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Microbiology of the food chain — Requirements and guidelines for conducting challenge tests of food and feed products —

Part 1: Challenge tests to study growth potential, lag time and maximum growth rate

Microbiologie de la chaîne alimentaire — Exigences et lignes directrices pour la réalisation des tests d'épreuve microbiologique —

Partie 1: Tests de croissance pour étudier le potentiel de croissance, le temps de latence et le taux de croissance maximal

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

A list of all the parts in the ISO 20976 series can be found on the ISO website.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

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Introduction

Under the general principles of the Codex Alimentarius on food hygiene, it is the responsibility of food business operators (FBOs) to control microbiological hazards in foods and to manage microbial risks. Therefore, FBOs implement validated control measures[11] within the hazard analysis and critical control point (HACCP) system, and conduct studies in order to investigate compliance with the food safety criteria throughout the food chain.

In the framework of microbial risk assessment (MRA), several complementary approaches are developed to estimate risks posed by pathogens or spoilage microorganisms in the food chain. MRA is adopted by regulators under the auspices of the international agency for setting food standards. Challenge testing is one of the recognized approaches used to validate control measures within the HACCP system, as well as to assess microbiological safety and quality of food, food production processes, food storage conditions and food preparation recommendations for consumers.

This document provides technical rules, calculations and approaches to investigate the ability of inoculated microorganism(s) of concern to grow, survive or be inactivated in raw materials and intermediate or end products under reasonably foreseeable food processes, storage and use conditions. The objective and the scope of the document are to determine the experimental design and the selection of the study conditions. Regulatory authorities can have different recommendations, and these differences have been included as much as possible. It is, however, possible that specific requirements should be incorporated to get regulatory approval of the challenge test.

As growth and inactivation kinetics are clearly different, the ISO 20976 series consists of two parts, under the general title, *Microbiology of the food chain — Requirements and guidelines for conducting challenge tests of food and feed products*:

— *Part 1: Challenge tests to study growth potential, lag time and maximum growth rate*

— *Part 2: Challenge tests to study inactivation potential and kinetics parameters* (to be developed)

The use of the ISO 20976 series involves expertise in relevant areas, such as food microbiology, food science, food processing and statistics. The statistical expertise encompasses an understanding of sampling theory and design of experiments, statistical analysis of microbiological data and overview of scientifically recognized and available mathematical concepts used in predictive modelling. Even though many mathematical models are available to describe and predict bacterial growth, the gamma-concept (γ-concept)[22] is particularly useful for further simulations using the data generated from the challenge test, e.g. to assess the growth at storage temperatures other than the one(s) tested, or in helping to design better food formulations and storage conditions, and thus improving the microbial quality and/or safety of the food under consideration.

For practical reasons, the term “food” includes feed.

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Microbiology of the food chain — Requirements and guidelines for conducting challenge tests of food and feed products —

Part 1:
Challenge tests to study growth potential, lag time and maximum growth rate

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1 Scope

This document specifies protocols for conducting microbiological challenge tests for growth studies on vegetative and spore-forming bacteria in raw materials and intermediate or end products.

The use of this document can be extended to yeasts that do not form mycelium.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 7218, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

ISO 11133, *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media*

ISO 18787:2017, *Foodstuffs — Determination of water activity*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
bacterial spore
resistant form of bacteria that is dormant until the *germination* (3.9) step

3.2
batch
group or set of identifiable food obtained through a given process under practically identical circumstances and produced in a given place within one defined production period

Note 1 to entry: The batch is determined by parameters established beforehand by the organization and may be described by other terms, e.g. lot.

[SOURCE: Commission Regulation (EC) No 2073/2005]

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3.3
cardinal value
estimated minimal, optimal and maximal values of physico-chemical factors (e.g. temperature, pH, a_w) that characterize the growth of a given microbial strain

3.4
control unit
unit of food identical to the *test unit* (3.24) but not artificially contaminated (used as a blank)

3.5
challenge test
study of the growth or inactivation of microorganism(s) artificially inoculated in food

3.6
experimental datapoint
result of analysis of a *test unit* (3.24) per unit weight ($\log_{10}$ cfu/g), per unit volume ($\log_{10}$ cfu/ml), or per unit area ($\log_{10}$ cfu/cm^2)

Note 1 to entry: For specific cases, the enumeration results may be expressed in $\log_{10}$ MPN.

3.7
exponential growth phase
phase in which the microbial population is exponentially multiplying as rapidly as possible; growth is dependent on the growth medium and environment (temperature, humidity, etc.)

Note 1 to entry: Figure 1 describes the three phases of microbial growth kinetics.

3.8
generation time
Tg
time it takes for the microorganisms to increase by a factor 2, also known as doubling time

3.9
germination
mechanism in which a *bacterial spore* (3.1) starts becoming a *vegetative cell* (3.25)

3.10
growth potential
Δ
difference between the decimal logarithm of the highest concentration of the target microbial population ($\log_{max}$) and the decimal logarithm of the initial concentration of this microbial population ($\log_i$)

Note 1 to entry: The $\log_{max}$ and $\log_i$ refer to concentrations and are expressed in $\log_{10}$ cfu/g or $\log_{10}$ cfu/ml or $\log_{10}$ cfu/cm^2

3.11
maximum growth rate
kinetics parameter to characterize the *exponential growth phase* (3.7), represented by the slope of the curve showing the evolution of the natural logarithm (μ_{max}) or decimal logarithm (V_{max}) of the population as a function of time, under constant growth conditions

3.12
inoculum
microbial suspension at the desired concentration used to contaminate *test units* (3.24)

3.13
lag phase
phase, directly after inoculation, during which the microbial population is adapting to the environment, before it enters the *exponential growth phase* (3.7)

Note 1 to entry: Figure 1 describes the three phases of microbial growth kinetics.

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3.14
lag time
λ
kinetics parameter in time unit to characterize the *lag phase* (3.13)

3.15
pH value
measure of the concentration of acidity or alkalinity of a material in an aqueous solution

[SOURCE: ISO 5127:2017, 3.12.2.29, modified — Notes 1 and 2 to entry have been removed.]

