Rapid methods in food microbiology

Sabina Purkrtová
Microbiological analysis procedures

CONVENTIONAL CELL CULTIVATION
- relatively easy to use, but time (requires several days), labour (lots of procedural steps) and material consuming
- many of them are recognised as approved for ISO and they are gold standard procedures
- Colony count method (CCM)
  - pour plate techniques
  - spread plate techniques
- Membrane filtration
- Most Probable Number (MPN)

RAPID METHODS
- immunonological method (based on antigen/antibody-binding)
- based on molecular biological method (based on PCR)
- others (ATP Photometry, Direct Epifluorescent Filter Techniques (DEFT), Electrical impedance method, Flow cytometry, etc.)
A bacterial antigen – a molecule on the surface of bacterial cell

Immunoglobulins Ig (also antibodies) are glycoprotein molecules produced by B cells plasma cells (white blood cells) according to antigen to mark remaining bacteria for destruction. The antibody immune response is highly complex and exceedingly specific
The various immunoglobulin isotypes differ in their biological features, structure, target specificity and distribution.
**Immunological method**

**Polyclonal antibodies**
The immune response to an antigen generally involves the activation of multiple B-cells all of which target a specific epitope on that antigen. As a result a large number of antibodies with different specificities and epitope affinities are produced.

**Monoclonal antibodies** are generated by a single B lymphocyte to one specific epitope. For production B cell is isolated from from the spleen and lymph nodes of immunised animals and fuse with immortal heteromyleoma. The produced hybridoma cells produce only one antibody within the supernatant.
Immunonological method

- immunonological method
(based on antigen/antibody-binding)
**Immunomagnetic beads separation**

ISO 16654:2001 Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Escherichia coli* O157

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**Selective enrichment**

1. **Day**: 25 g of sample + 225 ml of Modified Tryptone Soya Broth (mTSB) with novobiocine; homogenization; Incubation at 41.5 °C

**Immunomagnetic isolation**

- 8 and 16 hours after enrichment (2. day)

**Isolation on selective agar plates**

- MacConkey agar with sorbitol (instead of lactose) (SMAC)
- *E. coli* O157 is sorbitol negative – no fermentation – colourless colonies
- **Fluorocult® *E. coli* O157 Agar**
  - Chromogenic medium – chromogenic substrate for β-D-glucuronidase – *E. coli* O157 is negative -colourless colonies
  - 37 ± °C for 24 ± 3h

**Confirmation**

4. **Day** Isolation of a characteristic colony on Nutrient Agar and incubation at 37 ± °C for 24 ± 3h

5. **Day**: testing for positive indole reaction

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- 1 ml of homogenate + 100 µl of paramagnetic beads covered with antibody against *E. coli* O157
- Present cells *E. coli* O157 are trapped and separated by applying a magnet
- The homogenate with other bacteria is taken away.
- The beads with trapped *E. coli* O157 are washed (to add a washing buffer – to release a magnet – to mix – to apply a magnet)
- Plating out by 50 µl of paramagnetic beads with trapped *E. coli* O157 on 2 selective agar plates.

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**SMAC agar**

*Escherichia coli*

O157:H7 (colourless)
Enzyme-linked immunosorbent assay (ELISA)

How to detect the formation of complex antigen-antibody?

- Different systems with antibodies linked to enzyme (e.g. horseradish peroxidase, alkaline phosphatase) – then to add substrate – its transformation is measured (e.g. changes in the absorbance)
Enzyme-linked immunosorbent assay (ELISA)

When the complex antibody-antigen is formed, the secondary antibody conjugated with enzyme is to be bound to the antigen and not to be washed off.

After adding the substrate, the substrate is changed and give the signal (e.g. Measured as absorbance).
Enzyme-linked immunosorbent assay (ELISA)

- monoclonal or polyclonal antibodies coated microtitre trays to capture target antigen. The captured antigens detected using a second antibody which is conjugated to an enzyme. The addition of enzyme substrate enables the presence of the target antigen to be visualised.
- considerable specificity
- can be automated; commercially available (Salmonella, L. monocyctogenes, Campylobacter, ...etc.)
VIDAS or mini-VIDAS Analyzers

- the automated, multiparametric immunoanalyser
- based on an enzyme immunoassay which detect target antigens using the **ELFA** (Enzyme Linked Fluorescent Assay)

![Diagram of ELISA assay]

- 4-methyl-umbelliferone (fluorescent molecule) is released by alkaline phosphatase = fluorescence
Each test is composed of two parts:

1. The SPR® acts as a Solid Phase Receptacle for the reaction. The SPR is coated with anti-**target** antibodies adsorbed on its surface

2. The Strip contains all ready-to-use reagents necessary for the test: washing solution, alkaline phosphatase-labeled anti-**target** antibodies and substrate
VIDAS or mini-VIDAS Analyzers

Video: http://www.biomerieux-industry.com/food/vidas-listeria-monocytogenes-detection#VIDAS LMO2

bioMérieux
http://www.biomerieux.com/
**VIDAS or mini-VIDAS Analyzers**

*Salmonella spp.*

**Detection using VIDAS Easy SLM**

The sample = the sample diluted and enriched in **Xpress 2 broth** (different from ISO 6579)

The used broth must be convenient for the method.

Validated by ISO 16140 against ISO 6579 (AFNOR, N°BIO 12/16-09/05)
VIDAS kits

- **E. coli O157** - VIDAS® UP *E. coli* O157 (including H7)
- **E. coli O157** - VIDAS® ECO
- **E. coli O157 (confirmation)** - VIDAS® ICE
- **Salmonella spp.** - VIDAS® SLM
- VIDAS® ICS
- **Salmonella spp.** - VIDAS® Easy SLM
- **Listeria spp.** - VIDAS® LIS
- **Listeria spp.** - VIDAS® LSX
- **Listeria monocytogenes** VIDAS® LMO2
- **Staphylococcal enterotoxins** - VIDAS® SET2
- **Campylobacter spp.** - VIDAS® CAM
GLISA-Rapid Test

GLISA-Rapid Test (Gold Labelled ImmunoSorbent Assay) – Lateral Flow Test

Detection limit: $10^5$ CFU/ml in a sample (homogenate of selective enrichment or suspension of colony) (sometimes heat-treatment required - 20 min at 80 °C = cell lysis = better motility of the cell walls with antigens)

Singlepath© for detection of Listeria monocytogenes (Merck)
Merck’s Rapid Test Product Portfolio
8 Rapid tests for the most common pathogens

<table>
<thead>
<tr>
<th>Listeria:</th>
<th>Salmonella:</th>
<th>E. coli O157 / VTEC:</th>
<th>Campylobacter:</th>
<th>Bacillus cereus:</th>
<th>Legionella:</th>
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<tr>
<td>LEB Base Fraser</td>
<td>Tetrathionate</td>
<td>mEC + n</td>
<td>Bolton Broth</td>
<td>M.Y.P. Agar</td>
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<td>GVPC-</td>
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<tr>
<td>Listeria Agar</td>
<td>Rambach®</td>
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</table>

10-May-06
Rapid Detection of Pathogens
ISO Standard 6579 for Detection of Salmonella

Day 1: 25 g/ml test sample in 225 ml BPW
       16 - 20 h / 35 - 37 °C

Day 2: 0.1 ml BPW to
       10 ml RVS Broth
       24 h at 41.5°C

Day 3: XLD Agar
       24 h / 35-37°C
       1 plate 14 cm / 2 plates 9 cm

Day 3: Any other Salmonella Agar
       24 h / 35-37°C
       1 plate 14 cm / 2 plates 9 cm

Day 4: 1 ml BPW to
       10 ml MKTTn Broth
       24 h at 37°C

Day 4: XLD Agar
       24 h / 35-37°C
       1 plate 14 cm / 2 plates 9 cm

Day 4: Any other Salmonella Agar
       24 h / 35-37°C
       1 plate 14 cm / 2 plates 9 cm

Day 4: Interpreting of Growth on Plates

Day 5: For confirmation take 5 suspected colonies from each plate and streak onto Nutrient agar
       18 - 24 h / 35 - 37 °C

Day 6: Biochemical / Serological Confirmation Tests
       TSI / Urea / Lysin / β-Gal / VP / Indol

4 days

11 media
Rapid Testing

Singlepath® Salmonella

- For detection of *Salmonella* spp. in foods directly after 2-step enrichment

- No post-enrichment step needed:
  
  *sample directly taken from RVS*
  
  + 15 min heating,
  
  *transfer to sample port*
  
  *read results within 20 min*

- Advantage over competitor products:

  Detection of multiple *Salmonella* serological groups

  Lower detection limit: $10^5$ cfu / ml
### Rapid Testing

**Singlepath® Salmonella - Screening**

| Day 1 | 25 g/ml TEST SAMPLE
|       | In 225 ml Buff. Peptone Water
|       | 35 - 37°C for 16 - 20 h |
| Day 2 | 0.1 ml BPW to 9.9 ml RVS Broth
|       | 42°C for 24 h |
| Day 3 | NO
|       | Salmonella not present |
|       | YES
|       | Salmonella present |
| Day 4 | If positive, confirmation by culture and biochemical tests required
|       | STREAK OUT ONTO RAMBACH
|       | 37°C FOR 24 h |

Only 2 days

Only 2 media
Validation of procedure

Validation of an alternative method
• the validation of an alternative method is the procedure to demonstrate if the alternative method provides equivalent or better results compared to the reference methods.

• Validation is a process, within which the method is demonstrated to be suitable for its purpose. It documents methods validity!
• During validation process, methods performance characteristics are estimated.

The validation of qualitative and quantitative methods comprises two phases:
  1. a method comparison study of the alternative method against a reference method (performed by an expert laboratory).

  2. a interlaboratory study of the alternative method (organised by an expert laboratory).

   Expert laboratory (organising): laboratory having the qualified staff and skills to perform the method comparison study and organise the interlaboratory study.
Useful documents

ISO 16140:2003 – Microbiology of food and animal feeding stuffs – Protocol for the validation of alternative methods

ISO 17025:2005 – General requirements for the competence of testing and calibration laboratories
Accreditation bodies that recognize the competence of testing and calibration laboratories should use this International Standard as the basis for their accreditation.

ISO/IEC 17011:2004 Conformity assessment - General requirements for accreditation bodies accrediting conformity assessment bodies
If a laboratory wishes accreditation for part of all of its testing and calibration activities, it should select an accreditation body that operates in accordance with ISO/IEC 17011.

