ISO/TC 34/SC 9/WG 32 "Improvement of pre-enrichment step in ISO 11290-1"

Sub-group on methodology and criteria to compare and select (pre-)enrichment broths"

Draft document on the methodology to compare and evaluate broths for the (pre-) enrichment of *Listeria monocytogenes* and *Listeria* spp.: "SENSITIVITY" STUDY.

June 2023

CONTENT

1. INTRODUCTION

This document relies on ISO 17468 (under revision, ISO/DIS 17468) and ISO 16140-2 and 4 Standards. Given the high number of enrichment protocols to compare, ISO/TC 34/SC 9/WG 32 "Improvement of pre-enrichment step in ISO 11290-1" Sub-group on methodology and criteria to compare and select (pre-)enrichment broths" agreed to begin the comparison study by step 3 ("method evaluation") of ISO/DIS 17468: a « sensitivity study » on both artificially and naturally contaminated samples, in order to select options that would be submitted to LOD_{50} evaluation. As it is a preliminary study, it does not strictly respect ISO 16140-2 Standard, which is referred to in ISO/DIS 17468 and ISO 16140-4 Standards.

Participating laboratories need to follow the present document. **A workflow diagram of the general protocol of the assays is presented in Annex 1, as a summary**. Laboratories particularly interested in evaluating improvement of Half Fraser broth may use additional options of Annex 2. Finally, laboratories interested in evaluating impact of intensive stress of the inocula on broths performances with artificially contaminated samples may use additional protocol of Annex 3, which requires more complex preparation of the inocula.

All the assays should be conducted in parallel, as much as possible in repeatability conditions.

In order to harmonize work, a test report for the results will be send to each collaborating laboratory. Additionally a template indicating the foreseen study, as well as matrices used, will be filled by each collaborating laboratory.

ISO 17025 accreditation of participating laboratories is recommended, but not mandatory.

2. ALTERNATIVE ENRICHMENT PROTOCOLS TO BE COMPARED

The 7 proposed alternative enrichment procedures are based on results of WG32 enquiry, and proposals of WG32 experts and media suppliers. Indeed, this first possibility has been introduced during the ISO/TC 34/SC9 2022 annual meeting. In the case of a one-step enrichment procedure, Fraser (FB) and Half-Fraser (HFB) broths are replaced by the new broth. In the case of a two-step enrichment procedure, the new broth replaces HFB. However, each step (first and second) is evaluated, as well as the global procedure. The isolation stage of the current ISO 11290-1 is maintained: use of agar *Listeria* according to Ottaviani and Agosti (LOA), and a 2nd agar left to choice (this second agar is optional in the frame of this study).

For interpretation purposes, it is strongly asked to compare in parallel on the same day all the 7 proposed alternative enrichment procedures on replicate samples. Indeed, there is no minimum number of samples required, and each laboratory can choose the number of samples that it would be able to test. However, if for practical reasons this is too heavy for the participating laboratory, only some of the 7 procedures can be retained for comparison. As another alternative possibility, the same homogenized naturally contaminated sample is sent between laboratories that organize the work between them for testing only part of the broths; for artificially contaminated samples these laboratories may use the same strain, matrix and protocol.

In both cases, alternatives enrichment protocols must be compared on the same sample in parallel between themselves and to ISO 11290-1 reference method, which serves as the reference point for each comparison study.

2. 1. Enrichment culturing from Reference methods

2. 1. 1. ISO 11290-1 reference method

According to the ISO 11290-1 reference method, samples are homogenized in Half Fraser broth at 1:10 sample-to-media ratio, then incubated for 24-26 h at 30+/-1°C.

At 24 – 26 h, transfer **0.1 ml** of HFB into 10 ml of Fraser broth and incubate for 24 ± 2 h at 37°C ± 1°C.

Streak isolation plates from the primary and the secondary enrichment broths.

Please note that it is recommended, but not mandatory, to use Half Fraser and Fraser from the same manufacturer.

2. 1. 2. BLEB (FDA BAM Chapter 10 reference method)

This option is derived from the FDA Bacteriological Analytical Manual (BAM) method:

Samples are homogenized in Buffered *Listeria* Enrichment Broth (BLEB) at 1:10 sample-to-media ratio, then enriched at 30 +/-1°C for 48 ± 4 h, streaking enrichment onto agar plates at 24 ± 2 h and 48 ± 4 h. Selective agents are added in the BLEB after the first 4h ± 30 mn of incubation.

2. 1. 3. UVM1 (Health Canada MFLP-1 reference method)

This option is derived from the new Canadian reference method MFLP-01. It is a two-step enrichment procedure with a regrowth in Fraser:

Samples are homogenized in University of Vermont 1 (UVM1) at 1:10 sample-to-media ratio, then enriched at 30ºC +/-1°C for 48 ± 4 h, streaking enrichment onto agar plates at 48 ± 4 h.

At 24 ± 2 h, transfer of **0.2 ml** of UVM1 into 10 ml Fraser Broth and incubate for 24 ± 2 h at 37°C ± 1°C followed by isolation onto agar plates.

2. 2. Commercial broths proposed to be opened to standardization by media suppliers

2. 2. 1. LESS+ (Neogen)

It is a two-step enrichment procedure with a regrowth in Fraser:

Samples are homogenized in LESS+ broth at 1:10 sample-to-media ratio, then enriched at 30°C ± 1°C for 24 ± 2 h.

At 24 ± 2 h, transfer of **0.1 ml** of LESS+ broth into 10 ml Fraser Broth, and incubate for 24 ± 2 h at 37°C ± 1°C.

Streak isolation plates from the primary and the secondary enrichment broths.

2. 2. 2. LSB (*Listeria* Special Broth, Bio-Rad)

It is a two-step enrichment procedure with a regrowth in Fraser:

Samples are homogenized in LSB at 1:10 sample-to-media ratio, then enriched at 30°C ± 1°C for 24 ± 2 h.

At 24 ± 2 h, transfer of **0.1 ml** of LSB into 10 ml Fraser Broth, and incubate for 24 ± 2 h at 37°C ± 1°C.

Streak isolation plates from the primary and the secondary enrichment broths.

2. 2. 3. 24 LEB (Thermo Scientific™ Oxoid™ 24 *Listeria* Enrichment Broth) (ThermoFisher Scientific)

It is a two-step enrichment procedure with a regrowth in Fraser:

Samples are homogenized in 24LEB at 1:10 sample-to-media ratio, then enriched at 37°C ± 1°C for 24 ± 2 h.