3.16
primary model
mathematical model describing the changes of microbial counts as a function of time

3.17
organizing laboratory
laboratory with responsibility for managing the *challenge tests* (3.5)

3.18
sampling
selection of one or more units or portions of food such that the units or portions selected are representative of that food

3.19
sampling point
time at which the *test units* (3.24) are analysed and which are represented as *experimental datapoints* (3.6) on the kinetics graph

3.20
secondary model
mathematical model describing the effects of the environmental factors (e.g. temperature, pH, a_w) on the parameters of the *primary model* (3.16) (e.g. growth rate)

3.21
sporulation
mechanism by which *vegetative cell* (3.25) forms spore

3.22
stationary phase
phase in which the microbial population is at its maximum level

Note 1 to entry: Figure 1 describes the three phases of microbial growth kinetics.

3.23
test portion
measured (volume or mass) representative sample taken from the *test unit* (3.24) for use in the analysis

[SOURCE: ISO 6887-1:2017, 3.5, modified — The end of the definition has been changed from "taken from the laboratory sample for use in the preparation of the initial suspension" and the Note 1 to entry has been removed.]

3.24
test unit
measured (volume or mass) amount of the food used for inoculation

3.25
vegetative cell
state of microbial form that is capable of growing under favourable environmental conditions

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3.26
water activity
a_w
ratio of the water-vapour pressure in the foodstuff to the vapour pressure of pure water at the same temperature

[SOURCE: ISO 18787:2017, 3.1, modified — The definition has been condensed and the formula and Notes 1 and 2 to entry have been removed.]

4 Principle

4.1 General

The aim of the study shall be clearly defined (e.g. assessment/validation of the food shelf-life as a control measure, assessment of microbial stability). The experimental design shall be in accordance with that purpose and shall take into account the steps of the food chain for which microbial growth is assessed. The decision criteria shall be clearly defined (see 7.2).

Knowledge from the FBO on its products (e.g. characteristics or production process) shall be combined with expertise in food microbiology and analytical sciences to ensure the robustness of the study. The organizing laboratory shall have knowledge and skills in food microbiology, food science and technology, and statistics to design and conduct the studies, interpret the results and draw the conclusions. The analyses shall be conducted under a quality assurance system (e.g. in accordance with ISO/IEC 17025).

Challenge tests aim at studying the growth potential or growth kinetics (lag time and maximum growth rate) in order to assess, for example, the food shelf-life as a control measure or the microbial stability of a food.

Growth potential studies are most appropriate to:

— validate the microbiological shelf-life of a food under reasonably foreseeable conditions of use and storage between production and consumption, ensuring relevant microbiological criteria are met throughout the product shelf-life;

— assess if a product, tested under specific conditions, supports the growth of the inoculated microorganism.

Such challenge tests will only validate the specific food characteristics and conditions applied for the study. When microbiological criteria are not fulfilled or conditions (e.g. food formulation, physico-chemical characteristics, type and/or concentration of preservatives added, packaging, storage temperature) are changed, a new growth potential study needs to be carried out in order to validate the new conditions.

Growth kinetics studies are most appropriate for:

— assessing the effect(s) of intrinsic (e.g. pH, a_w, preservatives) and extrinsic characteristics (e.g. gas composition, temperature) that have a significant impact on the behaviour of the target microorganism;

— providing data for developed models to simulate the effect of such factors on microbial behaviour in the studied food under reasonably foreseeable storage conditions (time and temperature);

— comparing the simulation results to ensure that relevant microbiological criteria are met throughout the food shelf-life.

Growth kinetics studies are used to estimate and validate the microbiological shelf-life of a food. They are particularly suitable in the last steps of the food development, including reformulation, new packaging, and alternative processing conditions.

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A growth kinetics study can be more informative than a growth potential study. However, growth kinetics studies are more complex in terms of study design, execution, results interpretation and exploitation, particularly in cases where various factors are included.

The behaviour of a microbial population in a food, i.e. microbial growth kinetics, is dependent on the characteristics of the food (e.g. a_w, pH, preservatives concentrations), the food storage conditions (temperature, packaging format and gas composition), the food processes, the physiological state of the microorganism and interactions with the natural background microorganisms.

Microbial growth kinetics are defined by three major phases (see Figure 1).

a) Lag phase: This phase is characterized by the lag time (λ), which corresponds to the intersection between the exponential growth phase line (plotted in semi-log coordinates) and the horizontal line crossing through the initial cell concentration[15][18]. For spore-forming microorganisms, lag time includes spore germination and outgrowth.

   Lag time is dependent on the food characteristics (e.g. physico-chemical and microbiological), inoculation levels and storage conditions (e.g. temperature, relative humidity, gas composition). Lag time is also dependent on the physiological state of the microorganism contaminating the food and any stress experienced by these cells or spores.

b) Exponential growth phase: This phase is characterized by the growth rate (μ_{max} or V_{max}), which corresponds to the maximum increase in natural or decimal logarithm of cell number per unit of time.

   The growth rate corresponds to the slope of the curve showing the evolution of the natural logarithm (μ_{max}) or decimal logarithm (V_{max}) of the population over time during the exponential phase. The food characteristics (e.g. physico-chemical and microbiological) and storage conditions (e.g. temperature, relative humidity, gas composition) can significantly influence microbial growth rates. The growth rate of a microbial population is unaffected by its initial concentration and physiological states.

   The relationship between the generation time (Tg) and μ_{max} is given by Formula (1):

$$\mu_{max} = \ln(2) / Tg \quad (1)$$

   The slope of the curve plotting the $\log_{10}$ of the microbial population against time, V_{max}, and its relationship to maximum growth rate is given by Formula (2):

$$\mu_{max} = V_{max} \cdot \ln(10) \quad (2)$$

c) Stationary phase: In this phase, the microbial population is at its maximum level.

