produce, and fewer can grow in an oxygen-depleted atmosphere. Further, many of the organisms that can grow at low temperatures cannot grow at the higher temperature used for the APC test.
- It is important to remember that microorganisms detected by APC are usually not pathogens, APC results do <u>not</u> correlate well with the potential for pathogen contamination, and are not useful predictors of product safety.

- When to measure:
  1. Trend analysis of finished product microbial ecology
  2. Environmental indicator of sanitation processes
  3. Indicator of process control
  4. Have a reason to suspect that the microbiological quality of the product may be unacceptable.

- When not to measure:
  1. Indicator of safety
  2. Indicator of the presence or absence of pathogens
  3. Routine indicator of initial quality
  4. When baseline studies demonstrate that product or environmental conditions normally have a wide variability in microbial populations

Psychrotrophs

- Psychrotrophs are microorganisms capable of growing at refrigeration temperatures. They may or may not be able to grow at higher temperatures. The microorganisms capable of spoiling fresh produce under refrigerated conditions are psychrotrophs.
- Incubation parameter for psychrotroph growth is 7°C± 1°C for 4-10 days.
- Total Psychrotrophic Counts have been used by some as an indicator of microbial quality. Total Psychrotrophic Count is not generally considered a good indicator of potential pathogen contamination.

- When to measure:
  1. Profiling spoilage processes of refrigerated products

- When not to measure:
  1. Indicator of safety
  2. Indicator of the presence or absence of pathogens
  3. Indicator of initial quality
  4. When rapid results are necessary, because the test takes 4-10 days

Yeast/Mold

- A variety of yeast and molds are commonly found on fresh produce, usually at far lower numbers than bacteria. Yeast and molds tend to have the most effect on fruit quality, because of the higher sugar content and lower pH of many fruits.
- Yeasts and molds are typically grown at 20-25°C for 3-5 days. These organisms tend to grow more slowly than the bacteria detected by APC; slow enough that detection usually requires a test that inhibits the growth of bacteria.
- They are not important spoilage factors in fresh-cut vegetables because their growth is generally far slower than the enzymatic or psychrotrophic bacterial spoilage of the fresh-cut produce.
- It is highly unlikely that the yeast and molds typically found on fresh produce will cause illness, and they are not good indicators of potential pathogen contamination.

- When to Measure:
  1. Indicator of quality for fruit products
  2. Indicator of air quality in coolers and fruit packing facilities

- When not to measure:
  1. Indicator of safety
  2. Indicator of the presence or absence of pathogens
  3. When rapid results are necessary, because the test takes 3-5 days

Coliforms

- "Coliforms" includes a wide array of bacterial genera, and were so named because they were originally thought to grow only in an animal's or human's colon. It is now known that coliforms grow in a wide variety of environments.
- Incubation for coliforms occurs at 35±1°C for 24-48 hours.
- Because some coliforms are part of the natural flora of produce, they are not an accurate indicator of fecal contamination for these products. Consequently, coliform testing has limited value in fresh produce.

- When to measure:
  1. Indicator of potable water quality

- When not to measure:
  1. Indicator of safety of fresh produce
  2. Indicator of the presence or absence of pathogens in fresh produce
  3. Indicator of initial quality

Thermotolerant or "fecal" coliforms

- "Fecal coliforms" are coliforms that are able to grow at higher incubation temperatures

- Incubation for fecal coliforms is typically 44.5 - 45.5°C for 24-48 hours.
- There has been a movement to rename this group "thermotolerant coliforms" because not all so-called fecal coliforms are of fecal origin. Because of this, care must be taken in interpreting the significance of fecal coliform results. For example, it may be very appropriate to verify the adequacy of a manure compost operation using thermotolerant coliforms to ensure that their numbers are reduced; however, testing fresh produce for this group of organisms may have questionable value since they can be part of the normal flora of the plants.
- Detecting thermotolerant coliforms does not necessarily indicate the presence of either fecal matter or pathogens.

When to measure:
1. Indicator of proper compost treatment.