It is expected that all laboratories involved in each step of a validation process will have a Quality System or quality assurance (QA) program in place to ensure standardization of laboratory operations, as well as adequate quality control (QC) activities.
Validation of procedure

Test characteristics
The test characteristics for alternative methods are shown:

Qualitative methods
1. Selectivity (inclusivity/ exclusivity)
2. Relative accuracy
3. Detection level
4. Relative sensitivity/Relative specificity
5. The agreement between the methods

Quantitative methods
1. Selectivity (inclusivity/ exclusivity)
2. Lowest validated level with satisfactory precision
3. Repeatability
4. Reproducibility
5. Uncertainty of the method
Rapid Testing
Singlepath® Salmonella

Evaluation of Singlepath® Salmonella:

• Evaluated with 105 *Salmonella* and 58 non-*Salmonella* species
  - skimmed milk powder  - black pepper
  - pet food            - seafood
  - ground beef         - ground poultry

• Sensitivity and Specificity each >98%

• AOAC approval granted October 2004
Rapid Testing
Singlepath® Salmonella

Evaluation at University of Giessen: Comparison with CELSIS Path Stik

- Tested with spiked + native ground meat, poultry, milk powder
- Both tests have 98 % Sensitivity and Specificity (n = 50 food samples)
- Significant stronger bands than with PATHSTIK

  Singlepath showed better Detection Limit (10^5 CFU / ml)

Evaluation at Agriquality (NZ): Comparison with TECRA ELISA Salmonella

- Tested with roasted chicken, grated cheddar cheese, whole milk powder, non-fat milk powder, environmental swabs (n = 279 food samples)
- Both assays give 100 % identical results
- Both assays with >99 % Sensitivity / Specificity
DNA, RNA, PROTEINS

Deoxyribonucleic acid: (DNA)

A linear polymer that consists of four nucleotides:

Adenine Cytosine Guanine Thymine

Primer binding  A – T ,  C - G
PCR principle

PCR = Polymerase Chain Reaction (1983 by Kary Mullis)

*in vitro* amplification of the part of DNA (usually 100 bp – 1500 bp) bordered by two primers (syntetically prepared oligonucleotides)

Typical number of cycles = 30 - 45

https://www.youtube.com/watch?v=iQsu3Kz9NYo
PCR principle

**DENATURATION** of double-stranded DNA by heat (app. 94-95°C, initially 5, then in each step 2-3 minutes)

**ANNEALING** of PRIMERS on the free single strand DNA
- forward primer on the 3'-5' strand
- reverse primer sits on the 5'-3' strand

$T_a = \text{annealing temperature}$
- 50-65 °C – depends on the primers sequence
- Time: 30 s-60 s
- One of the most important factor for the specificity
- $T >> T_m$ = no annealing - no PCR product
- $T << T_m$ = non-specific annealing – nonspecific products

**ELONGATION** by DNA polymerase

*in vivo* starts to synthetize new DNA molecules from 3'-end of RNA primer

*in vitro* PCR from 3'-end the primers

72 °C – optimal temperature

45 s-3 minutes (final elongation 5-15 minutes)
PCR principle
Mistake in the beginning – the most influence

**Exponential phase**
duplication of PCR products amount in each cycle

**Linear phase**
The level of amplification is decreased (lower than duplication in each cycle)

**Plateau:**
Absence or degradation of some component
Reasociation can be preferred than primers anealing due to thermodynamical reasons

PCR principle

- Primers (forward, reverse)
- Mg\(^{2+}\) (cofactor for DNA polymerase)
- dNTP mix (dCTP, dATP, dTTP, dGTP) – building stones for newly synthetized DNA product
- DNA (genomic, plasmid DNA)
- Nuclease free water – without Dnase, Rnase presence (enzymes, which degrade DNA, RNA)
- Thermostable DNA polymerase

MASTERMIX

Total volume – 25 µl – 50 µl
**Polymerase**

**Taq DNA polymerase**
- from thermophilic bacterium *Thermus aquaticus* (now prepared by recombination)
- opt. temp.: 75-80°C (150 nts/s)
- active at the room temperature – to work on ice,
- inactive at >90°C, but reactivation when cooled
- only 5´exonuclease activity, not 3´exonuclease activity (proofreading)
- 1 mistake at 10-20 000 nts

**Other polymerases:**
- Proofreading polymerases (with the 3´exonuclease activity),
- *Tth* polymerase - reverse transcription (Mn$^{2+}$) or polymerase activity (Mg$^{2+}$)

**Hot start polymerases**
- Activity at the room temperature is inhibited - binding on anti-polymerase antibodies on the wax balls
- activation after heating at 94 °C (antibodies destroyed)
1) Releasing DNA from the cell
- lysis of the cell wall by detergents or by heat, digesting cell surface proteins by proteinase K
- RNAses treatment for RNA-free genomic DNA

2) Isolation DNA from the released cytoplasmatic contents
- Centrifugation the vaste in pellet (heat isolation)
- Ion-exchange chromatography (commercial kit) – binding DNA by ionic interactions to the column (DNA is negative charged) – proteins and RNA pass away – DNA is later released by using more concentrated salt solution (column is charged positively)
- Phase separation (aqueous/chlorophorm phase – CTAB method)
Detection of food-borne pathogens

DNA of microorganism = set of specific genes with specific sequences

*Primers are designed to be complementary to sequence of some specific gene*

– if the complementary sequence is present, PCR product will be performed

Main problems about *primers designing* and *applying of PCR*

- Is the gene of interest specific enough (can be found only in the detected microorganism)?
- Is the primers sequences complementary only to this gene?
- Is the primer sequence designed well? (requirements about easy and specific annealing – depends on the primers sequence)
- Primers can be designed commercially or by using software ([http://www.biocenter.helsinki.fi/bi/Programs/download.html](http://www.biocenter.helsinki.fi/bi/Programs/download.html))
- sequences and genomes needed - www.pubmed.com
PCR products detection

The PCR product has got specific length and specific sequence

**Visualiton by intercalating agents**
bind to double-stranded DNA - fluorescence signal

- **Ethidium bromide** – carcinogen/teratogen
  If intercalated the fluorescence of EtBr is significantly higher than in aqueous solution (UV absorbance maxima at 300 and 360nm/emitted as orange/yellow light at 590nm)

- **Sybr Green**
  DNA-Sybr Green complex absorbs blue light at 488 nm and emits green light at 522 nm

**Fluorescent tags** at 5’-end of primers
**Other tag** at 5’-end of primers
  e.g. biotin - for immunodetection detected by anti-bodies linked with an enzyme

**Microarray**

![SYBR® GREEN PCR ASSAY](image)
Conventional electrophoresis
a single electrical field causes biomolecules to migrate through a matrix according to its mass-to-charge ratio effectively separates DNA fragments up to ~20-30 kb (according to the gel concentration), larger fragments will comigrate (a large band at the top of the gel)
Electrophoresis

Primers (OT1559 a 18–1) complementary to DNA coding 16S rRNA *Campylobacter jejuni*, *Campylobacter coli* a *Campylobacter lari*.
Product is 287 bp

60 V, 100 minutes, M – 100 bp marker, (1-5: positive controls) 1- *C. jejuni* CCM 6212, 2- *C. jejuni* CCM 6214, 3 – *C. coli* CCM 6211, 4 – *C. lari* CCM 4897, 5 – *C. upsaliensis* ATCC 43954, 6- 15 : strains *C. jejuni*, Nt- no template control (negative control)
Electrophoresis

DUPLEX PCR = two pairs of primers

*C. jejuni*: C1 5' - CAA ATA AAG TTA GAGGTA GAA TGT-3'
C4 5' - GGA TAA GCA CTA GCTAGC TGA T-3'
Complementary to a part oxidoreductase, 159 bp

*C. coli*: 18F 5' - GGT ATG ATT TCT ACAAAG CGAG-3'
519R 5'- ATA AAA GAC TAT CGT CGC GTG-3'
Complementary to aspartatekinase and flanking ORF, 500 bp

Species specific PCR

60 V, 100 minutes, M – 100 bp marker, (1-5: positive controls) 1- *C. jejuni* CCM 6212, 2- *C. jejuni* CCM 6214, 3 – *C. coli* CCM 6211, 4 – *C. lari* CCM 4897, 5 – *C. upsaliensis* ATCC 43954, 6- 15 : strains *C. jejuni*, Nt- no template control (negative control)
Melting curve

Intercalating dye binds to double-stranded DNA - producing fluorescent signal when excited by light

When samples are heated to melting point, double-stranded DNA denatures into single strands, freeing dye into solution

- Melting temperature (Tm) is the point at which the DNA denatures
- Dependent on the length of fragment and G-C content
- More base pairs --> Increase in Tm
- Higher G-C content --> Increase in Tm

- Unbound dye has reduced fluorescent signal
- Loss in signal generates melt curve
Melting curve

Inflex point
- 1. derivation = 0
- In this case 2.derrivation is negative
Real-Time PCR

$C_T$ (threshold cycle) – the lowest cycle, when fluorescence crosses the determinated value

Video: https://www.youtube.com/watch?v=kvQWKcMdyS4
Food-borne pathogens detection by PCR

Identification of pure cultures - without problems

Detection/enumeration from food matrix directly or after enrichment ?????