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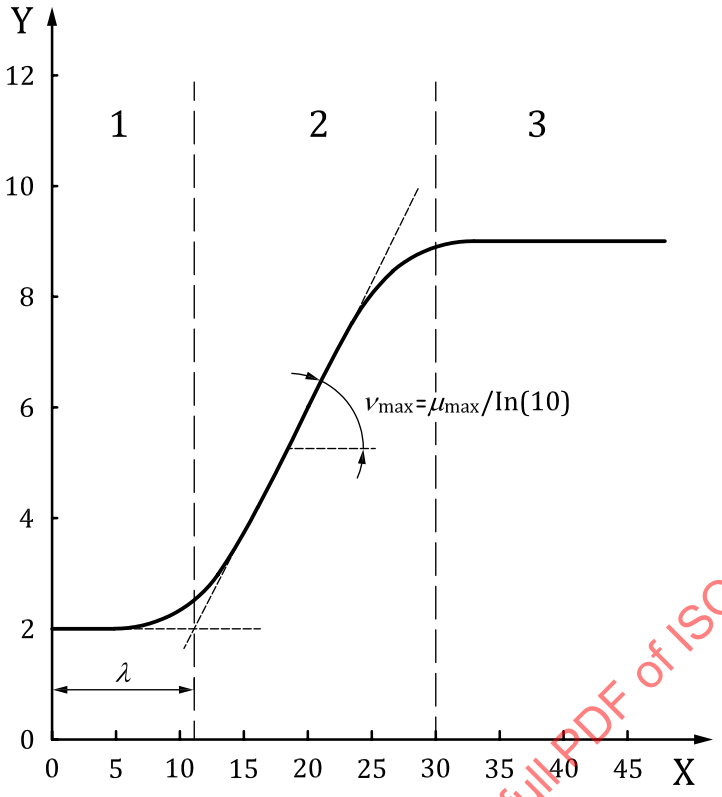

Key

Y $\log_{10}$ (cfu/g)
X time (days)
1 lag phase
2 exponential growth phase
3 stationary phase

Figure 1 — Microbial growth kinetics with three major phases

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4.2 Estimation of the growth potential

The food characteristics (e.g. physico-chemical and microbiological) and storage conditions (e.g. temperature, relative humidity, gas composition) can significantly influence the microbial growth potential.

The inoculum shall be adapted to conditions that mimic the microbial cell or spore injury induced by food handling/processing or any phenomena that can trigger subsequent adaptive responses to growth conditions, in order to mimic natural microbial behaviour in the food.

This type of test is designed to estimate the changes in concentration of the microbial population during the challenge test. These tests can be used to determine whether there is significant microbial growth in a foodstuff and to quantify the increase in the microbial population under a given set of storage conditions.

It is important to have a minimum of five sampling points that are evenly distributed across the entire shelf-life, to get an accurate estimation of the growth potential (see 14.2).

Growth potential does not provide information on the length of the lag phase, growth rate value or maximum stationary-phase level. This makes the growth potential unsuited for extrapolating the results to other conditions.

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The growth potential can be obtained using dynamic temperature profiles applied to the food during the study mimicking the food storage conditions.

4.3 Estimation of the growth kinetics parameters (lag time and maximum growth rate)

The growth kinetics characterization consists of the estimation of the lag time and maximum growth rate. The maximum growth rate is mainly used in assessing, determining and optimizing the microbiological shelf-life of the food.

Maximum growth rates can be used to directly calculate an increase in microbial counts under the conditions of the challenge test and/or as inputs for growth simulation.

The experimental design shall generate at least eight experimental data points distributed across all growth phases, with a minimum of five data points in the exponential phase. Growth kinetics shall be estimated at a constant temperature by fitting a recognized and commonly accepted mathematical model used for describing microbial growth.

5 Apparatus

Routine microbiology labware specified in ISO 7218 is required. Specific labware may also be needed to prepare the test portions, to store them under suitable conditions or monitor how their characteristics change during the challenge test study. These include the following.

5.1 Apparatus for packaging, the samples under air, under vacuum or under a protective modified atmosphere.

5.2 Chilled incubator, able to reach and hold setpoint temperatures to ±1 °C.

5.3 Climate-control chamber, able to reach and hold setpoint temperatures to ±1 °C and to adjust relative humidity to ±10 %.

5.4 pH meter, able to perform measurements to a tolerance of ±0,1 pH units. pH meters shall give readings to a resolution of 0,01 pH units.

5.5 a_w **meter**, meeting the requirements of the ISO 18787.

5.6 Headspace gas analyser, to measure gas composition (e.g. O_2, CO_2).

5.7 Logger for measuring temperature storage conditions of the test unit.

5.8 Logger for measuring relative humidity storage conditions of the test unit.

6 Culture media and reagents

Follow current laboratory practices as specified in ISO 7218.

For the preparation and performance testing of culture media and reagents, follow the procedures as specified in ISO 11133 and in the International Standard specific to the microbial population studied. Use internationally recognized and widely accepted methods or alternative methods validated according to internationally accepted protocols for the detection or enumeration of target microorganisms (e.g. ISO 16140-2).

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7 Study design and sampling

7.1 General

The study design shall address sources of variability, including:

— the inter-batch variability;

— the intra-batch variability;

— the variability in the artificial inoculation of the test units.

7.2 Setting decision criteria for growth potential

Depending on the aim of the challenge test, decision criteria shall be defined before the start of the study. A two-step approach is used for growth potential study: one step to determine if growth occurs and the second to determine how much growth is acceptable.

For example, for *Listeria monocytogenes*, some institutions consider that a food supports growth when the growth potential is greater than a cut-off value of 0,5 $\log_{10}$[10][12], whereas others set this cut-off value at 1 $\log_{10}$[13].

7.3 Number of batches and selection criteria

The number of batches to be included in the study depends on the variability of the food production process and food, especially in regard to the intrinsic (e.g. pH, a_w, preservatives) and extrinsic (e.g. gas composition) characteristics and microbiological properties of the food. This variability shall be documented. The characteristics of the studied batches shall be representative of the variability of the production process based on historical data.