When not to measure:
1. Indicator of safety of fresh produce
2. Indicator of the presence or absence of pathogens in fresh produce
3. Indicator of initial quality

Generic *E. coli*

- Generic *E. coli* are non-pathogenic. These organisms are ubiquitous to most animal, including human, digestive systems and are beneficial to digestive health.
- The minimum growth temperature for generic *E. coli* is about 7°C/45°F, so it is unlikely to become established or grow in a fresh-cut processing environment when the environmental temperature is maintained at <4°C/40°F.
- Testing for generic *E. coli* using traditional most probable number (MPN) and direct plating methods typically take 48 hours at 35±1° C for results.   Recent advances in microbiological testing have been able to reduce this time in some cases to a shorter period (e.g. 24 hrs for Colilert® water testing).
- Generic *E. coli* has long been used as an indicator of fecal contamination in water treatment because it is present in almost all fecal samples.
- Generic *E. coli* is generally considered a better indicator of the potential for fecal contact than APC or coliforms, but does not necessarily indicate the presence of pathogens.
- The levels of *E. coli* do not necessarily correspond to the initial level of fecal contamination in food products that support its growth, but may be indicative of conditions (e.g., temperature abuse) that could support the growth of mesophilic pathogenic bacteria.

- When to measure:
  1. Indicator of water quality
  2. Indicator of proper compost treatment

- When not to measure:
  1. Indicator of shelf life
  2. Indicator of initial quality
  3. Monitoring of in-plant environmental and sanitation practices of fresh-cut operations.

Pathogen Testing

- A pathogen is any agent (bacteria, virus, etc.) that may cause human or animal illness or disease.

- Technology has advanced to permit direct testing for many pathogens in a relatively rapid manner.
- It is recommended that the selection of pathogen tests be "risk based". That is, testing should be designed for pathogens that may be present based on historical or lot specific evidence. For example, the human pathogens *Staphylococcus aureus* and *Clostridium perfringens* are responsible for many foodborne illnesses every year, but neither has been identified as a pathogen of concern for fresh produce, so routine testing of fresh produce for either is unlikely to provide value.
- Considerations when testing for pathogens in fresh and fresh cut produce:

   o If present, the pathogens will usually be at such a low level, and so heterogeneously distributed, as to make it a "needle in a haystack" chance of detecting them by anything less than extensive product sampling.

   o A negative result does not necessarily mean that the product lot was pathogen-free. Properly designed, sampling, and testing for pathogens may be able to detect "gross contamination" (i.e., high frequency contamination events in the same field or produce lot at pathogen levels higher than normal), but is often unreliable in detecting the low levels of contamination that have typically been found when pathogens are detected in produce grown under GAPs (Good Agricultural Practices).

   o Since most test results will be negative, little data that can direct continuous improvement efforts are generated through pathogen testing.

   o Whenever testing for a pathogen, it is important to hold that product lot until cleared by the test results.

- When to test:
  1. When there is reason to suspect contamination with pathogens or fecal contact, either directly (e.g., animals) or indirectly (e.g., contaminated water or improperly treated compost).
  2. When there are significant numbers of generic *E. coli* in water that have contacted the edible portion of the plant.
  3. When there is evidence that prerequisite programs have not been properly or adequately followed.
  4. When there is evidence that a food safety process is out of control.

- When not to test:
  1. When there is no reason to suspect contamination

Environmental Testing

- Environmental monitoring programs are a commonly used tool to assess microbial contamination and to track sanitation effectiveness in a processing facility.
- Aerobic Plate Counts or coliforms are used by some to measure the effectiveness of environmental sanitation, as virtually all non-sporeforming bacteria are expected to be eliminated by an effective sanitation program. However, microbiological testing has largely been replaced by ATP testing procedures, which provide real-time results of sanitation effectiveness. While ATP tests do not reliably correlate with microbial levels, experience has demonstrated the superiority of ATP tests as a sanitation monitoring tool.
- Environmental monitoring for pathogens like *E. coli* O157:H7, *Salmonella* or *Shigella* is rarely done in fresh-cut operations because the typical environmental temperature

in a fresh-cut operation is less than 40°F, generally below the minimum growth temperature for most human pathogens, including the three mentioned, so such pathogens are not reasonably likely to be able to become established.