- How to isolate successfully DNA from few cells in 25 g/10 g????
  - Efficiency of DNA isolation (isolation by kits, heat-lysis, CTAB method)
- How many targets copies are needed for successfully PCR products detection ??
  - detection limits
  - presence of PCR inhibitors (inhibition of polymerase, primers annealing)
- How to distinguish DNA from dead and live cells ?? - Total DNA is detected (from dead and living cell)
- For distinguishing of dead DNA – e.g. application of EMA (ethidium monoazide) to the sample before DNA isolation – EMA irreversibly bound to DNA of dead cells – they are „invisible“ for PCR – no amplification
- Direct enumeration/detection only in some specific case – simple liquid sample when the required limit is higher than the limit of detection
- Standardly – for PCR is used enriched homogenate (sample diluted in enrichment broth and cultivated) – to obtain detectable level of microorganisms
BAX® System

- Salmonella
- Listeria monocytogenes
- Genus Listeria
- Enterobacter sakazakii
- E. coli O157:H7
- Staphylococcus aureus
- Campylobacter jejuni/coli/lari
- Vibrio cholerae/
- parahaemolyticus/vulnificus
- Yeast and molds

Real-time

BAX – three major steps

1. Prepare samples = perform heat-lysis in two stages
   - DNA present in the lysate

2. Process samples
   - Enrichment protocols vary depending on bacterial target and type of food you’re testing.
   - 5 µl for all bacterial targets
   - 20 µl for beef samples enriched in *E. coli* O157:H7 MP media
   - 20 µl for yeast & mold

3. Review results

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**Salmonella, Enterobacter sakazakii, E. coli O157:H7, E. coli O157:H7 MP, Campylobacter jejuni/coli, Yeast and Mold**

- 37° ±/- 2°C for 20 minutes, then 95° ±/- 3°C for 10 minutes

**Listeria and L. monocytogenes**

- 55° ±/- 2°C for 60 minutes, then 95° ±/- 3°C for 10 minutes
PCR tablets

- DNA-polymerase
- Primers
- Nucleotids
- Suplements
- SybrGreen or fluorescent probe
- Internal control + primer
- Mastermix in the tablet in PCR Eppendorf – minimal risk of contamination

For solving the problem of distinguishing dead and live cell the process of small volumes is used – only if live cells are present in the sample, they able to multiplied to the limit detection ($10^4$ CFU/ml in the enrichment suspension used for lysis step). If the result of detection is positive, confirmation by plating out is done (live and dead cells).
Amplification of specific fragments

Target organism DNA

+ BAX® system primers in tablets

PCR

Background DNA

+ BAX® system primers in tablets

PCR

no detectable amplification

Detection of specific fragments

Fluorescent signal drops with heating to generate a melt curve
Melting curves display peaks within a set temperature range for the control and target (if present).

- **Strong positive result**
- **Negative result**

**Melting curve**

**Raw Data**

**Processed Data**
Melting curve

Each target has a unique melting curve:

- Salmonella positive
- E. coli O157:H7 positive
- L. monocytogenes positive
- E. sakazakii positive
- E. coli O157:H7 MP positive
- C. jejuni/coli positive
- Salmonella positive
- Listeria positive
- L. monocytogenes positive
- E. sakazakii positive
Detection

Melt curve: weak INPC in presence of strong Salmonella positive

Real time: weak INPC in presence of strong Campylobacter positives

Direct protocol does not display a positive or negative result

PCR was successful, Indeterminate, Signal error
*Salmonella* AFNOR (ISO 16140) protocol

Sample 1:10 BPW

- **RVS**
  - 18 h, 37°C

- **Müller-Kauffmann**
  - 24 h, 41.5 / 37°C

- **XLD**
  - 24 h, 37°C

- **5 suspicious colonies on TSA**

- **Biochemical and serological confirmation**
  - 24 h, 37°C

- **BHI**
  - 3 h, 37°C

- **2. Plate**
  - 22 h, 37°C

- **1.5 d neg. and pos. result**

*Salmonella* (ISO 6579:2003) versus **BAX®-PCR**

3 d neg., 5 d pos. results
International certificates

AOAC-RI Performance Tested Method
Salmonella #100201; L. monocytogenes #070202; L. spp #030502, S.aureus # 120701; Campylobacter coli/lari/jejuni

AFNOR Certification
Salmonella #QUA 18/3-11/02
Certificates from AFNOR and AOAC are equal

NordVal Certification
Salmonella #2006-30-5408
The method is validated against ISO 6579:2002. BAX is also an AOAC method 2003.09. NordVal has studied the enclosures to the application and evaluated the results obtained in the validations by Afnor and AOAC Research Institute. ISO 16140 was used for the Afnor validation. NordVal has concluded that it has been satisfactorily demonstrated that the requirements of the NordVal validation protocol are fulfilled for foods, feeds and environmental samples. The limit of detection for the method is demonstrated to be 2-7 cfu/25 g or ml. The BAX Salmonella PCR System (BAX Classic and BAX Q7) can be used without further confirmation.
Fluorescence - principle

http://www.online-tensiometer.com/produkte/cleanospector/bilder/fluorescence_jabolinski_diagram.jpg

http://www.perceptive.co.uk/img/applications/applications_deft1.jpg

Absorption (\( \lambda \)) vs. Fluorescence emission (\( \lambda' \))
Dying with fluorescent kits
The different coloration is based on the different membrane permeability.

<table>
<thead>
<tr>
<th>Target</th>
<th>LIVE BacLight™ Bacterial Gram Stain Kit</th>
<th>LIVE/DEAD® BacLight™ Bacterial Viability Kit</th>
<th>LIVE/DEAD® Reduced Biohazard Cell Viability Kit #1</th>
<th>LIVE/DEAD® Yeast Viability Kit</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Target</strong></td>
<td>Bacteria</td>
<td>Yeasts</td>
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<td></td>
</tr>
<tr>
<td><strong>Result</strong></td>
<td>Living cells of G+ bacteria - green, living cells of G- bacteria - red</td>
<td>Living cells – mostly green, dead cells – mostly red (both dyes penetrate).</td>
<td>Active cell vacuoles – orange, living and dead cell walls – blue.</td>
<td></td>
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<td><strong>Fluorescent dyes</strong></td>
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<td>SYTO® 9</td>
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</tbody>
</table>

The LIVE/DEAD Bac Light Bacterial Viability Kits utilize mixtures of our SYTO®9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide. These stains differ both in their spectral characteristics and in their ability to penetrate healthy bacterial cells. When used alone, the SYTO 9 stain generally labels all bacteria in a population — those with intact membranes and those with damaged membranes. In contrast, propidium iodide penetrates only bacteria with damaged membranes, causing a reduction in the SYTO 9 stain fluorescence when both dyes are present. Thus, with an appropriate mixture of the SYTO 9 and propidium iodide stains, bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red. The excitation/emission maxima for these dyes are about 480/500 nm for SYTO 9 stain and 490/635 nm for propidium iodide. The background remains virtually nonfluorescent.
Fluorescence – application

DEFT - direct epifluorescent filter technique

Rapid Bioburden Testing in 3 Steps

1. Sample Preparation
Filter the desired sample volume through presterilized, disposable Milliflex filter units. Place membrane filter base onto a prefilled agar cassette and incubate.

2. Fluorescent Staining
Transfer membrane to a pad pre-wetted with fluorescent reagent and incubate for 30 minutes.

3. Counting CFUs
Count fluorescent colonies through the window of the Milliflex Quantum reader or use the camera to view the colonies on your computer screen.

Re-incubate for Microorganism Identification

Re-incubation Step
Place membrane on a prefilled agar media cassette and re-incubate. Collect and isolate the microorganisms and identify using any existing ID methodology.

Example: Tested sample of in-process, non-sterile water using the Milliflex Quantum system. After detection, membrane was re-incubated for full growth and identification.

Visual plate after staining.
View of membrane in the reader.

CFUs are not visible outside the reader.

After re-incubation, CFUs are visible to the eye

http://www.perceptive.co.uk/img/applications/applications_deft1.jpg
Fluorescence – applications

Fluorescent in situ Hybridization

Mixed population
Fixation
Hybridization

Probe
Fluorescent dye
Target (16S rRNA)
Ribosome
30S subunit containing 16S rRNA
Cytoplasm
Nucleoid
Ribosomes
Plasmid
Cell wall
Cytoplasmic membrane
Fluorescent oligonucleotide probes

Washing
Hybridized cells
FISH analysis

Fluorescence microscopy
Flow cytometry

http://www.biovisible.com/photopage.shtml

Escherichia coli with probe
Enterococcus faecium with probe

FISH – fluorescent hybridisation in situ
Flow cytometry – from 80ties used in medicine for counting a quite big blood cells. For counting cells of microorganisms – staining by fluorescent dyes is applied. Cells pass through a narrow glass capillary, where the laser enlightens them – the fluorescence and scattering is observed. Each passed cell has its own values of fluorescence and the scatter light, which depends on the size and complexity of the surface. The count of passed cells (up 1000 cells/s) and their properties can be determined according to the used dyes.
Counting of dead and live cells – e.g. BacLight®. Live cells – green fluorescence (FL1), dead cells – red fluorescence (FL3), after excitation by 488 argon laser.
ATP bioluminiscence

Principle: the detection of the present level of ATP (etekce úrovně přítomnosti ATP (direct proportion to the presence of microorganisms or their products residues). The enzyme luciferase catalyzes the transformation of d-luciferin to oxyluciferin (indicated by emission of light at 560 nm)
ATP bioluminiscence

ATP Hygiene Monitoring System - SystemSURE II
Cell-based sensors

Biosenzor = the whole cell/microorganism (CBS – cell-based sensors)

Patogenic microorganisms interact during the infection with mammalian cells – detection of cell or toxin by cell culture. The damage of cell culture is measured optically or electrically.

Bhunia, *Food Technol.* 65(2):38–43 (2011);
Identification of microorganisms by MALDI-TOF MS

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Definition of species in bacteria/archae

**The bacterial species definition**
- eminent practical significance
- but a very difficult issue
  - very wide and often changes in DNA
  - mutations during replication of DNA
  - horizontal gene transfer through mobile elements, plasmids, also between species

Under the same conditions some eucaryotic order would be a single bacterial species.