If inter-batch variability of food characteristics (e.g. physico-chemical and microbiological) is sufficient to induce differences in microbial growth behaviour (when either $\Delta\varphi_{\mathrm{pHaw}}$ or $\Delta\psi$ is over 0,2), it is necessary to study different batches in order to evaluate the variability within the microbial responses[14]. In that case, a minimum number of three batches should be used for both growth potential and the growth kinetics studies.

The use of a single batch shall be clearly justified, for example:

a) evaluating the impact of a new formulation of the food;

b) using a batch representing the most favourable growth conditions (worst case);

c) applicable for a growth kinetics study only if the impact of the inter-batch variability determined by the calculator tool (see Annex A) is not significant.

Annex A provides an appropriate calculation scheme for assessing the impact of the inter-batch variability of the food, pH and a_w on the behaviour of the microorganism under test. Annex A is only applicable in cases where the challenge tests are conducted to estimate growth rate and where the pH and a_w are the only relevant food characteristics having a significant impact on this kinetics parameter.

7.4 Preparation of the test units

Test units representative of the food matrix can be either:

— the complete content of the packaging units,

— aseptically-sampled portions from the packaging unit(s) or from the bulk food.

The tests units shall be maintained at an appropriate storage temperature before inoculation. The test units shall be inoculated as close as possible to the day of production unless otherwise defined by the

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objectives of the study. The test units shall have the same composition as the original food, especially for composite food.

The test units shall be packed using the same packaging conditions as the original food over the shelf-life under test. It is recommended to use the same packaging material, comparable gas mix and headspace-food ratio. If, for example, for practical reasons, it is not possible to use the original food packaging, or packaging material, the use of alternative packaging material shall be justified. The alternative packaging material shall have the same technical properties (e.g. gas permeability) as the original.

Prepare the number of units to be used for the inoculation of microorganisms (test units) and for the control (control units, see Clauses 10 and 11). It is recommended that additional test units are prepared to cover unforeseen incidents.

7.5 Number of test units to be inoculated

The minimum number of test units to be inoculated for analysis per sampling point will depend on the inter-batch variability (see Annex B). Depending on the intra-batch variability, the number of test units and/or sampling point may have to be increased.

The sources of variability in artificial inoculations include the type and structure of the food matrix, as well as the inoculation procedure. When the three batches are not simultaneously inoculated, the number of test units to be prepared for Time 0 (t_0) should be at least three per batch.

Annex B presents a flowchart for determining the minimum number of test units needed for inoculation, depending on the expected inter-batch variability, the type of challenge test and the minimum number of sampling points.

NOTE Microbiological growth can be followed by rapid inactivation (depending on the food composition and storage conditions). The sampling points are divided over the storage period in order to detect a possible decrease in levels after initial growth.

See Clause 11 for the number of test units to be prepared for the control tests.

8 Selection of strains

Each strain used shall be characterized biochemically and/or serologically and/or genetically in sufficient detail for its identity to be known. Strains previously isolated from the food matrix (raw materials, ingredients, end products) or from the production environment or from clinical/food/environmental samples in outbreaks involving the food are preferred compared to strains from a culture type collection. The original source of all isolates should be known and the isolates should be held in a local (e.g. laboratory that runs the challenge test), national or international culture collection to enable them to be used in future testing, if required. The growth ability of the strains should have been determined prior to the challenge tests by historical or published data or by testing them as described in 9.2 and 9.3.

The selected strain(s) shall be fit for purpose regarding their ability to grow under the tested conditions, including pH, temperature, a_w, etc. Whenever possible, use strains for which the cardinal values have been determined (e.g. temperature, pH, a_w, minimal inhibitory concentration for preservatives) to enable further predictive modelling using the gamma-concept[22].

It is recommended that a mix of strains of the same microbial species is used for estimation of growth potential so that variations among strains are taken into account. To estimate the growth rate, only one strain shall be used per challenge test.

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9 Preparation of the inoculum

9.1 General

Follow current laboratory practices as specified in ISO 7218.

For the preparation and performance testing of culture media and reagents, follow the procedures as specified in ISO 11133 and in the International Standard specific to the microbial population studied.

9.2 Preparation of the vegetative cell suspensions

Two successive cultures of the selected strain(s) should be conducted as follows.

— First, in a culture medium under conditions that enable optimal growth of the selected strain(s). The culture should have reached the end of the exponential phase or the early stationary phase, to standardize the physiological state of the microbial population.

— Second, in a culture medium that mimics the natural conditions of the food under test (at least temperature and, if relevant, pH and/or a_w) in order to adapt the strain(s) and to shorten the lag phase once inoculated in the food (i.e. worst-case scenario). The culture is grown until the end of the exponential phase or the early stationary phase is reached. Enumeration shall be performed unless previously determined under the same conditions.

In some cases, the inoculum needs to be submitted to treatments (injury and/or stress) in order to mimic the food production processes (see C.2). The impact of the induced stress shall be estimated (e.g. by enumerations on selective and non-selective media before and after the stress treatment).

NOTE There is no need for any adaptation and/or injury treatment if only the maximum growth rate is used in further simulations.

To avoid adding nutrients when inoculating the test units, serial dilutions of that second culture shall be performed in non-growth promoting diluent. If relevant, adjust the diluent to the conditions (e.g. pH, temperature, a_w) of the second culture medium in order to maintain the physiological state of the inoculum.

Inoculate the test units immediately.

When using a mix of strains, these strains should be previously individually enumerated and mixed in equivalent concentrations. The microbial count of the inoculum shall be determined by enumeration on the same medium that will be used for the challenge test. This count shall be adjusted to fit with the conditions described in Clause 10.

9.3 Preparation of the spore suspensions

To prepare the spore crop, inoculate the germinated strain(s) into an appropriate culture medium and incubate under conditions that will promote a high sporulation rate (aerobic/anaerobic, medium, temperature). The length of this step will vary (from days to weeks) depending on the microbial species or strain(s) and the sporulation conditions. The extent of sporulation shall be checked with a microscope prior to harvesting the spores. Spore crops should be enumerated and can be stored until needed for the challenge test study. An example of a protocol to produce spores is given in C.3.