- In fresh cut operations, environmental testing is often performed to detect the presence of *Listeria* which is able to grow at temperatures less than 40°F. While, *Listeria* may be present on produce in the field, experience has demonstrated that, when it occurs, it is predominantly an environmental contaminant of processing facilities with cold and/or wet environments. Consequently, fresh-cut processors, like most ready-to-eat product processors, use environmental testing for *Listeria* spp. as an indicator to detect potential harborage of the pathogenic species *Listeria monocytogenes.*

- Although *Listeria monocytogenes* is a potentially dangerous human pathogen, with a high mortality rate, fresh and commercially prepared fresh-cut produce has not been associated with a listeriosis outbreak in the U.S. since the early 1980's, when listeriosis was first recognized as a foodborne human disease. Consequently, fresh and commercially-prepared fresh-cut produce are not considered high risk for *L. monocytogenes* exposure. However, a prudent fresh-cut processor will maintain an environmental monitoring program in the processing area for *Listeria* spp.

- Monitoring for Listeria spp. in a raw material area is rarely useful, because transient positives of the organism are expected from field sources. However, with proper trimming, washing and other interventions, low levels of these transient pathogens are not expected to persist through the fresh-cut process.

- Any microbiological monitoring is not usually advised in areas where fresh produce is not exposed, e.g., after packaging, as the risk of contamination from the environment is not reasonably likely to occur.

- A single positive result for *Listeria* spp. in a non-food contact environmental sample of a processing area would not necessarily be a cause for concern because *Listeria* positives are often transient and non-repeating. However, a repetitive positive would be cause for investigation of the environment for potential harborage points, sanitation practices, and GMPs.

- Product testing for *L. monocytogenes* is not often recommended, for the same reasons as noted above for pathogen testing, unless there was reason to believe that the risk of *L. monocytogenes* presence was higher than normal.

- When to test:
    1. As part of a routine environmental monitoring program of fresh-cut processing areas for *Listeria.*
    2. If contamination is suspected.

- When not to test:
    1. For environmental monitoring of areas where conditions are not typically favorable for the harborage of *Listeria.*
    2. When ATP testing can be used to provide real-time results.
    3. When the target organism is not reasonably likely to colonize the environment.
    4. In raw product areas where transient *Listeria* positives from the field are expected.
    5. In areas where finished product is not exposed to a risk of environmental contamination (e.g., fresh-cut salads after packaging).

B. Test methods to use

The selection of test method is often dictated by the conditions of the test, such as the target organism, the material or surface to be tested, whether testing for presence/absence or for quantitative levels, and how soon the results are needed.

Standardization of test methods enables comparing and compiling data with others who may be conducting similar tests in different regions. This allows the management of such data in a database that brings more value than the individual test results by facilitating trend analysis, pattern identification, and input for risk analysis. For example, when evaluating the microbiological quality of a common water supply, pooling data for that water supply from a number of sources would accumulate data to establish a baseline of expected results, and more quickly to potentially identify a possible source of contamination. This type of testing has a great potential to identify and prioritize risks, and subsequently control strategies to reduce risks that warrant control.

Validated Methods

Ideally, the test method used has been validated for the target organism and for the material being tested to ensure accuracy, precision, and reproducibility.

Important points to consider in the selection of a method:

- It has been validated for the material of interest.
- It has been validated against an internationally recognized official method such as AOAC International or Bacteriological Analytical Method (BAM).
- It has been validated through an independent validation study (internal or third party)

The importance of selecting a properly validated method cannot be overstated. Historically there was little interest in testing fresh produce therefore many available testing methods have not been specifically validated for fresh produce applications. This is particularly true for more recently developed, rapid methods.

Types of Test Methods

While there are wide ranges of technologies for the detection of microorganisms, the three most common commercially available types are: cultural, immunoassay, and PCR.

- Cultural Methods
    - Cultural methods are typically tests that allow the target organism, if present, to grow to levels that can be seen or otherwise detected.
    - Historically, cultural methods have been the tests of choice for fresh produce. However, because of recent developments in the methods and validation studies, immunoassay and PCR methods are becoming more accepted.
    - Cultural methods can show the presence/absence of an organism (qualitative) or can provide information on the number of organisms present through plate counts or Most Probable Number/MPN (quantitative).
    - Produce plating methods can produce a live, isolated sample that can be further tested to verify the results. MPN methods require additional handling to produce a live, isolated sample for further testing. Plating methods are relatively insensitive, with a minimum level of detection of about 10-100 cfu/gm (colony forming units per gram of test material), unless paired with a cultural pre-enrichment. MPN methods can be more sensitive, with a minimum level of detection of about 1 cfu/g.
    - Time to obtain results can range from 12 hours to more than a week.