At 24 ± 2 h, transfer of **0.1 ml** of 24LEB into 10 ml Fraser Broth, and incubate for 24 ± 2 h at

37°C ± 1°C.

Streak isolation plates from the primary and the secondary enrichment broths.

2. 3. Modification of Half Fraser broth method

Modified Half Fraser broth (option 2 of Annex 2)

This is the preferred optional HFB enrichment method to be analyzed by participants.

This modification is from Bannenberg et al., 2021. Samples are analysed according to a modification of the ISO 11290-1 reference method:

Samples are homogenized in Half Fraser broth at 1:10 sample-to-media ratio, then enriched at 30°C ± 1°C for **26-28 h**, streaking enrichments onto agar plates.

At 26-28 h, transfer of **1.0 ml** of HFB into 10 ml Fraser Broth and incubate for 24 ± 2 h at 37°C ± 1°C. Streak FB for isolation onto agar plates.

It is recommended, but not mandatory, to use Half Fraser and Fraser from the same manufacturer.

Please note that the initial step is common with 2.1.1. and only one food suspension need to be performed.

2. 4. OPTIONAL: Modifications of Half Fraser broth

Laboratories particularly interested in evaluating improvement of Half Fraser broth may use options of Annex 2, in comparison with the reference method.:

3. SENSITIVITY STUDY

3. 1. Principle

The study is conducted on the candidate detection methods, using a wide range of naturally or artificially contaminated samples, however, the priority is to test, if possible, naturally contaminated samples, as they are more representative of reality, and are more easy to handle (no need of inoculation protocol).

All enrichment procedures are tested in parallel on the same samples.

This study is a multi-laboratory study conducted in different laboratories, located in different countries/different regions of the world to cover the largest diversity possible of matrices where the target microorganism can naturally be found and strains of the target microorganism.

In particular, each laboratory shall use its own samples, reagents and culture media to reflect their diversity. In particular, different media presentations (dehydrated, ready-to-use…) can be used for broths from references methods.

Laboratories may receive out of charge or buy ready-to-use commercial media from suppliers that proposed to open their broth to standardization. For reference methods, they can use ready-to-use or prepare broths from individual ingredients or from dehydrated powders.

3. 2. Study with naturally contaminated samples

The priority is to test, if possible, naturally contaminated samples.

When possible, samples are analysed without having been previously frozen. When necessary, the samples are stored frozen at –20°C and defrosted for one night at 5 +/- 3°C or at 18 to

27°C (laboratory ambient temperature) for a maximum of 3h before use. For practicability reasons, samples may be defrosted for up to 72h at 3 +/- 2°C.

As this is a preliminary study and given the importance to have homogenous test portions, the following protocol is proposed:

For non-homogenous matrices, 200 g of naturally contaminated solid sample (if there is not enough weight, add the same food type to the sample until sufficient weight) is mixed by stomaching with 200 mL Tryptone Salt or Buffered Peptone Water (1:2 dilution) to ensure homogeneity between test potions. 25 g or mL portions of this homogenate (taken with pipet or spoon or other material, depending on consistency) are immediately transfered to 225 g or mL test media to achieve final 1 in 10 dilution in the selected broths, and incubated according to chosen protocols.

For liquid, semi-liquid or powder samples, simply homogenate sample and transfer 25 g (or ml) of sample to 225 g or mL test media to achieve final 1 in 10 diluted in the selected broths.

If there is the information that the samples are contaminated at high levels, they can be mixed with the same matrix in order to lower the contamination level.

Optionally, naturally contaminated samples used in the study (either a test portion of a homogenised matrix, or the 1:2 dilution of non-homogenous matrices) can be enumerated according to ISO 11290-2 Standard. As low contamination levels are expected, it may be sufficient to spread twice 1 ml of the initial suspension.

Enrichment and analysis of the broths are performed as described before.

3. 3. Study with artificially contaminated samples

Matrices:

Special care is needed for matrices selection. The study should include the largest diversity

possible of matrices where the target microorganism can naturally be found, and the most representative matrices under regulation, in particular ready-to-eat food (RTE), food infant formula (PIF); New matrices types (vegetal-based RTE proteins, vegetal milk or fermented drinks…), matrices with frequent and dangerous inadequate use (frozen fruits or vegetables, diced bacon); Environmental samples, feed (silage…) and PPS. Difficult, such as for example low pH, low aw products or products with high levels of inhibitors/competitors (PIF, probiotics containing products, fermented +/- spiced RTE delicatessen such as chorizo, cheeses, raw milk products, acidic milk products with flora, cheese with garlic, spices and/or herbs…) should also be included. **It is of great importance to include as much as possible challenging matrices in the study.** Samples can be collected at both the production and distribution levels from various food processors and retail stores throughout the world, or at the primary production stage. For data reporting include detailed composition of samples, when possible.

Participating laboratory may include matrices they are familiar with analyzing. A common template for all participating laboratories will help to see what are the categories covered.

Strains and species:

Participating laboratories may use own available strains: wild isolates or culture collection strains. It is advised if possible to use strains isolated from the same matrix type or category, or origin (dairy, meat, seafood products, vegetables, environment, ...).

The study is first of all on *L. monocytogenes*, but may also include *Listeria* spp.

If possible, *L. monocytogenes* serovar, PCR geno-serogroup and MLST clonal complex (if known) should be indicated.

For participating laboratories wishing to test *Listeria* species, inoculate with *L. innocua*, *L.*

welshimeri or *L. seeligeri* species, with preference to include the last one because of lower growth in selective broths. The use of *Listeria* spp. is recommended for matrices that are commonly tested for the presence of all *Listeria* (e.g., environmental samples). *L. ivanovii* is also interesting as they are present in milk such as goat milk and could interfere with *L. monocytogenes* detection.

Preparation of inocula and contamination level:

High moisture samples:

Appropriate dilutions of *L. monocytogenes* or *Listeria* spp. cultures grown in Brain Heart Infusion (BHI) broth for 24 h, as two successive cultures of 6 h and 18 h at 37° C, may serve as standardized inocula for tests: 10ml BHI is inoculated with a fraction of an isolate *Listeria* colony or with a cryobead; after the first 6 h of incubation, 0,1ml of this first culture is transferred to a second BHI broth and incubated. The final BHI culture usually contained around 1×10^{9} CFU ml^{-1}. All decimal dilutions can be prepared in non-selective diluent such as Tryptone Salt (TS) solution. Plating 0.1ml the 6^{th} and 7^{th} serial dilution allows to verify the concentration of the inocula.