Prior to conducting the challenge test, enumerate the spore crop on the same medium that will be used for the challenge test after an appropriate heat treatment[9]. This concentration shall be adjusted to fit the conditions described in Clause 10.

10 Inoculation of the tests units

The inoculum procedure aims to achieve the same levels of microorganism per test unit without modifying the food's physico-chemical characteristics (pH and a_w included). To achieve this, it might be

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necessary to adjust the pH and a_w of the inoculum. The inoculum volume:sample mass (or volume) ratio should not exceed 1:100.

Before inoculation, food samples shall be equilibrated to the initial temperature of the test.

The inoculation level selected shall be justified for the studied food and microorganisms. To ensure the accuracy of the results, the level shall fit within the quantification limit of the enumeration method used. When the initial inoculum concentration is low, the limit of quantification can be lowered, for example, by increasing the number of the enumeration plates (see Clause 13). An inoculation level of at least five times the quantification limit of the enumeration method and no more than an initial concentration of 10^4 cfu/g (or per unit volume or area) is recommended.

Depending on the food under study, use an inoculation procedure to mimic contamination event(s) to be studied. For example, at the core (e.g. in a ground food) or at the surface (e.g. by applying several deposits locally over the surface or by spraying) or by bulk-inoculation (e.g. liquid or semi-liquid food).

The laboratory can identify a part of the heterogeneous food as the most favourable to microbial growth and inoculate that particular part, but the entire test unit shall be used to determine the concentration of the target microbial population in the test unit.

Modified atmosphere (including vacuum) packaged food can be opened, inoculated and repacked according to 7.4. The food can also be contaminated directly by injecting the inoculum through one or several adhesive septa so as not to affect the packaging atmosphere. In this case, the laboratory shall ensure that the packaging remains hermetically sealed throughout the duration of the test.

If the goal is to investigate microbial behaviour in the whole food, the inoculum shall be homogenously distributed in the test units.

If the goal is to characterize microbial behaviour in a specific part (e.g. core or surface) of the food, the inoculation should be done on this specific part.

When working on a particularly heterogeneous food, for example, composite food, the specific food component(s) being inoculated and the inoculation procedure shall be described and justified. The inoculation procedure shall not alter the ratio and structure of the multi-phasic and composite foods.

11 Controls

11.1 Food controls

The aim of food controls is to verify the representativeness of the test units in comparison to the studied food.

The food needs to be checked for relevant characteristics for the interpretation of the results (e.g. pH, a_w, preservatives concentrations, gas atmosphere, background microorganisms).

NOTE The background microorganisms can be mesophilic microorganisms, psychrotrophic microorganisms, lactic acid bacteria, yeasts and moulds, etc.

Food controls allow determining the level of natural contamination of the target microbial population.

11.2 Control units

The aims of the control units are:

— to ensure that the preparation of test units has not affected the characteristics of the studied food;

— to provide additional information to be used for the expression of the results (see Clause 14) and the test report (see Clause 15).

The control units can be prepared at the same time as the food controls (see 11.1).

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Add the non-growth promoting diluent described in Clause 9 (for the serial dilutions) to each control unit by following the same inoculation procedure as applied for test units (see Clause 10).

Prepare the minimum number of control units described in Annex B.

Only two sample points (t_0 and t_{end}) are considered in Annex B, but it is strongly advised that intermediate analysis points are included so that changes in relevant characteristics (e.g. pH, gas atmosphere, preservative concentrations, background microorganisms) during the challenge test are monitored.

The same units may be used for measurement of physico-chemical characteristics, gas atmosphere and determination of the concentration of the background microorganisms or any relevant component likely to affect the growth of the target microorganism.

It is recommended that additional control units be prepared to cover unforeseen incidents.

12 Storage of the test units

12.1 General

All test units shall be stored (5.2 and 5.3) at the selected temperature(s) for the appropriate time. The temperature throughout the duration of the challenge test shall be recorded. The integrity of the packaging shall be maintained throughout the duration of the challenge test.

The temperature chosen for storage of the test units should allow growth of the target microorganism and be as close as possible to the reasonably foreseeable food storage conditions.

For food affected by natural environment conditions (e.g. relative humidity), it may be necessary to use a climate-control chamber (5.3) that can mimic those storage conditions. In this case, the relevant environment conditions inside the chamber shall be recorded throughout the duration of the challenge test.

12.2 Estimation of growth potential

The storage conditions (time and temperature combination) applied during the challenge testing shall mimic the reasonably foreseeable food storage conditions (including time and temperature abuse) that the food is most likely to be subjected to, from manufacture until its final consumption.

Storage conditions could be based on observations from the countries where the food supply chain and consumers are located. For the periods during which storage conditions are fully controlled, the challenge test can be performed at the regulatory-controlled temperature or at an independently-set manufacturer-defined temperature.

NOTE To define the worst-case scenario, the storage conditions of each part of the cold chain considered can be, for example, the 75th percentile of the observed data.

12.3 Estimation of growth kinetics parameters (lag time and growth rate)

The test units shall be stored at one constant temperature throughout the test period. The temperature chosen shall allow growth of target microorganism and be, as close as possible, to reasonably foreseeable storage conditions.

The integrity of the packaging shall be maintained throughout the duration of the challenge test.

13 Analysis

Follow current laboratory practices as specified in ISO 7218.

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For the preparation and performance testing of culture media and reagents, follow the procedures as specified in ISO 11133 and in the International Standard specific to the microbial population studied.

Microbiological enumeration and physico-chemical parameters measurements shall be performed at different sampling points according to the study's design. For all types of challenge tests, an initial analysis shall be performed on the day the food is inoculated and at all subsequent sampling points relevant to the type of challenge test being performed.

Whenever possible, the entire test unit should be used for analysis.