The criterion for a bacterial or archael species

**a collection of strains that have**

- at least one diagnostic phenotypic trait
- ≥70% DNA–DNA hybridization of their purified molecules DNA
  - ~more than 97% 16S rRNA sequence identity.
Identification of bacteria

PHENOTYPE METHODS
• Morphology of colony and cell (Gram staining, shape)
• Requirements for temperature, atmosphere, nutrition
• Biochemical tests - presence of specific enzymes
  • catalase, oxidase, fermentation of glucose
  • the way how they utilize different compounds
• Immunochemical tests - specific antigens in the cell wall

METHODS BASED ON PROTEIN ANALYSIS
• MALDI-TOF MS

METHODS BASED ON GENOTYPE
• looking for and comparing of specific DNA sequences
  • conserved universal genes (e.g. 16S rRNA)
  • genes specific for genus, species
• done by PCR methods, DNA chips, sequencing etc.
Identification based on phenotype

**Physiological + biochemical features:**  
**Example:** *Bacillus cereus*

![Image of Bacillus cereus colonies](http://www.napavalley.edu/people/srose/PublishingImages/Bacillus%20cereus%202(Gram%20stain).jpg)

- **G+ rod endspores forming**
- **β-haemolysis**
- **moisty and easy to grow colonies**
- **catalase positive**

![Image of MYP agar](http://www.oxoid.com/uk/blue/prod_detail/prod_detail.asp?pr=CM0929&c=uk&lang=EN&org=9&img=CM0929&sec=G+rod\%20endspores\%20forming\%20\%20\%20\%20\%20β-haemolysis\%20\%20moisty\%20and\%20easy\%20to\%20grow\%20colonies)

**MYP agar** (Mannitol Egg Yolk Polymyxin Agar) pink colonies with a precipitation zone (no fermentation of manitol + enzyme lecininase)

Set of biochemical tests:  
**Microgen® Bacillus ID Panel**  
for the identification of *Bacillus* spp. and related genera  
(time for performing: 48 hours)
Principle of biochemical testing

to rank the bacterium to some group having the same basic features
• e.g. catalase positive gram positive cocci
• Family *Enterobacteriaceae*

the identification on the genus or species level
• sets of biochemical tests specific for some group of bacteria
Resistance to ATB/haemolysis

Resistance e.g. to novobiocin

Hemolysis on blood agar

**alpha-hemolysis** - oxidizing the iron in the hemoglobin - green discoloration of the medium around

**beta-hemolysis** - rupture of erythrocytes - complete clearing medium around

**gamma-hemolysis** – no effect – no change

Measuring the diameter of inhibition zone (less than 17 mm resistant, more or equal to 17 mm sensitive) – differing in *Staphylococcus* sp. (Clinical performance)

Fermentation of carbohydrates

Type of utilisation of glucose or carbohydrates
• an important feature of the bacterial metabolism
• widely used in the identification process of bacteria
• **fermentation** strictly means to be under anaerobic conditions (but in bacterial metabolism, which can be but not have to be the same as the anaerobic conditions of cultivation), so very often is used in a general way like „utilisation“

• fermentation (like „utilisation“) of carbohydrates to
  • acid and gas (Durham tubes)
  • acid without gas
  • non-utilised

• Testing of fermentation or oxidation of carbohydrates
  • e.g. OF test for glucose
  • by oxidation under aerobic conditions
  • by fermentation under anaerobic conditions (an oil layer)
Fermentation of carbohydrates

Fermentation of sugars to acid

Peptone Water w/Phenol Red (HiMedia M028I)
Ingredients g/l
Peptic digest of animal tissue 10.000
Sodium chloride 5.000
Phenol red 0.020
Final pH (at 25°C) 6.8±0.2

+ after sterilisation at the room temperature to add aseptically sugar discs and inoculate:
succrose, trehalose, rhamnose, xylose

Incubation: 30 °C, 24 h
Test for oxidation/fermentation of glucose (O/F glucose test) detection of acid production by acidobazic indicator change to yellow (acid present) in lower pH from green (no acid). Anaerobic atmosph. – covering by sterile mineral oil. If fermented anaerobically, then also runs aerobically.
Biochemical identification of G - bacteria

G – bacteria
- Cocci
- Bacilli (Rods)
- Oxidase positive/negative
- Glucose metabolism
  - fermentation
  - oxidation
  - Inactive
- Lactose metabolism for coliform
- Pigment formation for *Pseudomonas* sp.
Biochemical tests - divisions

STAPHYtest 24
genus *Staphylococcus* isolated from clinical material and for their distinguishing from related genera of other gram positive, catalase positive cocci

STREPTOtest 24
*Streptococcus, Enterococcus* and related gram positive, catalase negative cocci isolated from clinical material

ENTEROtest 24
species of family *Enterobacteriaceae*

NEFERMtest 24
Gramnegative non-fermenting bacteria and families *Vibrionaceae, Aeromonadaceae* and *Pleiomonas shigelloides* present mainly in clinical material.

ANAEROtest 23
ANAEROBIC BACTERIA (G + AND G-)
routine identification of anaerobic bacteria (G + AND G-) in clinical material and in food
Biochemical tests

**API® Gram positive Identification**
API Staph – Overnight identification of clinical staphylococci and micrococci
API 20 Strep – 4 or 24-hour identification of streptococci and enterococci
API Coryne – 24-hour identification of *Corynebacterium* and coryne-like organisms
API Listeria – 24-hour identification of all *Listeria* species

**API® Gram negative Identification**
API 20E – Species/subspecies identification of *Enterobacteriaceae* and group/species identification of non-fermenting Gram negative bacteria
API Rapid 20E – 4-hour identification of *Enterobacteriaceae*
API 20NE – 24 to 48-hour identification of Gram negative *non-Enterobacteriaceae*
API Campy – 24-hour identification of *Campylobacter species*

**API® Anaerobe Identification**
API 20A – 24-hour identification of anaerobes
Rapid ID 32 A – 4-hour identification of anaerobes
Biochemical tests

MicrogenTM GN-ID
Convenient 12 substrate identification system for commonly encountered *Enterobacteriaceae* (GNA).
Extended 24 substrate identification system for the complete range of *Enterobacteriacea* and oxidase positive non-fastidious Gram negative bacilli (GNA+B).

MicrogenTM Listeria-ID
Enhanced built-in haemolysis test.

MicrogenTM Bacillus-ID
24 substrate system for the identification of mesophilic *Bacillus* spp. isolated from foods and related samples.

MicrogenTM Staph-ID
12 substrate system minimizes handling and manipulation.
Substrates selected specifically for Staphylococcus and related organisms.

MicrogenTM Strep-ID
Substrates selected specifically for *Streptococcus*, *Enterococcus* and related species. Provides excellent differentiation of Enterococci spp. especially *E. casseliflavus*, *E. mundtii* and *E. gallinarum*.
ENTEROtest 24 — example of biochemical testing

Routine identification of clinically important species of family Enterobacteriaceae. The test is focused only on some species often isolated in clinical practice. Strains from other sources can be slightly different in the dominant biochemical profiles.

Intended exclusively for the identification of family Enterobacteriaceae, the preliminary ranking into family Enterobacteriaceae must be done (Gram staining, oxidase, glucose fermentation).

The results of reactions obtained in this modified micromethod may differ from the results obtained using conventional tests and results available in literature sources. In case of problem, to control positive/negative reactions by control strains or to use for this tests tube variant to check the functionality of the kit not to check the accuracy or success of the identification!
Preparation of suspension from **over-night culture growing on non-selective agar** (for staphylococci Columbia agar blood with 5 % of sheep blood is recommended)

Inoculation of microtitre plates, anaerobic reaction covered by mineral oil

Incubation

Reading results by both visual and instrumental mode, respectively

Standard off-line tests
INDOLtest or COLItest

Other off-line tests
OXItest, OFtest, VPtest, Acetoin test
**ENTEROtest 24 – example of biochemical testing**

| Identification                  | IND | H | URE | G | ARG | ORN | F | LYS | H2S | SCI | MAL | ONP | H | SAL | G | SOR | MLB | E | CEL | LAC | TRE | MAN | GLR | H | DUL | G | ADO | F | ART | E | SUC | D | INO | C | RAF | B | ESL | A | bXY |
|--------------------------------|-----|---|-----|---|-----|-----|---|-----|-----|-----|-----|-----|---|-----|---|-----|-----|---|-----|-----|-----|-----|-----|---|-----|---|-----|---|-----|---|-----|---|-----|---|-----|---|
| Yersinia aldovae               | -   | d |     |   |     |   |   |     |     |     |     |     |   | d   |   |     |     |   |     |     |     |     |     |   | d   |   |     |   |     |   |     |   |     |   |     |   |     |   |
| Yersinia bercovieri            | -   | d |     |   |     |   |   |     |     |     |     |     |   | d   | (+) |     |     |   |     |     |     |     |     |   |   |     |   |     |   |     |   |     |   |     |   |     |   |     |   |
| Yersinia enterocolitica ssp. enterocolitica | d | d |     |   |     |   |   |     |     |     |     |     |   | d   |     | (+) |     |   |     |     |     |     |     |   | d   | d | (+) | d |     |   |     |   |     |   |     |   |     |   |     |   |
| Yersinia frederiksenii         | +   | d |     |   |     | (-) |   |   |     |     |     |     |   | d   |     |     | (+) |   |     |     |     |     |     |   | d   | d | (+) | d |     |   |     |   |     |   |     |   |     |   |     |   |
| Yersinia intermedia            | +   | (+) |     |   |     |   |   |     |     |     |     |     |   | (+) |     |     | (+) |   |     |     |     |     |     |   | d   |     |     |   |     |   |     |   |     |   |     |   |     |   |     |   |
| Yersinia kristensenii          | d   | d |     |   |     |   |   |     |     |     |     |     |   | d   |     | (-) |     |   |     |     |     |     |     |   | (-) | d |     |   |     |   |     |   |     |   |     |   |     |   |     |   |
| Yersinia mollaretii            | -   | d |     |   |     |   |   |     |     |     |     |     |   | d   | (-) |     |     |   |     |     |     |     |     |   | d   |     |     |   |     |   |     |   |     |   |     |   |     |   |
| Yersinia pestis                |     |     |     |     |     |     | d | d   | d |     | d |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |   |     |   |     |   |     |   |     |   |     |   |
| Yersinia pseudotuberculosis    |     |     |     |     |     |     | d | d   | d |     | d |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |   |     |   |     |   |     |   |     |   |     |   |
| Yersinia rohdei                |     |     |     |     |     |     | d | d   | d |     | d |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |   |     |   |     |   |     |   |     |   |     |   |
| Yersinia ruckeri               |     |     |     |     |     |     | d | d   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |   |     |   |     |   |     |   |     |   |     |   |

**Explanations:**
- + = 90–100% of positive reactions
- d = 26–74% of positive reactions
- (±) = 75–89% of positive reactions
- = 75–89% of positive reactions
- = negativní reakce
- = orientation identification

**Performance of the kit:**
The kit was tested on a set of 97 strains, including INDOLtest a VPTest:
The identification of 93% of the strains was correct.
The identification of 7% of the strains was to the genus level.
ENTEROtest 24 – example of biochemical testing

The way of identification:
the obtained profile is compared with profiles in database
• The most similar profiles are found
  • Identification score = % of similarity
• But what about the different reactions?
  • T-index is calculated
    • how is the probability of the different reactions?
      • It depends on their importance – typical reactions should be the same
• Reliability of the result
  • the combination of identification score and T-index =
  • Excellent, very good, good, acceptable, intermediary strain, needs some additional tests, genus level, non-existing taxon

| Identification            | IND | HRE | ARG | FON | ENS | DSS | SCI | MAL | ONP | HSA | GRO | MLB | CEL | DCA | TRE | MAN | GLR | DUL | ADO | FER | ERE | SUC | INO | RAF | ESL | bXY |
|---------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Cronobacter sakazakii    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |    |
| (-)                       |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |    |