Additionally, it is recommended to perform the inoculum count by plating it on several plates (6-10 plates). This will confirm that the inoculum is homogenous and that all individual inocula contain culturable cells (avoiding false-negative results).

Theoretical contamination level should be reported on test report.

Alternatively, certified reference materials containing defined levels of cells can be used to prepare inocula.

Some examples of certified reference materials commercially available are:

-BIOBALL (30 CFU) from bioMérieux described below;

-Certified reference material developed by NCTC and available from Sigma-Aldrich: lenticule of NCTC 11994 (WDCM 00019) containing 30 - 120 CFU.

For CRMs, follow manufacturer's instructions for reconstitution.

For contamination of matrices other than milk, meat or seafood products (for example fresh vegetables), it is advised to prepare the dilution that will be used to inoculate the samples in whole milk.

Individually inoculate 25g samples (as much as alternative enrichment procedures to be tested in parallel) distributed in stomacher bags. A contamination level of approximately if possible, to around 2-10 CFU/ sample is advised, with an inocula volume lower than 1ml.

The target is to be if possible close to the lower limit, but not lower.

For difficult matrices (see before) a higher initial level of inoculation may be required to allow for recovery in the target range. It is recommended to not exceed 10 CFU/test portion.

After gentle manual mixing, each bag is stored at 1-5°C for 2 days maximum (if not possible to ensure this temperature, freezing is recommended), or frozen at –20°C for 2 weeks, and defrosted for one night at 5 +/- 3°C or at 18 to 27°C (laboratory ambient temperature) for a maximum of 3h before use. For practicability reasons, samples may be defrosted for up to 72h at 3 +/- 2°C.

<u>Low moisture samples and powders</u>:

It is advised to use <u>freeze-dried</u> certified reference materials of *L. monocytogenes* or *Listeria* spp. cultures commercially available containing well-defined levels of target analyte in a

stable, but stressed state to spike samples.

As an example, BIOBALL (30 CFU) from bioMérieux may be used:

- *Listeria monocytogenes* ref. 56039 (WDCM 00019 NCTC 11994)

- *Listeria innocua* ref. 56038 (WDCM 00017 NCTC 11288)

In particular, by putting one BIOBALL of 30 CFU in the Re-Hydration Fluid of 1,1ml - ref. 56021 (x 20 vials) we obtain for example 4 aliquots of 250 µl each charged to approximately 5 to 7 CFU

It is also possible to use 2 BIOBALL of 30 CFU in the Re-Hydration Fluid of 1,1ml to obtain for example 9 aliquots of 120 µl each charged to approximately 5 to 7 CFU.

Notes: It is recommended to use an inoculum of minimum 100 µl and to leave a minimum of 100 µl at the bottom of the tube; Re-hydrated BIOBALL® can be used immediately or for up to 8 hours after rehydration if stored at 2ºC to 8ºC; Refer to the certificate of analysis of each lot to know the average of the lot on which to make the calculation.

Individually inoculate 25g samples (as much as alternative enrichment procedures to be tested in parallel) distributed in stomacher bags. A contamination level of 2-10 CFU/ sample is advised. The target is (if possible) to be close to the lower limit, but not lower. In order to align with ISO 6887-4 Standard, and to avoid osmotic shock, first put the broth into the stomacher bag, then add the powder, and finally the inocula. The bag is shaken until the inoculum appears to be evenly distributed throughout the food item, and immediately submitted to enrichment procedure (indeed, low a_w may induce decline in *Listeria* population during storage).

Alternatively follow ISO 16140-2 Standard: C.3.2 Artificial contamination of low moisture

foods with a lyophilized culture. No 2-week stabilization is required. Immediately enrich test portions after inoculation.

Surfaces environmental samples:

As an example (Gnanou Besse et al., 2019), environmental samples (for example, gauze pads, sponges, swab…) immerged in 20 ml diluent (a mixture of 16ml TS solution and 4ml of a bacteriostatic mixture) in which a competitive background microflora potentially isolated from food production environment is artificially added, including for example: *L. innocua*, added at a concentration similar to *L. monocytogenes*, and a mixture of *Staphylococcus epidermidis* (about 10^4 CFU), *Bacillus cereus* (about 10^3 CFU) and *Pseudomonas fragi* (about 10^3 CFU) may be added in each sample. A contamination level of approximately 2-10 CFU *L. monocytogenes* / sample is advised, with an inocula volume lower than 1ml. The microflora within the sample is stabilized by the addition of a bacteriostatic mixture. The bacteriostatic effect is negated when the sample is diluted with media during examination. Four milliliters of a solution containing 10 g of boric acid, 2 g of glycerol and 0.150 g of potassium sorbate per 200 ml of distilled water is added to 16 ml of TS diluent. A contamination level of approximately 5-10 CFU *L. monocytogenes* / sample is advised, with an inocula volume lower than 1ml. Each sample is stored at least 48 h at 2 - 8 °C, and then 1/10 diluted in selective broths.

Alternatively, a protocol based on an AOAC protocol used for validation can be employed. Stainless steelsurface may be used. *Enterococcus faecalis* and vegetative *Bacillus subtilis* may be the competing species. These competing species are inoculated with the amount at least 10-times of the amount of *Listeria* on each surface. Bacterial strains are cultured overnight and

diluted to appropriate concentrations in brain heart infusion (BHI) broth for inoculation. Each surface, in one or several identical pieces, is divided to 30 squares for each enrichment scheme. The squares for swab sampling and sponge sampling may be 1 inch × 1 inch (2,5 × 2,5 cm) and 4 inch × 4 inch (10 × 10 cm), respectively, and are inoculated with 20 µl and 320 µl volumes of liquid cultures, respectively. Each surface should be inoculated with higher amount of CFU in total to obtain only a few cells after sampling, depending of temperature and humidity of the laboratory. Laboratory conducting the assays should perform a pre-study for recovery to adjust inoculation level, beginning for example at 50 CFU in total. The cultures are evenly spread onto each square with sterile spreaders, and are allowed to dry for 24 h at room temperature in biosafety cabinets. Each square is subsequently sampled, resulting in an environmental sample. For each comparison, aliquots spread onto different squares are from the same inoculum, and thus drying time, temperature and humidity are identical among the enrichment compared. To sample, even and firm pressure is used to push the swab or sponge across a square 10 times vertically, then the device is turned and pushed 10 times horizontally, and then 10 times diagonally.