The physico-chemical and microbiological analyses shall be performed using internationally recognized and widely accepted methods or alternative methods validated according to internationally accepted protocols. In cases involving inoculation on the food surface or on a composite or heterogeneous food, the test portion should be the entire test unit and shall not be reused. If the test portion is large (e.g. 100 g), the 1 in 10 dilution may be achieved in two steps. For example, prepare a 1:2 dilution followed by 1:5 dilution in accordance with ISO 6887-1 or ISO 16140-2.

The sources of analytical uncertainty are essentially the sampling and the analytical method. When the variability of the food matrix or the artificial inoculation is high, the number of test units to analyse per enumeration sampling point should be increased accordingly. The number of analyses per sampling point should also be increased when the aim is to reduce the analytical uncertainty.

NOTE 1 In cases where the food has no background microorganisms, a non-selective agar can be used to enumerate the target microbial population, as this will favour the recovery ability of injured cells.

NOTE 2 The microbiology laboratory can adapt the volumes plated on the culture medium for isolation (in accordance with ISO 7218) in order to lower the limit of quantification of the method used.

NOTE 3 When running challenge tests, it is not mandatory to run full confirmation tests on typical colonies as part of the enumeration technique procedure.

NOTE 4 Surface sampling can be performed by methods such as rinsing or swabbing (e.g. ISO 17604, ISO 18593).

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14 Expression of the results

14.1 General

Convert the enumeration results of the test portions to $\log_{10}$ cfu per weight or volume or surface.

14.2 Growth potential (Δ)

If three or more batches are studied, the growth potential shall be calculated for each batch according to the formula: $\Delta = \log_{max} - \log_i$ where $\log_{max}$ is the highest value observed for the batch and $\log_i$ is the initial value of the same batch. The final growth potential corresponds to the highest growth potential value from the batches.

If one batch is studied, for each sampling point calculate the mean of the experimental datapoints. Calculate growth potential according to the formula: $\Delta = \log_{max} - \log_i$ where $\log_{max}$ is the highest of these mean values and $\log_i$ is the initial mean value.

Suspected outliers shall be investigated.

Express the growth potential in $\log_{10}$ at the end of the challenge test. If $\log_i$ is the highest value from all the test units sampled, then the growth potential is equal to zero.

The challenge test is rejected at Time 0 (t_0) if the standard deviation (due to measurement uncertainty and contamination heterogeneity) is greater than the limit of 0,3 $\log_{10}$ cfu/g.

D.1 gives examples of growth potential estimations.

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14.3 Growth kinetics parameters (lag time and growth rate)

When more than one food batch has been challenge tested, growth kinetics parameters shall be estimated for each batch.

Growth kinetics parameters are estimated on the full set of experimental datapoints by fitting a recognized primary model. D.2 gives some examples of growth kinetics parameters estimated by fitting a microbial growth model. Tools are freely available online for this purpose (e.g. Curve Fitting from Sym'Previus[23], DMFit from ComBase[24][1]).

Lag time can be expressed in hours or days. Maximum growth rate can be expressed in h^{-1} or d^{-1}. The standard error of the growth kinetics parameters shall be provided to assess the accuracy of the estimation.

NOTE The growth rate is estimated at a fixed temperature. The estimated growth rate can be used for further simulations in both static (e.g. a different pH than the pH of the tested food matrix) and dynamic (e.g. temperature change or fluctuation effects along the food chain, see Annex E) conditions using predictive modelling based on the gamma-concept[19][20][22].

15 Test report

15.1 General

The test report shall state the method used and the results obtained with the standard errors mentioned in 14.2 and 14.3. It shall also give details of all operational steps that are either not specified or regarded as optional in this document, and shall report any deviations that might have influenced the results.

The test report shall include all the data needed for interpreting the challenge test.

15.2 Aim of the study and type of challenge test

The aim of the study shall be clearly described (e.g. assessment/validation of the food shelf-life as a control measure, assessment of microbial stability).

Information describing the characteristics of the food under test shall be provided, such as:

— test batch identification and date of manufacture;

— characteristics of the food relevant to the test, e.g. food composition and structure when known (including all ingredients, food additives);

NOTE A photograph of the food is very useful to illustrate food composition and structure, as well as the packaging.

— physico-chemical, microbiological and packaging characteristics of the food (e.g. pH, a_w, microorganisms, preservatives concentrations, gas composition);

— storage conditions recommended on the packaging and expected shelf-life of the food and the foreseeable storage conditions throughout the food chain.

Based on this information, the rationale for the selection and design of the type of challenge test and the target microorganisms shall be justified.

Predetermined decision criterion (e.g. the target microorganism will be considered to have grown if the growth potential exceeds 0,5 $\log_{10}$ cfu/g) shall be also mentioned.

1) Curve Fitting from Sym'Previus and DMFit from ComBase are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

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15.3 Experimental protocol

The experimental protocol shall include the following:

— information on the microbial strain(s) used:

  — identification and original source of the strain(s) used and justification for their selection (see Clause 8);

  — conditions under which the inoculum was prepared, including the preparation of the different cultures and the dilution medium/media used, any adaptation/stress procedures used as relevant to the food under test (e.g. adaptation to low temperature, pH or a_w stress);

  — inoculum concentration;

  — impact of the induced stress treatment, when appropriate (see 9.2);

— number of batches studied and the justification;

— information on the sample unit preparation:

  — mass or volume or surface of the inoculated test units;

  — ratio of inoculum to the test sample and the contamination mode (e.g. at-core or at-surface);

  — packaging characteristics (e.g. material, permeability properties, gas composition);

  — storage conditions (time, temperature and, if relevant, relative humidity);

— sampling plan and analyses.

15.4 Sample analysis

The information regarding sample analysis shall include the following:

— sample storage conditions (time, temperature and if relevant, relative humidity);

— number of test portions analysed at each sampling point;

— weight or volume or area of the test units;

— reference to each method used for microbial analysis (target microbial population and other microorganisms, if performed) and their limit of quantification and, if needed, limit of detection;

— reference to each method used for physico-chemical analyses (pH and a_w, preservative concentrations, gas composition in the packaging on control samples when performed) and apparatus used.