Explanations:
+ = 90–100% of positive reactions
d = 26–74% of positive reactions
(-) = 11–25% of positive reactions
(+) = 75–89% of positive reactions
* = orientation identification
MALDI-TOF MS

1) sample preparation
- microbial culture or its proteins extract is smearing onto a steel plate and covered over by matrix
- matrix enables the sample (A) to be desorped and ionised as pseudomolecule ionts [A+H] +

2) MALDI-TOF MS analysis
unique mass spectral fingerprint of desorbed microbial cell components (mainly intracellular proteins), different among genera, species or also some strains

3) identification:
comparison of mass spectrum to those of reference strains in database

Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry

http://pubs.rsc.org/en/content/articlehtml/2014/RA/C4RA05604C
http://cmr.asm.org/content/26/3/547.figures-only
**MALDI-TOF MS: PRINCIPLE**

**MALDI (Matrix Assisted Laser Desorption Ionization)**

1. Irradiation
2. Desorption
3. Desolvation & Ionization

![Diagram of MALDI-TOF MS principle](image)

**Soft ionisation method:**
- low level of sample fragmentation

**Time of flight** is a function of the specific ion mass (m/z)

\[
\frac{m}{z} = 2eU \frac{t^2}{L^2}
\]

- *m* mass, *z* charge,
- *L* length of drifting zone, *e* elementary charge, *U* speeding voltage


MALDI-TOF MS: SAMPLE AND MATRIX

Sample preparation
Direct transfer
- intact cells spread directly onto a steel plate
- lysis of cells occurs during the contact with acid matrix and by laser desorption
- most bacteria

Proteins extraction
- previous extraction of proteins by organic acids and/or alcohol (e.g. ethanol and 70% formic acid)
- yeasts, moulds, some species of bacteria (depending on the cell wall composition)

Matrix:
- able to absorb the energy of the laser (usually 337 nm)
- to crystallise with samples - usually acid character (proton ionisation of sample), dissolved in organic solvent

CHC: α-Cyano-4-hydroxycinnamic acid
(organic solvent: 50% acetonitrile with 2,5 % trifluoroacetic acid)

SA : 3,5-Dimethoxy-4-hydroxycinnamic acid (sinapic acid)
DHB: 2,5-Dihydroxybenzoic acid

Matrix:
- able to absorb the energy of the laser (usually 337 nm)
- to crystalise with samples - usually acid character (proton ionisation of sample), dissolved in organic solvent

http://cmr.asm.org/content/26/3/547.figures-only
MALDI-TOF MS: MICROORGANISMS

Mass spectrum protein profile
z equals usually to 1+ (so m/z usually corresponds to molecule mass)
the range usually used for identification: 2000 -20 000 m/z
the intensity of single peaks corresponds to the abundance of the protein
Which proteins dominates in the protein profile ?
abundant, basic and mediumly hydrophobic
: mainly ribosomal proteins
: further cold-shock and heat-shock proteins, chaperons etc.

Analysis is recomended (and validated) to be performed from colonies grown onto non-selective agar

Bacillus cereus

Visualisation of mass spectrum protein profile – (software mMass 5, Strohalm et al., 2010)
MALDI-TOF MS: ANALYSIS

Comparison of mass spectrum protein profile of unknown sample with these of reference strains present in database by software

BioTyper:
The statistical analysis for correlation includes peak positions, intensities and frequencies across the complete range of microorganisms. Score value:
0 (none similarity) - 1000 (absolute similarity) But it is expressed in decadic logarithm

\[ \log(\text{score value}) : 0-3 \]

<table>
<thead>
<tr>
<th>Range</th>
<th>Description</th>
<th>Symbols</th>
<th>Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.300 ... 3.000</td>
<td>highly probable species identification</td>
<td>( +++ )</td>
<td>green</td>
</tr>
<tr>
<td>2.000 ... 2.299</td>
<td>secure genus identification, probable species identification</td>
<td>( ++ )</td>
<td>green</td>
</tr>
<tr>
<td>1.700 ... 1.999</td>
<td>probable genus identification</td>
<td>( + )</td>
<td>yellow</td>
</tr>
<tr>
<td>0.000 ... 1.699</td>
<td>not reliable identification</td>
<td>( - )</td>
<td>red</td>
</tr>
</tbody>
</table>

Commercial databases from different MALDI-TOF MS producers

**Bruker Daltonics** – MALDI BIOTYPER

**Shimadzu** - Shimadzu Launchpad software + SARAMIS database

**Biomérieux** - VITEK® MS

Other databases compatible with different hardware systems (e.g. Andromas)
Bacillus cereus

<table>
<thead>
<tr>
<th>Rank (Quality)</th>
<th>Matched Pattern</th>
<th>Score Value</th>
<th>NCBI Identifier</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (+++</td>
<td>Bacillus cereus DSM 31T DSM</td>
<td>2.554</td>
<td>1396</td>
</tr>
<tr>
<td>2 (++</td>
<td>Bacillus cereus 994000168 LBK</td>
<td>2.203</td>
<td>1396</td>
</tr>
<tr>
<td>3 (++</td>
<td>Bacillus weihenstephanensis DSM 11821T DSM</td>
<td>2.158</td>
<td>86662</td>
</tr>
<tr>
<td>4 (++)</td>
<td>Bacillus mycoides DSM 2048T DSM</td>
<td>2.155</td>
<td>1405</td>
</tr>
<tr>
<td>5 (++)</td>
<td>Bacillus cereus 4080 LBK</td>
<td>2.147</td>
<td>1396</td>
</tr>
<tr>
<td>6 (++)</td>
<td>Bacillus thuringiensis DSM 2046T DSM</td>
<td>1.975</td>
<td>1428</td>
</tr>
<tr>
<td>7 (+)</td>
<td>Bacillus pseudomycoides DSM 12442T DSM</td>
<td>1.787</td>
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<tr>
<td>8 (+)</td>
<td>Bacillus bataviensis DSM 15601T DSM</td>
<td>1.369</td>
<td>220685</td>
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<tr>
<td>9 (-)</td>
<td>Brevibacterium linens IMET 11075T HKJ</td>
<td>1.347</td>
<td>1703</td>
</tr>
<tr>
<td>10 (-)</td>
<td>Acinetobacter towneri DSM 14962T HAM</td>
<td>1.345</td>
<td>202956</td>
</tr>
</tbody>
</table>

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<td>0.000 ... 1.699</td>
<td>not reliable identification</td>
<td>(-)</td>
<td>red</td>
</tr>
</tbody>
</table>

Bacillus cereus
MALDI-TOF MS - PROCEDURE

Direct method: smearing sample in four parallels in lower and higher cells concentrations – after drying to cover over by matrix (1-2 μl) and let to crystallise at room temperature

Matrix: solution of α-Cyano-4-hydroxycinnamic acid (10 mg/ml) in 50% acetonitrile with 2,5 % trifluoroacetic acid (prepared with 10% TFA solution)

Protein standard (1 μl): Bruker Bacterial Test Standard (Bruker Daltonics, SRN) – proteins extracted from z Escherichia coli DH5alpha BRL + some others

Equipment
Bruker Autoflex Speed
Database MALDI Biotyper 3.1
MALDI-TOF MS: PROTEIN STANDARD

Bruker Bacterial Test Standard (referred to as 'Bruker BTS') contains a carefully manufactured extract of *Escherichia coli* DH5 alpha that shows a characteristic peptide and protein profile in MALDI-TOF mass spectra. The extract is spiked with two additional proteins that extend the upper boundary of the mass range covered by Bruker BTS. The overall mass range covered by Bruker BTS is 3.6 to 17 kDa.

**Table 1: Calibration points with a mass tolerance limit of ±300 ppm**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Reference mass (average mass)</th>
<th>± 300 ppm range</th>
</tr>
</thead>
<tbody>
<tr>
<td>RL29 [M+2H]²⁺</td>
<td>3637.8 Da</td>
<td>3636.7 Da – 3638.8 Da</td>
</tr>
<tr>
<td>RS32 [M+H]⁺</td>
<td>5096.8 Da</td>
<td>5095.3 Da – 5098.3 Da</td>
</tr>
<tr>
<td>RS34 [M+H]⁺</td>
<td>5381.4 Da</td>
<td>5379.8 Da – 5383.0 Da</td>
</tr>
<tr>
<td>RS33meth [M+H]⁺</td>
<td>6255.4 Da</td>
<td>6253.5 Da – 6257.3 Da</td>
</tr>
<tr>
<td>RL29 [M+H]⁺</td>
<td>7274.5 Da</td>
<td>7272.3 Da – 7276.7 Da</td>
</tr>
<tr>
<td>RS19 [M+H]⁺</td>
<td>10300.1 Da</td>
<td>10297.0 Da – 10303.2 Da</td>
</tr>
<tr>
<td>RNAse A [M+H]⁺</td>
<td>13683.2 Da</td>
<td>13679.1 Da – 13687.3 Da</td>
</tr>
<tr>
<td>Myoglobin [M+H]⁺</td>
<td>16952.3 Da</td>
<td>16947.2 Da – 16957.4 Da</td>
</tr>
</tbody>
</table>

**Analyte name**  | **Rank (Quality)** | **Matched Pattern** | **Score Value**
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>F4</td>
<td>1 (+++ )</td>
<td><em>Escherichia coli</em> DH5alpha BRL</td>
<td>2.439</td>
</tr>
</tbody>
</table>
EU legislative for food-borne pathogens

Sabina Purkrtová
European Food Law

Reg.(EC) No 178/2002
European Food Law

Food Hygiene
Reg.(EC) No 852/2004
Reg.(EC) No 853/2004
Reg.(EC) No 854/2004
Reg.(EC) No 882/2004

Organic / Origin
Reg.(EC) No 834/2007
Reg.(EC) No 510/2006
Reg.(EC) No 1898/2006

Food Contact Materials
Reg.(EC) No 1935/2004
Reg.(EC) No 2023/2006
Directives (approx. 10)

Feed
Reg.(EC) No 322/2003
Reg.(EC) No 183/2005
Reg.(EC) No 1812/2005

GMO
Reg.(EC) No 1829/2003
Reg.(EC) No 1830/2003

Labelling
Dir. 2000/13 EC et al
Reg.(EC) No 1924/2006

Food Additives
Num. Reg.