Also, the International Standard ISO 18593: 2018, describing surface sampling technique (contacts plates, stick swabs, sponges and cloths) for the detection or enumeration of bacteria in food processing area and equipment can be followed.

3.4. Analysis

Examination of the broths:

Isolation:

For each enrichment step of each enrichment procedure, one loop of the broth (after its homogenisation: stomaching or manual mixing) is isolated on LOA agar (of the same

manufacturer and brand for all procedures tested in parallel), further incubated for 24 h ± 2 h and 48 h ± 4 h at 37 ± 1°C, observed for typical colonies with each incubation period. Use the same material for isolation, or same loop volume for isolating all broths tested in parallel.

Optionally a second *Listeria* selective agar of own choice can be used and incubated according to its technical specifications or the manufacturer's instructions.

The aspect of the plates, in particular presence and aspect of characteristic *Listeria* spp. and *L. monocytogenes*, as well as presence of background microflora, are recorded on the test report document.

If possible, take some pictures in particular if particular colonies are observed – atypical colonies (slow PIPLC, impact of enrichment, etc…).

Semi-quantification:

The method herein is derived from Drop plate technique according to DIN 10161:2016-12: Microbiological analysis of meat and meat products – Aerobic count at 30°C – Drop plating method, and on a protocol from Valencia University (Spain), with origin from Miles Misra, 1938, and the proposed protocol example can be used:

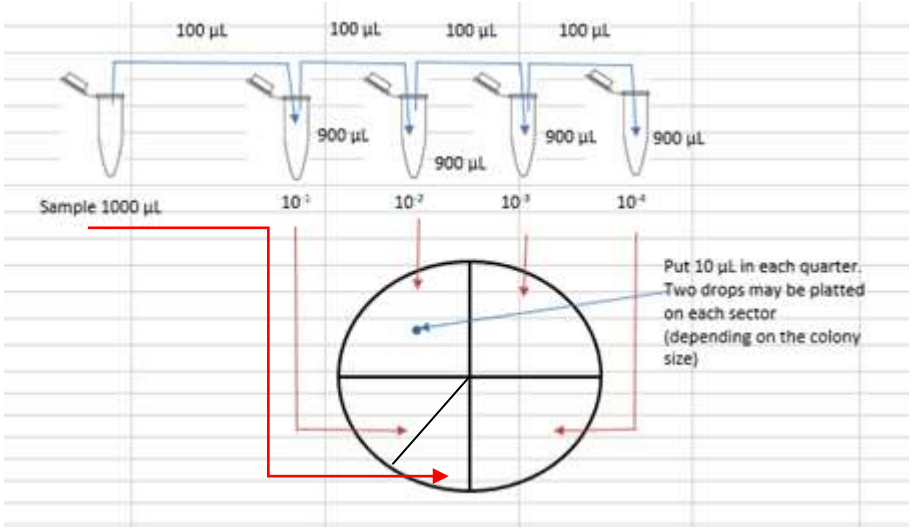
After the first 24h of enrichment, serial decimal dilutions of the enriched broths are performed after its homogenisation (stomaching or manual mixing):

Dilution is done according to ISO 6887, 0.01 ml of every dilution is spot dropped onto one sector of a LOA agar plate (of the same manufacturer and brand for all procedures tested in parallel), previously dried for 15 to 30 mn in laminar flow cabinet, split into 4, 6 or 8 sectors (the user can add some additional plate if one is not enough), and incubated for 24 h ± 2 h and 48 h ± 4 h at 37 ± 1°C. The drops are to be spread with a little loop in each sector, by beginning with the most diluted. Last steps are the counting of characteristic *Listeria spp*. and

L. monocytogenes colonies in 2 sectors having 1-50 colonies and the calculation of the weighted average. When for one broth no colony appears, results are expressed as <100 CFU/ml. If 4 decimal dilutions are performed, and uncountable colonies appear on all sectors, results are expressed as > 5. 10^7 CFU/ml.

Results are recorded on the test report document.

See example scheme:



Confirmation of the colonies:

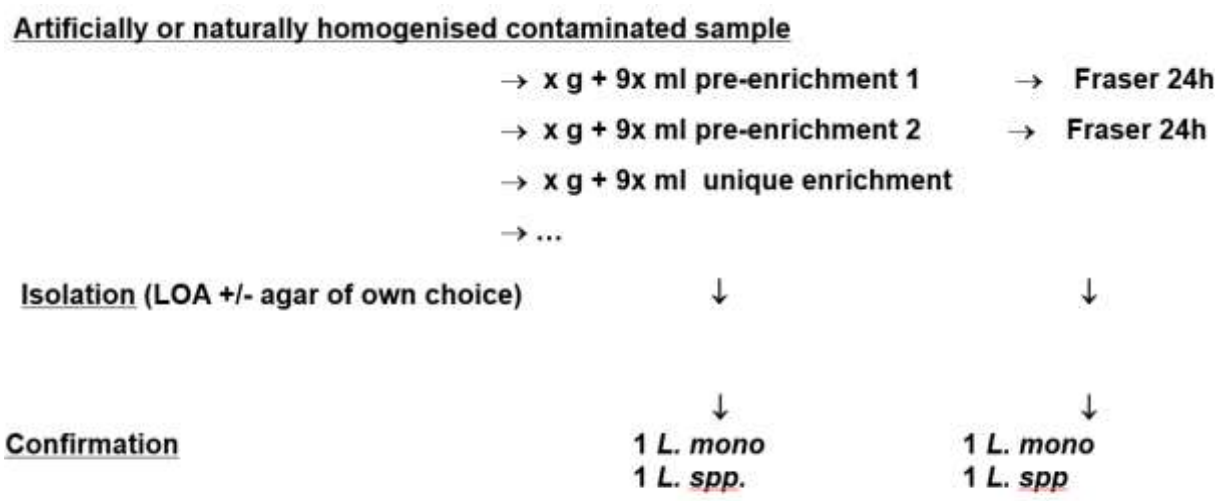
For each enrichment procedure, after each enrichment step, one characteristic *Listeria* spp. and *L. monocytogenes* colony are picked from LOA isolation plates, and purified on non-selective agar for further confirmation.