15.5 Results

The results shall include the following:

— all raw data obtained according to 15.4;

— the temperature of the test units and, if relevant, the relative humidity of storage conditions during the study;

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— if the purpose of the challenge test is to estimate growth potential:

  — the report shall give the overall value and the value of the individual batches of the growth potential calculated according to 14.2 and the standard deviation shall be provided when more than one batch is analysed;

— if the purpose of the challenge test is to estimate the growth kinetics parameters:

  — the fitting tool and the primary model used for the fitting;

  — the graph showing the experimental datapoints and the fitted curve;

  — the estimated lag time and growth rate, and their associated standard errors according to 14.3;

  — the simulation of the behaviour of target microorganisms (tool, primary and secondary models used in the simulation, inoculum level, maximum population density, time/temperature scenario, intrinsic and extrinsic characteristics of the food).

15.6 Conclusions

The results shall be compared to the pre-defined decision criteria.

Based on the results, conclusion(s) shall be provided according to the aim of the study.

15.7 Reference documents

List the bibliographic references used to write the report for the study, if relevant.

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Annex A
(informative)

Inter-batch variability assessment based on pH and a_w

This annex proposes a method for assessing the impact of pH and a_w inter-batch variability on the growth rate of a given microorganism. It is only applicable in cases where the challenge tests are conducted to estimate growth kinetics parameters.

When this variability has a significant effect on microbial growth pattern, more than three batches should be tested.

A tool called the Inter-Batch Physico-Chemical Variability calculator and its user guide are available at http://standards.iso.org/iso/20976/-1/ed-1/en.

The physico-chemical inter-batch variability of the food has a significant impact on the growth of the studied strain if at least one of the parameters ($\Delta\varphi_{pHaw}$ or $\Delta\psi$) is over 0,2 (see calculator user guide for calculation details). Generally, this value corresponds to variation of 10 % of the μ_{max}.

EXAMPLE

Bacteria studied: *Listeria monocytogenes*.

Cardinal temperatures: T_{min} = −1,72 °C, T_{opt} = 37 °C.

Cardinal pH: pH_{min} = 4,71, pH_{opt} = 7.

Cardinal a_w: a_{wmin} = 0,913, a_{wopt} = 0,997.

Inter-food-batch pH: $\overline{pH}$ = 6,7 and s_{pH} = 0,1.

Inter-food-batch a_w: $\overline{a_w}$ = 0,96 and s_{a_w} = 0,005.

If the challenge test is carried out at a temperature of 8 °C, this gives:

φ_T = 0,420, $\varphi_{pH,i}$ = 0,000, $\varphi_{pH,s}$ = 0,010, $\varphi_{a_w,i} = 0{,}033$, $\varphi_{a_w,s} = 0{,}175$, ψ_i = 0,246, ψ_s = 0,421, and $\Delta\varphi_{pHaw}$ = 0,152, $\Delta\psi$ = 0,175.

As $\Delta\varphi_{pHaw}$ and $\Delta\psi$ are both less than 0,2, the impact of inter-batch variability in physico-chemical parameters on *L. monocytogenes* growth can be considered negligible, which means the challenge test can be run using one batch only.

If the challenge test is carried out at a temperature of 4 °C, this gives:

φ_T = 0,619, $\varphi_{pH,i}$ = 0,000, $\varphi_{pH,s}$ = 0,010, $\varphi_{a_w,i} = 0{,}033$, $\varphi_{a_w,s} = 0{,}175$, ψ_i = 0,364, ψ_s = 0,628, and $\Delta\varphi_{pHaw}$ = 0,152, $\Delta\psi$ = 0,264.

As $\Delta\psi$ is greater than 0,2, the impact of inter-batch variability in physico-chemical parameters on *L. monocytogenes* growth is not negligible. As a result, the challenge test will need to be run on several batches in order to conclude on the microbial response (growth, no growth) of *L. monocytogenes* in this food at this temperature.

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Annex B
(normative)

Minimum number of units to prepare for the challenge test study

Growth kinetics studies are most appropriate to assess, determine and optimize the microbiological shelf-life (quality and safety) of the food. For example, characteristics such as pH, a_w, temperature can be changed for formulation of the food.

Growth potential studies are most appropriate when characteristics conditions (shelf-life, food characteristics) are known.

The minimum number of test units needed for the study is given in Figure B.1.

	Growth potential	**Growth rate**
Minimum number of batches	3 batches[a]	3 batches[a]
Minimum number of test units per batch	1 test unit[a]	1 test unit
Minimum number of sampling points	5 sampling points including t_o and t_{end}	8 sampling points including 5 in exponential phase
Minimum number of test units	3 batches × 1 test unit × 5 sampling points **= 15 test units**	3 batches × 1 test unit × 8 sampling points **= 24 test units**
Minimum number of control units	3 batches × 1 control unit × 2 sampling points (t_o and t_{end}) (+ temperature control[b]) **= 6 control units**	3 batches × 1 control unit × 2 sampling points (t_o and t_{end}) (+ temperature control[b]) **= 6 control units**
Minimum total number of units	**21 units**	**30 units**

Key

a The use of a single batch shall be clearly justified, for example, to assess a new formulation of the product or using a batch representing the most favourable growth conditions (worst case). In that case, the number of test units per batch is extended to 3.

b If the three batches are not run at the same time, one minimum temperature control per batch is needed.

Figure B.1 — Minimum number of test units needed for the challenge test study

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Annex C
(informative)

Examples of protocols to prepare inocula

C.1 General

This annex provides examples of protocols for inducing microbial stress or adaptive responses. The selected injury protocol should mimic natural contamination and conditions encountered during food processing.

The conditions for applying stress depend on the type of microorganism and the selected strain.

C.2 Examples of protocols inducing injury of vegetative cells

C.2.1 General

The following protocols have been developed, as examples, to injure bacterial populations of *Listeria monocytogenes*[17], leading to a loss of recoverability close to 1,5 $\log_{10}$.