Residues / Contaminants
Reg.(EC) No 1441/2007
Reg.(EC) No 1881/2006

COMMISSION REGULATION (EC) No 2073/2005

of 15 November 2005

on microbiological criteria for foodstuffs

(Text with EEA relevance)

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Community,

Having regard to Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of foodstuffs (1), and in particular Articles 4(4) and 12 thereof,
Presumption:
Microbiological hazards in foodstuffs form a major source of food-borne diseases in humans.

Aim:
Foodstuffs should not contain micro-organisms or their toxins or metabolites in quantities that present an unacceptable risk for human health.

Method:
The safety of foodstuffs is mainly ensured by a preventive approach, such as implementation of good hygiene practice and application of procedures based on hazard analysis and critical control point (HACCP) principles.
According to Article 4 of Regulation (EC) No 852/2004, food business operators are to comply with microbiological criteria.

Criteria:
The Communication from the Commission on the Community Strategy for setting microbiological criteria for foodstuffs describes the strategy to lay down and revise the criteria in Community legislation, as well as the principles for the development and application of the criteria.

The Scientific Committee on Veterinary Measures relating to Public Health (SCVPH) issued an opinion on 23 September 1999 on the evaluation of microbiological criteria for food products of animal origin for human consumption. It highlighted the relevance of basing microbiological criteria on formal risk assessment and internationally approved principles.
The European Food Safety Authority (EFSA) is the keystone of European Union (EU) risk assessment regarding food and feed safety. In close collaboration with national authorities and in open consultation with its stakeholders, EFSA provides independent scientific advice and clear communication on existing and emerging risks. EFSA is an independent European agency funded by the EU budget that operates separately from the European Commission, European Parliament and EU Member States.

the Codex Alimentarius guideline
HACCP

**Table 1. HACCP Principles**

<table>
<thead>
<tr>
<th>Principle</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Principle 1</td>
<td>Conduct a hazard analysis.</td>
</tr>
<tr>
<td>Principle 2</td>
<td>Determine the critical control points.</td>
</tr>
<tr>
<td>Principle 3</td>
<td>Establish critical limits.</td>
</tr>
<tr>
<td>Principle 4</td>
<td>Establish monitoring procedures.</td>
</tr>
<tr>
<td>Principle 5</td>
<td>Establish corrective actions.</td>
</tr>
<tr>
<td>Principle 6</td>
<td>Establish verification procedures.</td>
</tr>
<tr>
<td>Principle 7</td>
<td>Establish record-keeping and documentation procedures.</td>
</tr>
</tbody>
</table>

**Table 2. Global Food Safety Initiative Recognized Schemes**

<table>
<thead>
<tr>
<th>Scheme</th>
<th>Description</th>
<th>Website</th>
</tr>
</thead>
<tbody>
<tr>
<td>British Retail Consortium (BRC) Global Standard Version 5</td>
<td></td>
<td><a href="http://www.brcglobalstandards.com">www.brcglobalstandards.com</a></td>
</tr>
<tr>
<td>Dutch HACCP (Option B)</td>
<td></td>
<td><a href="http://www.foodsafetymanagement.info">www.foodsafetymanagement.info</a></td>
</tr>
<tr>
<td>Food Safety System Certification (FSSC) 22000 (conditional recognition)</td>
<td></td>
<td><a href="http://www.fssc22000.com">www.fssc22000.com</a></td>
</tr>
<tr>
<td>International Food Standard Version 5</td>
<td></td>
<td><a href="http://www.ifs-certification.com">www.ifs-certification.com</a></td>
</tr>
<tr>
<td>Safe Quality Foods (SQF) 2000 Level 2</td>
<td></td>
<td><a href="http://www.sqf.com">www.sqf.com</a></td>
</tr>
</tbody>
</table>

Application of HACCP

Controlled/Regulated by:
- BCMOH
- CFIA
- BCMAL
- Consumer
- Health Authority

Hazard Types
- B - Biological
- C - Chemical
- P - Physical

Red: where potential hazards are present
Green: where potential hazards can be controlled
Application of HACCP

Form 3: Process Flow Diagram
1. FOOD SAFETY CRITERIA
‘food safety criterion’ means a criterion defining the acceptability of a product or a batch of foodstuff applicable to products placed on the market;

2. PROCESS HYGIENE CRITERIA
‘process hygiene criterion’ a criterion indicating the acceptable functioning of the production process. Such a criterion is not applicable to products placed on the market. It sets an indicative contamination value above which corrective actions are required in order to maintain the hygiene of the process in compliance with food law;

3. Rules for sampling and preparation of test samples


<table>
<thead>
<tr>
<th>Food category</th>
<th>Stage where the criterion applies</th>
<th>Analytical reference method:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sampling plan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = number of units comprising the sample;</td>
<td></td>
<td>Limits:</td>
</tr>
<tr>
<td>c = number of sample units giving values between m and M</td>
<td></td>
<td>- presence/absence</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- m/M</td>
</tr>
</tbody>
</table>
(10) The SCVPH issued at the same time a separate opinion on *Listeria monocytogenes*. That opinion recommended that it be an objective to keep the concentration of *Listeria monocytogenes* in food below 100 cfu/g. The Scientific Committee on Food (SCF) agreed with these recommendations in its opinion of 22 June 2000.

**Risk analysis:**
Although listeriosis is rare, the disease is often severe with high hospitalisation and mortality rates. In the EU about 1,470 human cases were reported in 2011, with a mortality rate of 12.7%.
Listeriosis is usually contracted by eating foods that contain the *Listeria monocytogenes* bacteria such as fish, cold meats and soft cheeses. Ready-to-eat foods are often the source of listeria infections as their long shelf life is conducive to bacterial growth and these foods are usually consumed without any additional cooking.

Ready-to-eat foods are foods intended by the producer for direct consumption without the need for cooking or other processing.
## 2073/2005 – *Listeria monocytogenes*

### Chapter 1. Food safety criteria

<table>
<thead>
<tr>
<th>Food category</th>
<th>Micro-organisms/their toxins, metabolites</th>
<th>Sampling-plan ((n), (c), (m), (M))</th>
<th>Limits ((^{(1)}))</th>
<th>Analytical reference method ((^{(2)}))</th>
<th>Stage where the criterion applies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ready-to-eat foods intended for infants and ready-to-eat foods for special medical purposes (^{(4)})</td>
<td><em>Listeria monocytogenes</em></td>
<td>10, 0, 0, 0</td>
<td>Absence in 25 g</td>
<td>EN/ISO 11290-1</td>
<td>Products placed on the market during their shelf-life</td>
</tr>
<tr>
<td>Ready-to-eat foods able to support the growth of <em>L. monocytogenes</em>, other than those intended for infants and for special medical purposes</td>
<td><em>Listeria monocytogenes</em></td>
<td>5, 0, 100 cfu/g (^{(3)})</td>
<td>EN/ISO 11290-2 (^{(6)})</td>
<td>Before the food has left the immediate control of the food business operator, who has produced it</td>
<td></td>
</tr>
<tr>
<td>Ready-to-eat foods unable to support the growth of <em>L. monocytogenes</em>, other than those intended for infants and for special medical purposes (^{(4)}) (^{(5)})</td>
<td><em>Listeria monocytogenes</em></td>
<td>5, 0, 100 cfu/g (^{(3)})</td>
<td>EN/ISO 11290-2 (^{(6)})</td>
<td>Products placed on the market during their shelf-life</td>
<td></td>
</tr>
</tbody>
</table>

- Ready-to-eat deli meats and hot dogs
- Refrigerated pâtés or meat spreads
- Unpasteurized (raw) milk and dairy products
- Soft cheese made with unpasteurized milk, such as queso fresco, Feta, Brie, Camembert
- Refrigerated smoked seafood
- Raw sprouts
- Smoked salmone etc..

**Risk population**
- Pregnant women
- Elderly people
- Immunosuppressed people
- Children
(12) The SCVPH issued an opinion on Norwalk-like viruses (NLVs, noroviruses) on 30-31 January 2002. In that opinion it concluded that the conventional faecal indicators are unreliable for demonstrating the presence or absence of NLVs and that the reliance on faecal bacterial indicator removal for determining shellfish purification times is unsafe practice. It also recommended using *E. coli* rather than faecal coliforms to indicate faecal contamination in shellfish harvesting areas, when applying bacterial indicators.

<table>
<thead>
<tr>
<th>n = 1, c=0</th>
<th>230 MPN/100g of flesh and intravalvular liquid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live bivalve molluscs and live echinoderms, tunicates and gastropods</td>
<td></td>
</tr>
<tr>
<td>ISO TS 16649-3</td>
<td><em>E. coli</em></td>
</tr>
<tr>
<td>Minced meat</td>
<td>ABS. in 10 g (from 1.1.2006)</td>
</tr>
<tr>
<td>------------------------------------------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>Minced meat and meat preparations intended to be eaten raw</td>
<td>25 g</td>
</tr>
<tr>
<td>Minced meat and meat preparations made from poultry meat intended to be eaten cooked</td>
<td></td>
</tr>
<tr>
<td>Meat products made from poultry meat intended to be eaten cooked</td>
<td></td>
</tr>
<tr>
<td>Minced meat and meat preparations made from other species than poultry intended to be eaten cooked</td>
<td>10 g</td>
</tr>
<tr>
<td>Mechanically separated meat (MSM)</td>
<td>10 g</td>
</tr>
<tr>
<td>Meat products intended to be eaten raw, excluding products where the manufacturing process or the composition of the product will eliminate the salmonella risk</td>
<td>25 g</td>
</tr>
<tr>
<td>Gelatine and collagen</td>
<td>25 g</td>
</tr>
</tbody>
</table>

**ABS** = absent
# Food safety criteria - *Salmonella*

<table>
<thead>
<tr>
<th>Minced meat (n=5, c=0)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cheeses, butter and cream made from raw milk or milk that has undergone a lower heat treatment than pasteurisation</td>
<td>ABS. in 25 g</td>
</tr>
<tr>
<td>Milk powder and whey powder</td>
<td></td>
</tr>
<tr>
<td>Ice cream, excluding products where the manufacturing process or the composition of the product will eliminate the salmonella risk</td>
<td></td>
</tr>
<tr>
<td>Egg products, excluding products where the manufacturing process or the composition of the product will eliminate the salmonella risk</td>
<td></td>
</tr>
<tr>
<td>Ready-to-eat foods containing raw egg, excluding products where the manufacturing process or the composition of the product will eliminate the salmonella risk</td>
<td>ABS. in 25 g or 25 ml</td>
</tr>
</tbody>
</table>