Confirmation of *Listeria* spp. and *L. monocytogenes* are performed according to laboratory own choice procedure (including standard tests, certified rapid confirmation methods, rapid alternative confirmation methods used to confirm colonies picked from agars respecting LOA formula in the frame of certified rapid detection methods…)

The differentiation of *Listeria* species (other than *L. monocytogenes*) is optional.

Results are recorded on the test report document.

See scheme of broths analysis:



(+ after the first 24h enrichment, semi-quantitative Drop plate technique on LOA in parallel to isolation)

3.5 Criteria for pre-selection of alternative protocols

As it is a preliminary study, criteria for pre-selection of alternative protocols will be based on Table 3 of ISO 16140-2 Standard: positive and negative agreement, positive and negative deviation. Based on these data, the values for sensitivity of the alternative method and of the reference method, as well as the relative trueness (to take with caution since for this criteria, the actual ISO Standard is supposed to represent the true value) will be calculated.

Acceptability thresholds will depend on the number of samples analysed.

Criteria for selection will be submitted to subgroup WG2-WG3 'Review evaluation/validation protocols for ISO standards'

Additionally several points have to be considered:

-General outcome of sensitivity study by the laboratories that have tested the broths

-semi-quantitative growth performances in each broth after 24h

-One enrichment step or two-step enrichment procedure

-Media already open

-Harmonization of reference methods

-Potential preference for Half Fraser modified

-Need to add selective agents after a delay

-Total duration to obtain the results

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5. ANNEXES

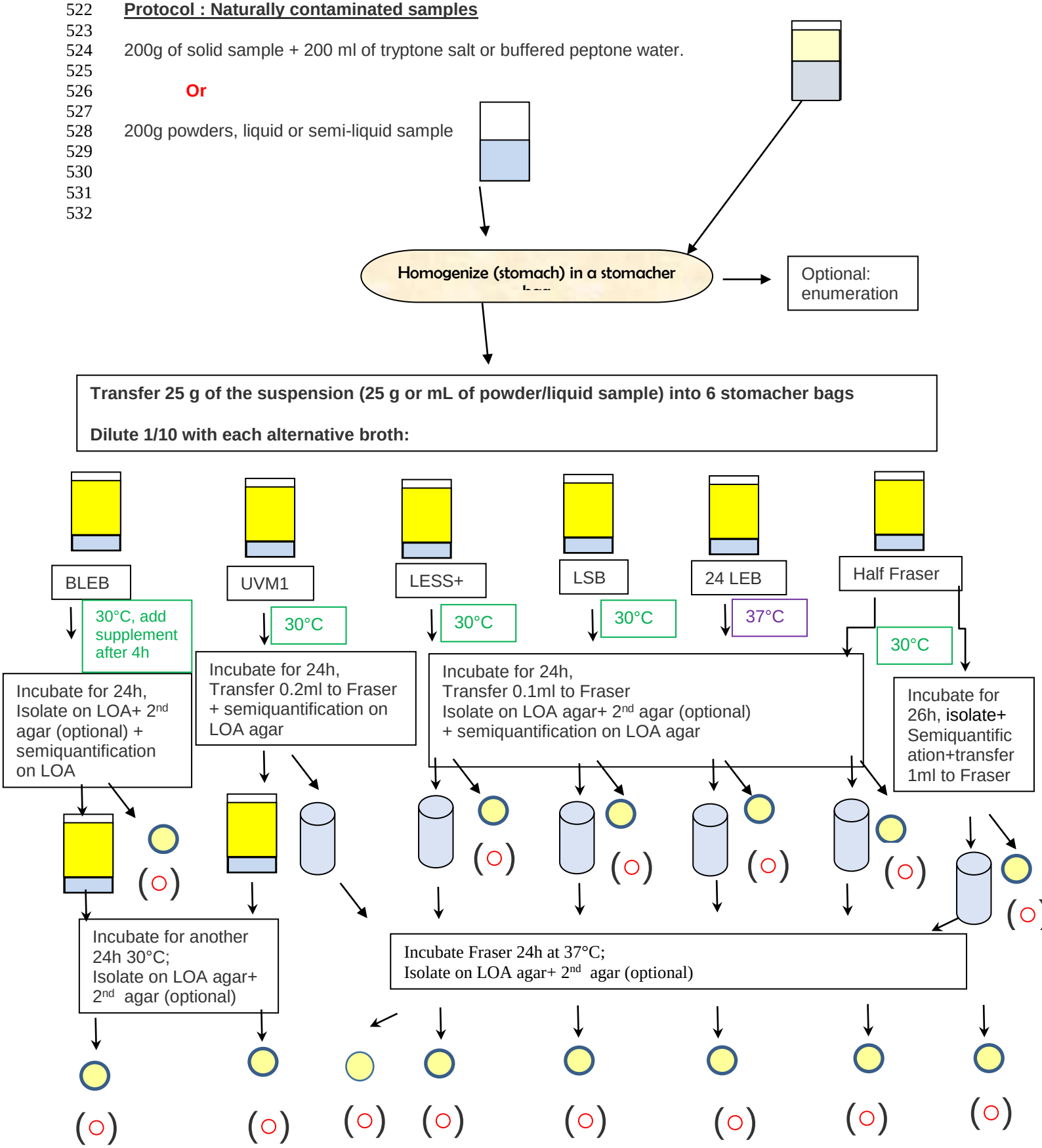
ANNEX 1: WORKFLOW DIAGRAM OF THE ASSAYS FOR THE GENERAL PROTOCOL

Protocol : Naturally contaminated samples

200g of solid sample + 200 ml of tryptone salt or buffered peptone water.