C.2.2 Preparation of cultures prior to the injury treatments

The following protocol is used to obtain a suspension of exponential growth phase cells:

a) inoculate the target strain in a non-selective broth;

b) incubate the broth in appropriate conditions, to reach approximately 10^9 cells/ml;

c) perform serial dilutions in a non-selective broth to obtain 10^3 cells/ml;

d) perform a second sub-culture in appropriate conditions to reach the end of the exponential-phase culture, with approximately 10^8 cells/ml.

C.2.3 Injury protocols

To stress a cell suspension, the following protocol is used:

a) wash the exponential-phase culture:

   1) centrifuge broth at 5 000*g* for 10 min at 4 °C;

   2) discard the supernatant and suspend the cell pellet in suitable diluent (for example, physiological saline solution, 0,85 % NaCl);

   3) run a second centrifugation at 5 000*g* for 10 min at 4 °C and discard the supernatant; use the pellet for preparing the injured cells;

b) select an appropriate injury protocol.

EXAMPLE 1 Hydrochloric acid injury (e.g. the pellet is suspended in suitable diluent adjusted to pH 3 with HCl and the cell suspension is stored at 25 °C for 34 min).

EXAMPLE 2 Lactic acid stress (e.g. the pellet is suspended in suitable diluent adjusted to pH 4,6 with lactic acid and the cell suspension is stored at 25 °C for 48 h).

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EXAMPLE 3 Sodium hydroxide stress (e.g. the pellet is suspended in suitable diluent adjusted to pH 12 with NaOH and the cell suspension is stored at 25 °C for 22 min).

EXAMPLE 4 Hypochlorite stress (e.g. the pellet is suspended in suitable diluent at 2,4 ppm sodium hypochlorite and the cell suspension is stored at 25 °C for 1,5 min).

EXAMPLE 5 Cold-shock stress (e.g. the pellet is suspended in suitable diluents and the cell suspension is stored at −25 °C for 48 h).

EXAMPLE 6 Heat-shock stress (e.g. the pellet is suspended in suitable diluent at 25 °C and diluted to 1:100 in suitable diluent, the cell suspension is submitted to heat treatment of 55 °C for 3 min and cooled by dilution in a cold suitable diluent).

EXAMPLE 7 Sodium chloride stress (e.g. the pellet is suspended in suitable diluent containing 250 g/l NaCl and the cell suspension is stored at 25 °C for 25 h).

EXAMPLE 8 Nutrient starvation stress (e.g. the pellet is suspended in suitable diluent and centrifuged at 5 000*g* for 10 min at 4 °C; the pellet is suspended in suitable diluent and stored at 30 °C for 24 h).

C.2.4 Injury measurement

The injury efficiency is usually evaluated by enumerating the injured culture on selective and non-selective agars. A difference greater than 0,5 $\log_{10}$ cfu/ml is expected when sufficient stress has been applied.

C.3 Example of protocol to produce spores

C.3.1 General

As an example, the following protocol has been developed to produce spores of strains of the mesophilic genus *Bacillus* bacteria[16][21].

C.3.2 Preparation of the cell suspension prior to sporulation

The following protocol is used:

a) run two subcultures in a non-selective broth under appropriate conditions;

b) check the purity of the second subculture with appropriate method(s);

c) enumerate the suspension using appropriate non-selective conditions.

C.3.3 Preparation of the spore suspension

The following protocol is used:

a) prepare a sporulation agar (for example, a pH 7,4 nutrient agar supplemented with $MnSO_4$ 0,04 g/l, $CaCl_2$ 0,1 g/l) in 90 mm or 140 mm-diameter Petri dishes;

b) inoculate the cell suspension onto the sporulation agar by surface plating;

c) incubate under appropriate conditions to obtain a sufficiently high sporulation rate (ideally 80 % to 90 % spores);

d) check the extent of sporulation under a microscope;

e) run three successive washing steps by centrifugation (e.g. 15 min at 5 000*g* to 7 000*g*) in sterile distilled water;

f) suspend the pellet in 10 ml of sterile distilled water;

g) run a heat treatment at 80 °C for 10 min to inactivate remaining vegetative cells;

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h) check the spore concentration by enumeration on an appropriate non selective agar and under appropriate conditions (time, temperature, pour or surface plating);

i) store the spore suspension at 4 °C.

If appropriate, further treatments can then be applied to mimic the conditions encountered by the spores during the food processes, or the conditions encountered in the raw materials used in the food processes.

The spores can be stored at 4 °C (or frozen temperatures in some cases) in sterile distilled water or in a volume fraction of 90 % (volume fraction) ethanol. During this storage period, it is necessary to verify the recoverability of the spores by enumeration.

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Annex D
(informative)

Examples of how to estimate growth potential, lag time and maximum growth rate from results of challenge tests

D.1 Example applications of challenge tests designed to estimate growth potential

D.1.1 When three batches are inoculated simultaneously

D.1.1.1 Growth/no growth

Aim of the challenge test: To assess the ability of the food to support the growth of the target microbial population.

Decision criterion: The food supports the growth if a growth potential higher than 0,5 $\log_{10}$ cfu/g is observed.

The parameters are given in Table D.1.

Table D.1

	$\log_{10}$ cfu/g					
	Time 0	**T 5 days**	**T 10 days**	**T 15 days**	**T 20 days**	**Δ**
Batch 1	2,00	1,90	1,90	2,00	1,90	0,00
Batch 2	1,70	1,80	1,70	1,90	1,80	0,20
Batch 3	2,18	2,20	2,10	2,20	2,20	0,02
Standard deviation	0,24					

Target inoculation level: 2 $\log_{10}$ cfu/g.

Limit of quantification of the method used: = $\log_{10}$ cfu/g = 1.

The standard deviation of the log counts at Time 0 is below the limit of 0,30. Therefore, all inoculations are acceptable.

Estimated growth potential is 0,2; corresponding to the maximum value observed for batch 2. This is lower than the decision criterion of 0,5 $\log_{10}$ cfu/g.

Conclusion: The food does not support growth under the time/temperature conditions assessed.

D.1.1.2 Quantification of growth potential

Aim of the challenge test: Quantification of growth potential.

The parameters are given in Table D.2.

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