ABS = absent
### Food safety criteria- *Salmonella*

<table>
<thead>
<tr>
<th>Minced meat</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cooked crustaceans and molluscan shellfish</td>
<td>ABS. in 25 g</td>
</tr>
<tr>
<td>Live bivalve molluscs and live echinoderms, tunicates and gastropods</td>
<td>ABS. in 10 g (from 1.1.2006)</td>
</tr>
<tr>
<td>Sprouted seeds (ready-to-eat)</td>
<td>ABS. in 25 g (from 1.1.2010)</td>
</tr>
<tr>
<td>Pre-cut fruit and vegetables (ready-to-eat)</td>
<td>ABS. in 10 g</td>
</tr>
<tr>
<td>Unpasteurised fruit and vegetable juices (ready-to-eat)</td>
<td>ABS. in 10 g</td>
</tr>
</tbody>
</table>

**ABS = absent**

Analytical reference method for *Salmonella* spp.: EN/ISO 6579  
**Microbiology of food and animal feeding stuffs –**  
Horizontal method for the detection of *Salmonella* spp.
<table>
<thead>
<tr>
<th>1.28 Fresh poultry meat</th>
<th>( ^{10} ) Salmonella typhimurium ( ^{21} )</th>
<th>5</th>
<th>0</th>
<th>EN/ISO 6579 (for detection) White-Kauffmann-Le Minor scheme (for serotyping)</th>
<th>Products placed on the market during their shelf-life</th>
</tr>
</thead>
<tbody>
<tr>
<td>( ^{10} ) Salmonella enteritidis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^{10}\) This criterion shall apply to fresh meat from breeding flocks of \textit{Gallus gallus}, laying hens, broilers and breeding and fattening flocks of turkeys.

\(^{21}\) As regards monophasic \textit{Salmonella typhimurium} only 1,4,5,12:i:- is included.

According to the Community Summary Report on trends and sources of zoonoses, and zoonotic agents and food-borne outbreaks in the European Union in 2008 \(^{5}\) by the European Food Safety Authority approximately 80% of human salmonellosis cases are caused by \textit{Salmonella enteritidis} and \textit{Salmonella typhimurium} which is similar to preceding years. Poultry meat remains a major source of human salmonellosis.

Setting a criterion for \textit{Salmonella enteritidis} and \textit{Salmonella typhimurium} would provide the best balance between reducing human salmonellosis attributed to the consumption of poultry meat and the economic consequences of the application of that criterion. At the same time, it would encourage food business operators to take measures at previous stages of poultry production that may contribute to the reduction of all serotypes of salmonella with public health significance. Focusing on those two serotypes would also be consistent with the Union targets set for primary production of poultry.
Food safety criteria – *Salmonella* + **Enterobacter sakazakii**

<table>
<thead>
<tr>
<th>Infant formula</th>
<th>n = 30, c=0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dried infant formulae and dried dietary foods for special medical purposes intended for infants below six months of age, as referred to in the <em>Enterobacteriaceae</em> criterion in Chapter 2.2 of this Annex</td>
<td></td>
</tr>
<tr>
<td><strong>Salmonella</strong> - EN/ISO 6579</td>
<td>ABS. in 25 g</td>
</tr>
</tbody>
</table>

Analytical reference method for *Enterobacter sakazakii*:
Milk and milk products -- Detection of *Enterobacter sakazakii*

Note: According by 365/2010 due change in taxonomy *Enterobacter sakazakii* should be changed to *Cronobacter* spp.
(8) Given a recent change in taxonomy the name of Enterobacter sakazakii in Regulation (EC) No 2073/2005 should be changed to Cronobacter spp. (Enterobacter sakazakii).

The analytical reference method set out for Enterobacteriaceae in pasteurised milk and other pasteurised liquid dairy products ISO 21528-1 has been shown to be difficult to use for routine analyses in own checks since it is very laborious and time consuming. Due to the methodological development the analytical reference method of Enterobacteriaceae in pasteurised milk and other pasteurised liquid dairy products should be changed to ISO 21528-2 which is quicker and easier to perform.
(5) On 20 October 2011 the European Food Safety Authority (‘EFSA’) adopted a Scientific Opinion on the risk posed by Shiga toxin-producing *Escherichia coli* and other pathogenic bacteria in seeds and sprouted seeds (3). In its Opinion, EFSA concludes that the contamination of dry seeds with bacterial pathogens is the most likely initial source of the sprout-associated outbreaks. In addition, the Opinion states that, due to the high humidity and the favourable temperature during sprouting, bacterial pathogens present on dry seeds can multiply during sprouting and result in a public health risk.

ISO/TS 13136:2012
Microbiology of food and animal feed - - Real-time polymerase chain reaction (PCR)-based method for the detection of food-borne pathogens -- Horizontal method for the detection of Shiga toxin-producing *Escherichia coli* (STEC) and the determination of O157, O111, O26, O103 and O145 serogroups
Food safety criteria: Histamine

<table>
<thead>
<tr>
<th>Analytical reference Method: HPLC</th>
<th>Sampling plans</th>
<th>Limits (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Products placed on the market during their shelflife</td>
<td>n</td>
<td>c</td>
</tr>
<tr>
<td>Fishery products from fish species associated with a high amount of histidine</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>Fishery products which have undergone enzyme maturation treatment in brine, manufactured from fish species associated with a high amount of histidine</td>
<td>9</td>
<td>2</td>
</tr>
</tbody>
</table>

Histamine in fishery products from fish species *(biogenic amine - part of an immune response to foreign pathogens)* associated with a high amount of histidine:
— satisfactory, if the following requirements are fulfilled:
1. the mean value observed is ≤ m
2. a maximum of c/n values observed are between m and M
3. no values observed exceed the limit of M,
— unsatisfactory, if the mean value observed exceeds m or more than c/n values are between m and M or one or more of the values observed are >M.
### Food safety criteria

#### Staphylococcal enterotoxins

<table>
<thead>
<tr>
<th>European screening method of the CRL (Community reference laboratories) for Milk and Milk products</th>
<th>Sampling plans</th>
<th>Limits (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Products placed on the market during their shelflife</td>
<td>n</td>
<td>c</td>
</tr>
<tr>
<td>Cheeses, milk powder and whey powder, as referred to in the coagulase-positive staphylococci criteria in Chapter 2.2 of this Annex (2.2. Process hygiene criteria for milk and dairy products)</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

**Based on immunodetection**

1. Extraction/concentration of enterotoxins: The sample is mixed and homogenised with distilled water. The toxins diffuse in water and are recovered, after two centrifugations, in the supernatant. This aqueous phase is concentrated overnight by dialysis.

2. Immuno-enzymatic detection (Vidas SET2 or Ridascreen SET Total)

The Vidas SET2 detection is based on an Enzyme Linked Fluorescent Assay (ELFA) test.

The Ridascreen SET Total is a sandwich type enzyme immunoassay (ELISA) for combined detection of the Staphylococcus enterotoxins (SET) types A, B, C, D and E. The surface of the microtiter plate is coated with specific, purified antibodies which can bind the enterotoxins contained in a food sample.
## Process hygiene criteria: Milk and dairy products

### Milk powder and whey powder
(The criterion does not apply to products intended for further processing in the food industry)

<table>
<thead>
<tr>
<th>Enterobacteriaceae ISO 21528-2</th>
<th>n</th>
<th>c</th>
<th>m (cfu/g)</th>
<th>M (cfu/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>End of the manufacturing process</td>
<td>5</td>
<td>0</td>
<td>10 (m=M)</td>
<td></td>
</tr>
</tbody>
</table>

Action in case of unsatisfactory results: Improvements in production hygiene.

<table>
<thead>
<tr>
<th>Coagulase-positive staphylococci EN/ISO 6888-1 or 2</th>
<th>n</th>
<th>c</th>
<th>m (cfu/g)</th>
<th>M (cfu/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>2</td>
<td>10</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

Action in case of unsatisfactory results: Improvements in production hygiene. If values > 10⁵ cfu/g are detected, the batch has to be tested for staphylococcal enterotoxins.

E. coli, Enterobacteriaceae (other food categories) and coagulase-positive staphylococci:
— satisfactory, if all the values observed are ≤ m,
— acceptable, if a maximum of c/n values are between m and M, and the rest of the values observed are ≤ m,
— unsatisfactory, if one or more of the values observed are >M or more than c/n values are between m and M.
**Process hygiene criteria: Meat and products thereof**

<table>
<thead>
<tr>
<th>Aerobic colony count</th>
<th>ISO 4833:2013</th>
<th>m</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcases after dressing but before chilling</td>
<td>Log cfu/cm² Daily mean log</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carcases of cattle, sheep, goats and horses</td>
<td>3,5</td>
<td>5,0</td>
<td></td>
</tr>
<tr>
<td>Carcases of pigs</td>
<td>4,0</td>
<td>5,0</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><em>Enterobacteriaceae</em></th>
<th>ISO 21528-2:2004</th>
<th>m</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcases after dressing but before chilling</td>
<td>Log cfu/cm² Daily mean log</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carcases of cattle, sheep, goats and horses</td>
<td>3,5</td>
<td>5,0</td>
<td></td>
</tr>
<tr>
<td>Carcases of pigs</td>
<td>2,0</td>
<td>3,0</td>
<td></td>
</tr>
</tbody>
</table>

Action in case of unsatisfactory results: **Improvements in slaughter hygiene and review of controls**

The limits (m and M) apply only to samples taken by the destructive method. The daily mean log is calculated by first taking a log value of each individual test result and then calculating the mean of these log values.
### Process hygiene criteria: Meat and products thereof

<table>
<thead>
<tr>
<th>Salmonella</th>
<th>EN/ISO 6579</th>
<th>n</th>
<th>c</th>
<th>m</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcases after dressing but before chilling</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carcases of cattle, sheep, goats and horses</td>
<td>50</td>
<td>2</td>
<td></td>
<td></td>
<td>Absence in the area tested per carcase</td>
</tr>
<tr>
<td>Carcases of pigs</td>
<td>50</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carcases after chilling</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poultry carcases of broilers and turkeys</td>
<td>50</td>
<td>7</td>
<td></td>
<td></td>
<td>Absence in 25 g of a pooled sample of neck skin</td>
</tr>
</tbody>
</table>