Or

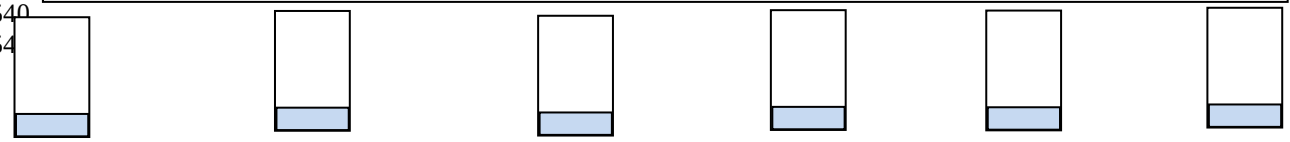
200g powders, liquid or semi-liquid sample



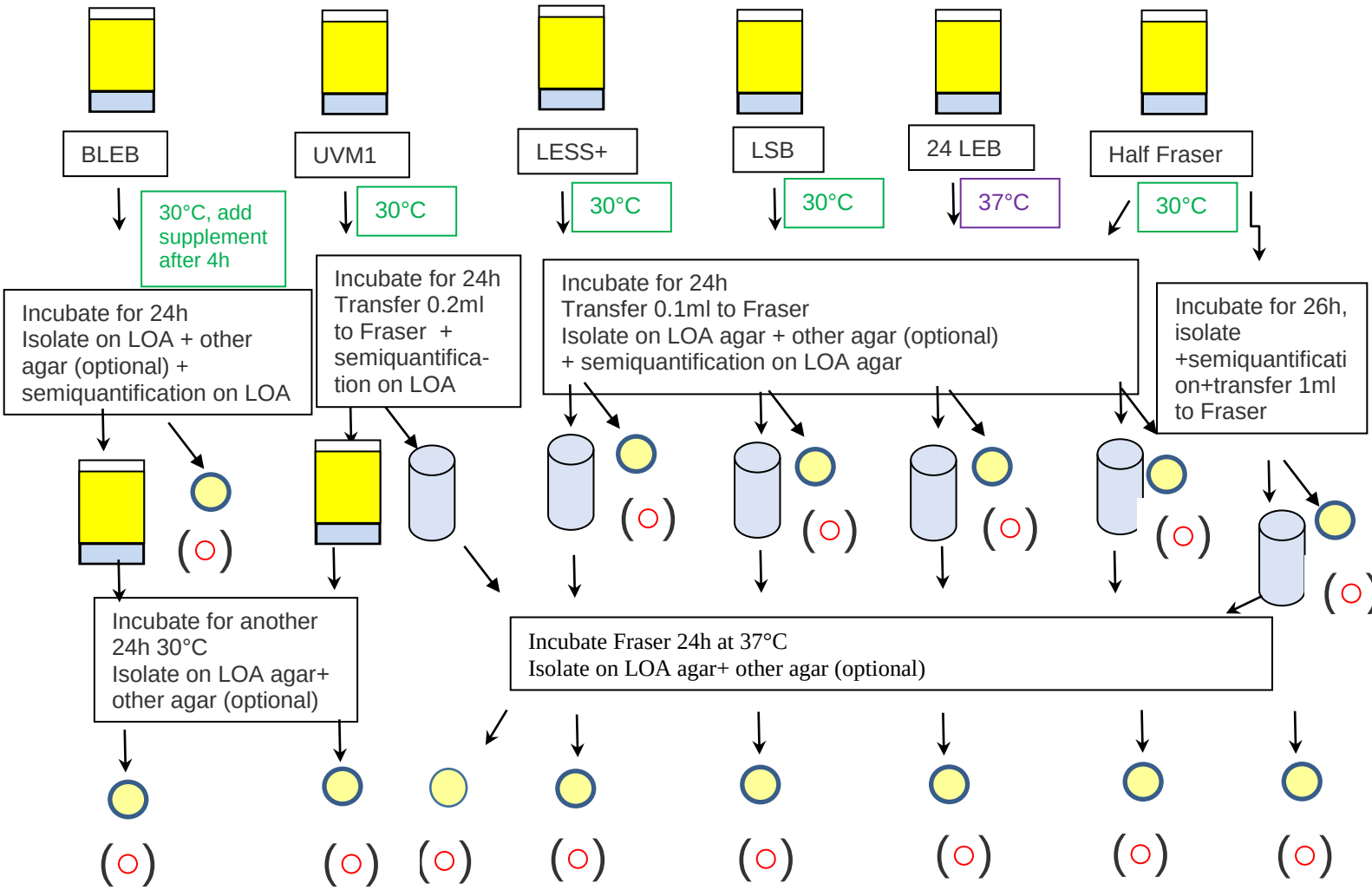
After each enrichment step, one characteristic *Listeria* spp. and *L. monocytogenes* colony are confirmed with laboratory own choice procedure (see 3.4)

Protocol : Artificially contaminated samples

Verify absence of *L. monocytogenes* or *Listeria spp*. according to ISO 11290-1 or certified alternative method
Weight 25 g samples in 6 stomacher bags
Contaminate individually each sample and store according to 3.3



Dilute 1/10 with each alternative broth, and (stomach)



After each enrichment step, one characteristic *Listeria spp.* and *L. monocytogenes* colony are confirmed with laboratory own choice procedure (see 3.4)

ANNEX 2: ALTERNATIVE ENRICHMENT PROTOCOLS TO BE COMPARED: MODIFICATIONS OF HALF FRASER BROTH

All proposed enrichment procedures are compared in parallel to ISO 11290-1 reference method (option 1).

Given the importance of incubation temperature on growth potential and stress recovery (Cornu et al. 2002; Barre et al. 2016), modified Half Fraser broth, described below, may be incubated at 30°C or 37°C.

In the case of media preparation from individual ingredients, it is important to use the same ingredients (batch and trademark) for both modified and unmodified Half Fraser.

In case of using ready-to-use commercial media, it is recommended, but not mandatory, to use Half Fraser and Fraser broths from the same manufacturer.

In that case, if media provider is able to manufacture on request also the modified Half Fraser, it is recommended, but not mandatory, that it uses the same ingredients (batch and trademark) for both modified and unmodified Half Fraser, and with exactly the same amount for common ingredients (except those related to the modification of the formula).

In any cases it is necessary to have the same quality controls as for unmodified media.

In particular, performance testing of the culture media shall be available from the manufacturer or shall be carried out in accordance with ISO 11133, or Annex B.5 of EN ISO 11290-1:2017, which includes in particular definitions for productivity and selectivity.

ISO 11290-1 reference method (Option 1)

According to the ISO 11290-1 reference method, samples are 1/10 homogenized in Half Fraser broth, then incubated for 24-26 h at 30°C. Following incubation, 0.1 ml of the culture is transferred into 10 ml of Fraser broth and incubated for a further 24 h at 37°C. It is recommended, but not mandatory, to use Half Fraser and Fraser from the same manufacturer.