- Immunoassay:

- One common, commercially available type is ELISA (**E**nzyme-**L**inked **I**mmuno **S**orbent **A**ssay, i.e. "dip stick" or "pregnancy test" type method or 96-well methods).
- Uses antibodies to detect specific proteins that are expected to be unique the target microorganism.
- Methods are typically presence/absence tests but some can be quantitative.
- Immunoassay methods are only sensitive if paired with a cultural enrichment.
- Results are usually obtained in 24-48 hrs, including time for cultural pre-enrichment.
- Additional cultural handling is required to produce a live, isolated sample for further testing
- Have been known to be susceptible to false negative and false positive results with various produce matrices.

- PCR (Polymerase Chain Reaction) Methods;
  - This type of test recognizes pieces of DNA or RNA that are expected to be unique to the target microorganism.
  - PCR methods are typically presence/absence tests but some can be quantitative.
  - PCR tests can be rapid and sensitive methods, particularly if paired with a cultural pre-enrichment.
  - Results are usually obtained in 24-48 hours, including time for cultural pre-enrichment, although results can be obtained in less than a day if pre-enrichment is not used.
  - Additional cultural handling is required to produce a live, isolated sample for further testing.
  - Validated PCR tests rarely cross-react with other non-target microorganisms.

Confirmation Testing

During initial screening of a food product for a pathogen, most microbiological tests, particularly presence/absence tests, are designed to provide either a "negative" result, where no further testing is required, or a "presumptive positive" result, which requires further testing. A presumptive positive result is NOT a positive result until confirmation testing is performed.

Many test kits that are designed to detect the presence of one specific target organism (such as *Salmonella*) can also detect organisms that are similar to, but not, the target organism. This situation yields a "false positive" or "presumptive positive" result.

A false positive is when the test, taken to completion, yields a result that the target organism is present, when it really is not. A key aspect of the method validation process is to determine the frequency and causes of false positive results, so that users of the test can be aware and take steps to detect false positive results.

A presumptive positive, on the other hand, is when the test detects an organism that might be the target and cannot quickly yield a result that the target organism is not present. Many tests are designed with a screening feature, that can indicate that no organism matching the target organism is present, quickly clearing the tested material. When a presumptive positive result occurs, the test must be taken to completion to "confirm" whether the detected organism is the target organism or only an organism that behaves similarly in the test. It is important to understand that a presumptive positive that is confirmed as "negative" is not a false positive; a presumptive positive is only a preliminary indication that the target organism may be present.

There may be occasions that justify using a second validated test to further confirm the results of the first test, such as when a test result is unexpected. However, it is important that such confirmation testing be performed only with the original sample or enrichments from the original sample. Testing a different sample, even if it is a "split sample" of the original, cannot be used to negate a positive finding in the first sample.

III.   DEVELOPING A TESTING PROGRAM

A. Determining the quantity of samples

Selecting an appropriate number of samples to test, and understanding the level of confidence in the result that those samples represent, is one of the biggest weaknesses in microbiological testing of fresh produce today. Too frequently, a testing protocol or customer specification will state the maximum acceptable number (e.g., <1000 coliform/g, or none detected in 25 g) and either provide no number or some low number of samples to test, expecting that any sample tested will provide 100% confidence in the result. The International Commission on Microbiological Specifications for Foods (ICMSF, volume 7) examined the statistical confidence of test results on the basis of samples tested, and reported that the probability that a test result will give a false sense of security depends on what level of contamination is present, what percent of the lot is actually contaminated at that level, and how many samples are tested.

Table 1, adapted from ICMSF volume 7, Table 7-1, shows the probability of accepting a contaminated lot (i.e., getting an acceptable test result on a lot that is actually contaminated) on the basis of contamination rate and the number of samples tested.