Action in case of unsatisfactory results: Improvements in slaughter hygiene and review of process controls, origin of animals and of the biosecurity measures in the farms of origin

The 50 samples are derived from 10 consecutive sampling sessions in accordance with the sampling rules and frequencies laid down in this Regulation. The number of samples where the presence of salmonella is detected. The c value is subject to review in order to take into account the progress made in reducing the salmonella prevalence. Member States or regions having low salmonella prevalence may use lower c values even before the review.
### Process hygiene criteria: Meat and products thereof

<table>
<thead>
<tr>
<th>Aerobic colony count (7)</th>
<th>ISO 4833</th>
<th>n</th>
<th>c</th>
<th>m</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>End of the manufacturing process</td>
<td>cfu/g</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minced meat</td>
<td>5</td>
<td>2</td>
<td>5x10⁵</td>
<td>5x10⁶</td>
<td></td>
</tr>
<tr>
<td>Mechanically separated meat (MSM)</td>
<td>5</td>
<td>2</td>
<td>5x10⁵</td>
<td>5x10⁶</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>E. coli (8)</th>
<th>ISO 16649-1 or 2</th>
<th>n</th>
<th>c</th>
<th>m</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>End of the manufacturing process</td>
<td>cfu/g</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minced meat</td>
<td>5</td>
<td>2</td>
<td>50</td>
<td>500</td>
<td></td>
</tr>
<tr>
<td>Mechanically separated meat (MSM)</td>
<td>5</td>
<td>2</td>
<td>50</td>
<td>500</td>
<td></td>
</tr>
<tr>
<td>Meat preparations</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Action in case of unsatisfactory results: Improvements in production hygiene and improvements in selection and/or origin of raw materials

(7) This criterion does not apply to minced meat produced at retail level when the shelf-life of the product is less than 24 hours.

(8) *E. coli* is used here as an indicator of faecal contamination.
Process hygiene criteria: Milk and dairy products

<table>
<thead>
<tr>
<th><strong>Enterobacteriaceae</strong></th>
<th>ISO 21528-1</th>
<th>n</th>
<th>c</th>
<th>m</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>End of the manufacturing process</td>
<td></td>
<td></td>
<td></td>
<td>cfu/ml</td>
<td></td>
</tr>
<tr>
<td>Pasteurised milk and other pasteurised liquid dairy products</td>
<td>5</td>
<td>2</td>
<td>▶ C1 &lt;1/ml ◄</td>
<td>▶ C1 5/ml ◄</td>
<td></td>
</tr>
</tbody>
</table>

Action in case of unsatisfactory results: Check on the efficiency of heat- treatment and prevention of recontamination as well as the quality of raw materials

(4) The criterion does not apply to products intended for further processing in the food industry.

ISO 21528-1:2004
Microbiology of food and animal feeding stuffs -- Horizontal methods for the detection and enumeration of Enterobacteriaceae -- Part 1: Detection and enumeration by MPN technique with pre-enrichment
### Process hygiene criteria: Milk and dairy products

<table>
<thead>
<tr>
<th><strong>E. coli</strong></th>
<th>ISO 16649-1,2:2001</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cheeses made from milk or whey that has undergone heat treatment</strong></td>
<td>5</td>
</tr>
</tbody>
</table>
| At the time during the manufacturing process when the *E. coli* count is expected to be highest  
For cheeses which are not able to support the growth of *E. coli*, the *E. coli* count is usually the highest at the beginning of the ripening period, and for cheeses which are able to support the growth of *E. coli*, it is normally at the end of the ripening period. | | | | |
| **Butter and cream made from raw milk or milk that has undergone a lower heat treatment than pasteurisation** | 5 | 2 | 10 | 100 |
| Action in case of unsatisfactory results: Improvements in production hygiene and selection of raw materials | | | | |

**E. coli** is used here as an indicator for the level of hygiene

ISO 16649-1:2001  
Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of beta-glucuronidase-positive *Escherichia coli* – Part 1: Colony-count technique at 44 degrees C using membranes and 5-bromo-4-chloro-3-indolyl beta-D-glucuronide  
Part 2: Colony-count technique at 44 degrees C using 5-bromo-4-chloro-3-indolyl beta-D-glucuronide
Process hygiene criteria: Milk and dairy products

<table>
<thead>
<tr>
<th>Coagulase-positive staphylococci</th>
<th>n</th>
<th>c</th>
<th>m</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(cfu/g)</td>
<td></td>
</tr>
</tbody>
</table>

At the time during the manufacturing process when the number of staphylococci is expected to be highest

<table>
<thead>
<tr>
<th>Cheeses made from raw milk (EN/ISO 6888-2)</th>
<th>n</th>
<th>c</th>
<th>m</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>2</td>
<td>10⁴</td>
<td>10⁵</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cheeses made from milk that has undergone a lower heat treatment than pasteurisation (7) and ripened cheeses made from milk or whey that has undergone pasteurisation or a stronger heat treatment (7) (EN/ISO 6888-1 or 2)</th>
<th>n</th>
<th>c</th>
<th>m</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>2</td>
<td>100</td>
<td>1000</td>
</tr>
</tbody>
</table>

End of the manufacturing process

<table>
<thead>
<tr>
<th>Unripened soft cheeses (fresh cheeses) made from milk or whey that has undergone pasteurisation or a stronger heat treatment (7) (EN/ISO 6888-1 or 2)</th>
<th>n</th>
<th>c</th>
<th>m</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>2</td>
<td>10</td>
<td>100</td>
</tr>
</tbody>
</table>

Action in case of unsatisfactory results: Improvements in production hygiene and selection of raw materials.
If values >10⁵ cfu/g are detected, the cheese batch has to be tested for staphylococcal enterotoxins. Excluding cheeses where the manufacturer can demonstrate, to the satisfaction of the competent authorities, that the product does not pose a risk of staphylococcal enterotoxins.
## Process hygiene criteria: Milk and dairy products

<table>
<thead>
<tr>
<th>Enterobacteriaceae</th>
<th>n</th>
<th>c</th>
<th>m (cfu/g)</th>
<th>M (cfu/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>End of the manufacturing process</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ice cream and frozen dairy desserts (Only ice creams containing milk ingredients) (ISO 21528-2)</td>
<td>5</td>
<td>2</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td><strong>Improvements in production hygiene</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dried infant formulae and dried dietary foods for special medical purposes intended for infants below six months of age (ISO 21528-1)</td>
<td>10</td>
<td>0</td>
<td><strong>Absence in 10 g</strong></td>
<td></td>
</tr>
</tbody>
</table>

Improvements in production hygiene to minimise contamination. If *Enterobacteriaceae* are detected in any of the sample units, the batch has to be tested for *E. sakazakii* and *Salmonella*.
# Process hygiene criteria: Milk and dairy products

**1441/2007**

COMMISSION REGULATION (EC) No 1441/2007

of 5 December 2007

amending Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs

(Text with EEA relevance)

---

**Testing for presumptive *Bacillus cereus***

<table>
<thead>
<tr>
<th>2.2.11 Dried infant formulae and dried dietary foods for special medical purposes intended for infants below six months of age</th>
<th>Presumptive <em>Bacillus cereus</em></th>
<th>5</th>
<th>1</th>
<th>50 cfu/g</th>
<th>500 cfu/g</th>
<th>EN/ISO 7932 (10)</th>
<th>End of the manufacturing process</th>
<th>Improvements in production hygiene. Prevention of recontamination. Selection of raw material.</th>
</tr>
</thead>
</table>
## Process hygiene criteria: Egg product

**Egg products**  
(The criterion does not apply to products intended for further processing in the food industry)

<table>
<thead>
<tr>
<th><strong>Enterobacteriaceae</strong> ISO 21528-2</th>
<th>n</th>
<th>c</th>
<th>m (cfu/g)</th>
<th>M (cfu/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>End of the manufacturing process</td>
<td>5</td>
<td>2</td>
<td>10</td>
<td>100</td>
</tr>
</tbody>
</table>

Checks on the efficiency of the heat treatment and prevention of recontamination

## Process hygiene criteria: Fishery

**Shelled and shucked products of cooked crustaceans and molluscan shellfish**

<table>
<thead>
<tr>
<th><strong>E. coli</strong> ISO TS 16649-3</th>
<th>n</th>
<th>c</th>
<th>m (cfu/g)</th>
<th>M (cfu/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>End of the manufacturing process</td>
<td>5</td>
<td>2</td>
<td>▶️C1 1/g ◀️</td>
<td>▶️C1 10/g ◀️</td>
</tr>
</tbody>
</table>

Improvements in production hygiene.

**Coagulase-positive staphylococci** EN/ISO 6888-1 or 2  

<table>
<thead>
<tr>
<th>n</th>
<th>c</th>
<th>m (cfu/g)</th>
<th>M (cfu/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>2</td>
<td>10</td>
<td>100</td>
</tr>
</tbody>
</table>

Improvements in production hygiene.
### Process hygiene criteria: Vegetables, fruits and products thereof

<table>
<thead>
<tr>
<th>E. coli</th>
<th>ISO 16649- 1 or 2</th>
<th>n</th>
<th>c</th>
<th>m</th>
<th>M</th>
<th>cfu/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manufacturing process</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-cut fruit and vegetables (ready-to-eat)</td>
<td>ISO 16649-1 or 2</td>
<td>5</td>
<td>2</td>
<td>100</td>
<td>1000</td>
<td></td>
</tr>
<tr>
<td>Unpasteurised fruit and vegetable juices (ready-to-eat)</td>
<td>ISO 16649-1:2001</td>
<td>5</td>
<td>2</td>
<td>100</td>
<td>1000</td>
<td></td>
</tr>
<tr>
<td>Improvements in production hygiene, selection of raw materials</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ISO 16649-1:2001
Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of beta-glucuronidase-positive *Escherichia coli* – Part 1: Colony-count technique at 44 degrees C using membranes and 5-bromo-4-chloro-3-indolyl beta-D-glucuronide
Part 2: Colony-count technique at 44 degrees C using 5-bromo-4-chloro-3-indolyl beta-D-glucuronide
Thank you for your attention

Questions to be sent to
Sabina.Purkrtova@vscht.cz