Modified Half Fraser broth (Option 2)

This modification is from Bannenberg et al., 2021. Samples are analysed according to the ISO 11290-1 reference method: they are 1/10 homogenized in Half Fraser broth, but then incubated for 26-28 h at 30°C. Following incubation, 1 ml of the culture is transferred into 10 ml of Fraser broth and incubated for a further 24 h at 37°C. Please note that the initial step is common with option 1 and only one food suspension is performed.

Modified Half Fraser broth (Option 3)

Samples are analysed according to the ISO 11290-1 reference method: they are 1/10 homogenized in Half Fraser broth, but then incubated for 26-28 h at **37°C**. Following incubation, 1 ml of the culture is transferred into 10 ml of Fraser broth and incubated for a further 24 h at 37°C.

Modified Half Fraser broth (Option 4)

Modifications consist in increasing the incubation time to 26-28 h at 30°C and the transfer volume to Fraser from 0.1 to 1.0 ml, and in formula improvement (NaCl 0 g/L, YE 10 g/L) (Dupont, 2010).

Modified Half Fraser broth (Option 5)

Modifications consist in increasing the incubation time to 26-28 h, temperature to **37°C** and the transfer volume to Fraser from 0.1 to 1.0 ml, and in formula improvement (NaCl 0 g/L, YE 10 g/L) (Dupont, 2010).

ANNEX 3: ALTERNATIVE SPIKING PROTOCOLS WITH INTENSIVE STRESS

HEAT STRESS:

Bannenberg et al. (2021) showed that intensive stress resulting to one $\log_{10}$ (1D) reduction in the *L. monocytogenes* population had a significant impact on lag phase duration and could result in problems of detection because of delayed growth in pre-enrichment:

The reference cells without stress pre-treatment showed a short lag duration, which ranged from 1.4 to 2.7 h. However, significant variation in the ability to recover after 60 ◦C heat stress was observed among the tested strains and resulted in a lag duration from 4.7 to 15.8 h. Such stress could be used to prepare inocula for food samples submitted to heat treatment.

Stress protocol:

Working cultures are diluted 1:100 in 50 ml BHI broth pre-heated at 60 ◦C in a water bath for the time of one D60-value reduction (see Table S1).

Afterwards, the cultures are quickly cooled on ice for 15 s and decimally diluted in Peptone Physiological Salt solution for further use until the required contamination level (the decimal reduction due to heat stress has to be taken into account, and, if possible, verified ahead through a preliminary study).

The inoculum obtained will have enhanced lag phase duration.

A contamination level of 10-20 cells/sample is advised, to avoid heterogeneity between test portions due to the impact of individual cell lag time variability.

For facilities reasons, use the strains cited in the article: either strains from international collections, or the Dutch collection (that may be available on request via a Mutual Transfer Agreement). It is strongly advised in particular to include at least strain H7962 and/or the

627 reference strain WDCM00021, or a mixture of both.

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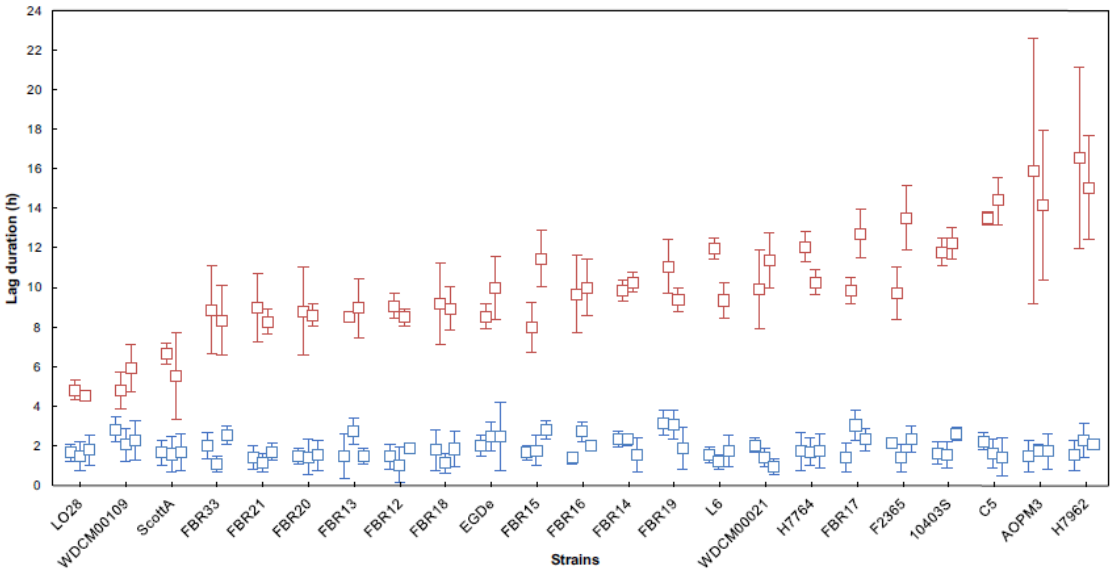
629 Characteristics of the strains, from Bannenberg et al. (2021):



630 **Fig. 1.** Lag duration of 23 strains of *L. monocytogenes* in half Fraser enrichment broth with reference cells in blue (with no additional stress pre-treatment applied) and 60 °C heat stress pre-treatment in red (aiming for one D_{60}-value reduction). The 3-phase model was used to fit the growth kinetics and the lag duration was

Table S1 List of *L. monocytogenes* strains used in this study with their serotype, isolation source, D_{60}-value in minutes and the pH used for one $\log_{10}$ reduction (during 24h at 10°C).

Strain	Serotype	Source	D_{60}-value (minute)	pH for log reduction
WDCM00021	4b	Human isolate meningitidis (used in ISO 11290:2017)	0.5	2.25
ScottA	4b	Human isolate milk outbreak	0.6	2.00
WDCM00109	1/2a	Guinea pig (used in ISO 11290:2017)	0.6	3.35
LO28	1/2c	Healthy pregnant carrier	0.6	-
FBR13	1/2a	Frozen endive a la creme	0.6	-
10403S	1/2a	Human skin isolate	0.7	-
H7764	1/2a	Deli turkey	0.8	-
FBR12	1/2a	Frozen vegetable mix	0.8	-
FBR17	4d	Frozen fried rice	0.9	-
FBR20	1/2a	Frozen vegetables for soup	1.1	-
H7962	4b	Hotdog	1.2	2.25
FBR18	1/2a	Ice cream	1.2	-
FBR21	4d	Fresh yeast	1.2	-
FBR33	1/2c	Pancake	1.2	-
FBR19	1/2a	Frozen meat	1.3	-
AOPM3	4b	Human isolate	1.4	-
FBR15	1/2c	Ice cream packaging machine	1.5	-
EGDe	1/2a	Rabbit	1.6	3.20
C5	4b	Smoked meat	1.6	-
F2365	4b	Jalisco cheese	1.7	-
FBR16	1/2a	Ham (after cutting machine)	2.0	-

ACID STRESS:

Bannenberg et al. (2021) showed that intensive stress leading to 1D reduction in the *L. monocytogenes* population had a significant impact on lag phase duration and could result in problems of detection because of delayed growth in pre-enrichment.