Composition of lot		% probability of accepting a defective lot at the number of sample units tested from that lot							
% acceptable	% defective	3	5	10	15	20	30	60	100
98	2	94%	90	82	74	67	55	30	13
95	5	86	77	60	46	36	21	5	1
90	10	73	59	35	21	12	4	<	<
80	20	51	33	11	4	1	<		
70	30	34	17	3	<	<			
60	40	22	8	1					
50	50	13	3	<					
40	60	6	1						
30	70	3	<						
20	80	1							
10	90	<*							

Table 1.   *< means less than a 0.5% probability

From the table, one can see, for example, that if a test result is based on 3 samples tested, there is less than a 0.5% chance of missing the contamination if the lot is 90% contaminated, but a 94% chance of missing the contamination if the lot is 2% defective (2 of every 100 leaves or fruit or other unit). At contamination rates less than 2%, one is virtually guaranteed to miss the contamination if testing only 3 samples. The table also shows that if one is trying to detect lots that are 2% contaminated, testing 100 samples would still leave you with a 13% chance of missing the contamination (collecting 100

samples and compositing them may or may not improve the chances of detecting contamination, depending on the test and whether it has been validated for compositing). While increasing the number of samples to be tested would seem an obvious solution, the table shows that one would have to test an impractical number of samples to detect low levels of contamination with any reasonable level of confidence. Taken to the extreme, the only way to achieve near 0% chance of missing a lot contaminated at very low levels would be to test everything. So, if one wants to determine if a lot is contaminated based on, for example, 5 samples, one can either accept good confidence of detecting gross contamination (1% chance of missing a lot contaminated at 60%) or poor confidence of detecting low level contamination (90% chance of missing a lot contaminated at 2%).

B. Ensuring proper sample collection

The accuracy of a test is as dependent on proper sampling technique as on the test itself. Ideally, sample-handling procedures are defined for the specific test method, and all training on sample collection recorded. The following can be used as general guidelines:

- **Training in sample collection** – The sample collector must be trained on how the sample is to be collected, including where and when in the process, how much sample, and specific methods and techniques for collecting the sample.

- **Aseptic technique** – The sample collector must be trained in aseptic sampling procedures. This minimizes the potential for contamination from other sources, including the individual collecting the sample, and from causing a false positive reaction. When aseptic sampling is not practical, such as sampling water at the end of irrigation line, the use of sterile containers and sanitized gloves or handling utensils and careful handling procedures will help minimize the potential for sample contamination.

- **Traceability** – It is essential that all samples, regardless of number, be clearly and accurately identified. At a minimum, a sample should have the following identification information: sampling date and time, sample location or other relative identification, and the person performing the sampling. Depending on the product, additional information such a lot code and sample ID number may be required.

- **Temperature control** – Unless specified otherwise by the test method, fresh produce, water and environmental samples should be chilled (32°-40°F) as soon after collecting the sample as practical and kept cold, without freezing, until tested. A time/temperature recorder, or other device to verify proper temperature control, is recommended if the samples are shipped or held for more than a few hours before testing.

- **Time Dependency** – Even at low temperatures, microorganisms in the sample may grow or die if held for too long before testing, potentially causing erroneous results. Samples should be tested as soon after collection as practical, but should be within 24 or 48 hrs, depending on the test, with < 30 hrs (1 day) highly recommended, especially for environmental swab samples.

- **Sample Handling** – Even when all sampling procedures and techniques are followed, the result will only be as good as the final sample handling. Samples must be handled in an aseptic manner with sterile supplies. Only sterile bags and dilution bottles are to be used. All media need to be properly sterilized before use and when possible, use pre-made media. Work areas must be sanitized and supplies such as pipettes, used in an aseptic manner.

- **Negative Control** – Negative controls should be included as part of sample collection to ensure that proper technique was employed and cross contamination was avoided. The negative control samples should be handled in a manner identical to that of all other samples within the lot. Collection data, storage and handling should be identical to that of true samples to be tested.

C. Selecting a Sample Site

The selection of a sample site must reflect the intended goal of the testing program. This may include the product itself, product-contact environmental, non-product contact environmental, field or water samples.

Some examples of sample site selection follow:

- Example 1: Raw agricultural commodity testing prior to harvest:

    If it is a general field-testing program, samples must be taken from areas that clearly represent the field. On the other hand, if there is a need to identify the possible effect of a localized contamination, such as animal intrusion in a field, then sampling should be restricted to only the affected areas.

- Example 2: Measuring the effectiveness of antimicrobial treatments in process wash water at various concentrations:

    If the goal is to validate the effectiveness of an antimicrobial treatment in process wash water, samples taken at the point of the flume where antimicrobial is added may provide misleading results. Sampling should be conducted near the end of the flume in addition to the beginning to clearly identify the treatment's efficacy.

- Example 3: Process verification of sanitation effectiveness.

    Testing can be used for process verification; i.e., was this run of the process as effective as expected? In order to measure how effective sanitation is in a processing environment, product-contact and non-product contact surfaces may be selected as sampling sites.

When identifying sample sites, one must consider:
- Does the site reflect the product in its "intended use" state?
- Can a representative sample be obtained at the site with reasonable control of preventing contamination?
- Is there a more representative site?

D. Actions based on results

Prior to implementing any testing program, identification of what the results will mean and any subsequent actions that will need to be taken must be clearly identified. Unless there is a reason to suspect the result was not accurate (e.g. lab error identified by not following a written laboratory protocol), all results must be considered valid and actionable. One must remember that microorganisms may not be uniformly distributed in samples.

Examples:

a. APC in a fresh diced onion sample can have an initial count of 95,000 cfu/g, but when ten samples are analyzed, this 95,000 cfu/g sample is found to range from 75,000 cfu/g to 200,000 cfu/g. All results would be valid.

b. A sample of spinach may have a generic *E. coli* count of 20 cfu/g. But when nine additional samples are tested, all are observed to be <10 cfu/g. Finally, the product is tested using an MPN method and a result of <2.2 MPN/g is observed. The 20 cfu/g is still a valid result. None of the <10/g results rule out the initial result. Looking closer, the average of the 20 cfu/g and the nine <10/ cfu/g is 2 cfu/g, which is also consistent with the MPN reading of <2.2 MPN/g. The 20 cfu/g remains a valid result.

Knowing there is a possibility that an unexpected or undesired result may (and will) occur, a clearly defined course of action must be in place. This may include an applicable pasteurization or sterilization treatment, or destruction of the product.

In all cases, including regulatory sampling, it is highly recommended that product subject to pathogen testing should remain on "hold" status, within company custody and control, until the results of all testing are complete and all results are negative for the product. If there are multiple samples taken of a lot, and one sample is found positive, none of the negative results negates the positive result. The lot is positive for the pathogen and the appropriate predetermined action must be implemented.

SUMMARY

Microbiological testing during the processing of fresh produce is a tool that may be of value in verifying the integrity of the product as it passes though each segment of the supply chain. However, if testing is used, it would be but one component in the development of any "Field to Fork" food safety program that includes programs such as Good Agricultural Practices (GAP), Good Manufacturing Practices (GMP), HACCP, Traceability and Recall Management.

A proper testing program must have clearly defined the intended purpose of the test, the organism of concern, logical and defined sampling locations in the supply chain, the use of appropriate and validated methods, and defined actions based on the potential results.

If not properly designed and implemented, microbiological testing can provide unreliable information that can easily be taken out of context and create unwarranted concerns or false assurances about the safety of the product.

Though microbiological testing cannot assure the absence of pathogens, microbiological testing can provide important information about an environment, a process, and even a specific product lot, when sampling plans and methodology are properly designed and performed.

RESOURCES

The following resources may provide additional information and assistance in the development of a microbiological sampling and testing program for fresh product applications. Note: These resources are not all inclusive.

Bacteriological Analytical Manual *Online,* (2009) 8th Ed., Food and Drug Administration, Washington DC

Compendium of Methods for the Microbiological Examination of Foods, (2001) 4th Ed., American Public Health Association, Washington DC

ICMSF (International Commission on Microbiological Specifications for Foods) (1996) Characteristics of Microbial Pathogens, 5, Microbial Ecology of Food Commodities, Blackie Academic & Professional, London

ICMSF (International Commission on Microbiological Specifications for Foods) (2005) Microorganisms in Foods, 6, Microbial Ecology of Food Commodities, Kluwer Academic/Plenum Publishers, New York, USA

ICMSF (International Commission on Microbiological Specifications for Foods) (2002) Microorganisms in Foods, 7, Microbiological Testing In Food Safety Management, Springer, New York, USA

Official Methods of Analysis of AOAC International (2007) 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD

Standard Methods for the Examination of Water and Wastewater, (2005) 21st Ed., American Public Health Association, Washington DC

ACKNOWLEDGEMENTS

April 21, 2010