Such stress could be used to prepare inocula for samples submitted to acidic conditions.

Stress protocol:

Working cultures are stressed in acidified BHI broth at 10 ◦C for 24h. The BHI broth is acidified with 2.5 M HCl until the pH value that gave one $\log_{10}$ (D) reduction after 24 h at 10 ◦C (see Table S1 of the reference article).

This low temperature of the stress protocol is chosen to simulate the temperature in the cold food chain.

Acid stressed cells are subsequently decimally diluted in PPS until the contamination level required (the decimal reduction due to acid stress has to be taken into account, and, if possible, verified through an upfront preliminary study).

The inoculum obtained will have enhanced lag phase duration.

A contamination level of 10-20 cells/sample is advised, to avoid heterogeneity between test portions due to the impact of individual cell lag time variability.

For facilities reasons, use strains cited in the article: either strains from international collections, or the Dutch collection (that may be available on request via a Mutual Transfer Agreement). It is strongly advised in particular to include at least strain H7962 and/or the reference strain WDCM00021, or a mixture of both.

658 Characteristics of the strains, from Bannenberg et al. (2021):

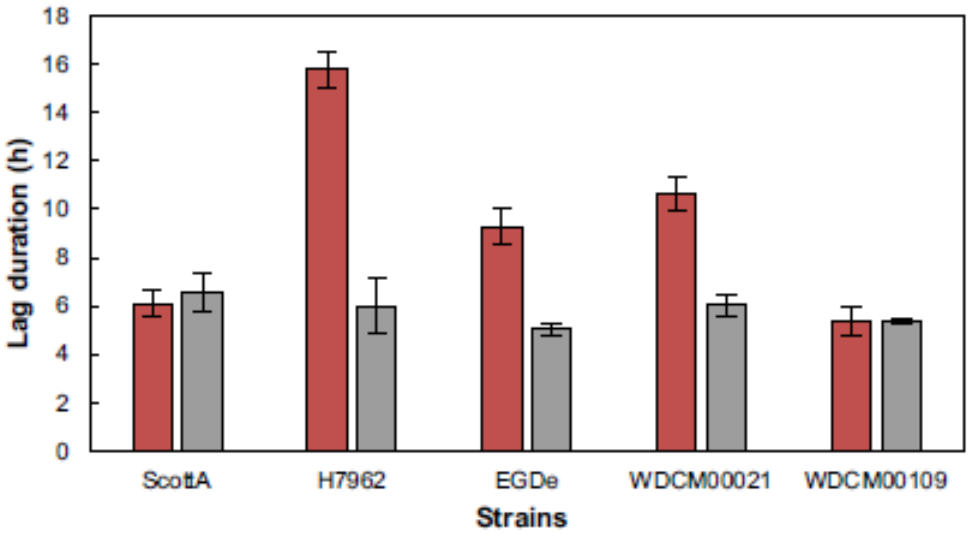


Fig. 3. Comparison of the lag duration after 60 °C heat stress (red) and 10 °C low-temperature acid stress (grey) in half Fraser enrichment broth. Growth

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OXIDATIVE STRESS:

Jasson et al. (2007) established procedures provoking sub-lethal injury of *L. monocytogenes* to analyze method performance testing. Criteria for the choice of stress procedure employed was easy to handle, repeatable and relevant for stress conditions in foods, but also on microorganism itself. The oxidative stress was chosen for *L. monocytogenes*. It delivered the highest percentage of sub-lethal cells, lead to inactivation (data not shown) and the variation in the obtained results was the smallest.

As hydrogen peroxide may enter in the composition of various disinfectants, often associated with peracetic acid, such stress could be used to prepare inocula for processed samples or environmental samples.

Stress protocol:

Working cultures are stressed by the addition of 1000 µM hydrogen peroxide and incubated at 37 °C for 1 h. Stressed cells are subsequently decimally diluted in unselective diluent.

For practicability reasons, the strains used in the article (see Table below) could be used: some of these LFMFP strains are deposited and allocated an LMG number and can be ordered at the official Belgian Culture Collection of Micro-organisms (BCCM) (https://bccm.belspo.be/about-us/bccm-lmg) or from international collections, if available..

Characteristics of the strains, from Jasson et al. (2007):

Sub-lethal cells were defined as cells able to multiply and form colonies on non-selective media (TSAYE) but not on selective media (LOA):

$$\%\ \text{sub} - \text{lethal injury} = \frac{\text{counts on non selective media} - \text{counts on selective media}}{\text{counts on non selective media}} \times 100.$$

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688 *Please note that as inactivation data are not available, pre-assays should be conducted before*

689 *using this protocol to determine decrease in the population.*

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Listeria monocytogenes	**LFMFP-UGent number**	**Culture collection number or unique coding**	**Origin**
Listeria monocytogenes 4b (4d, 4e)	394	LMG 23194	Wijnendaele cheese
Listeria monocytogenes 4b(4d,4c)	235	LMG 23905	Cooked ham
Listeria monocytogenes 4b(4d,4c)	034	LMG 13305	Soft cheese
Listeria monocytogenes 4b(4d,4c)	447	LMG 23356	Jalisco cheese (USA)

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698 Mean value of percentage sub-lethal injury ±sta

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Strain	% sub-lethal injury
	ALOA [a]
	H_2O_2
LFMFP 394	38.9±0.8[c]
LFMFP 447	19.7±2.6
LFMFP 182	68.3±5.0[c]
LFMFP 34	82.0±0.7[c]
LFMFP 45	75.3±12.5[c]
LFMFP 235	82.0±2.4[c]

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