Measurement methods in food microbiology
Sampling for microbiological analysis

- Need valid samples for microbiological analysis
  - Valid sample
    - Representative
    - Collected aseptically
    - Store properly
    - Sterile equipment
# Microbiological level in foods

We need to know the presence, types and number of microorganisms and/or their products:

- Quality of safety (Infective dose)
- Quality of wholesomeness

<table>
<thead>
<tr>
<th>Number of microorganisms related to:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall quality</td>
</tr>
<tr>
<td>Keeping quality</td>
</tr>
<tr>
<td>Safety</td>
</tr>
<tr>
<td>Handling practice</td>
</tr>
</tbody>
</table>
Measurement methods in food microbiology

- **Direct methods**
  - Examine or count number of microorganisms
  - Cells (Cells/g or ml)

- **Indirect methods**
  - Allow bacteria to grow and measure results under the condition established
  - Reflection (number of M/O) based on growth and activity of microorganism
  - CFU (Colony Forming Unit) (CFU/g or ml)
## Measurement methods in food microbiology

<table>
<thead>
<tr>
<th></th>
<th><strong>Direct</strong></th>
<th><strong>Indirect</strong></th>
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</thead>
<tbody>
<tr>
<td><strong>Advantage</strong></td>
<td>Rapid</td>
<td>Accurate</td>
</tr>
<tr>
<td></td>
<td>Minimum and inexpensive equipment</td>
<td>Predict keeping quality</td>
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<tr>
<td></td>
<td>Determine morphology</td>
<td>Predict safety</td>
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<tr>
<td><strong>Disadvantage</strong></td>
<td>Count total cells (living/Death)</td>
<td>Use for sample contain small #</td>
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<tr>
<td></td>
<td>Use only sample contain high #</td>
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<tr>
<td></td>
<td>Use small volume of sample</td>
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<tr>
<td></td>
<td>Reduce precision</td>
<td></td>
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<tr>
<td></td>
<td>food particle interference</td>
<td></td>
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<tr>
<td></td>
<td>Count CFU</td>
<td>Slow</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Need media and equipment</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Accuracy depend on growth and activity of M/O</td>
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</tbody>
</table>
Direct methods

- Direct microscopic examination of M/O
  - Examination of living organism
    - Wet mounts
    - Hanging drop mount
  - Examination of stained films
    - Dry mounts
      - Simple stain
      - Differential stain
- Direct microscopic clump count
- Electronic cell count
Direct microscopic examination of microorganisms

Examination of living organism

- Determine whether or not bacterial cells are motile
  - gliding
  - tumbling
- Wet mouths
  - slide = coverslip
- Hanging drop mount
  - slide = grease = coverslip
- Motility medium with indicator
Direct microscopic examination of microorganisms

Examination of stained films

Simple stain
- Routinely use for determining numbers, size, shape, and arrangement of cells
- All organisms are stained the same color
- Acidic or basic dye
  - Acidic dye → the alkaline portion (cytoplasm)
  - Basic dye → the acidic portion (cell wall)
- Methylene blue or Crystal violet

Dry mount → Methylene blue 1-2 min
Rinse with water → 100X
Direct microscopic examination of microorganisms

- Differential stain
  - Distinguish between different types of bacteria
- Gram stain
  - Differentiate 2 classes of bacteria
    - Gram positive
    - Gram negative
  - Principle: Different cell wall structure between Gram+ and Gram - bacteria resulted in different staining
    - Gram + ➔ Blue (Crystal violet)
    - Gram - ➔ Red (Safranin)
- Gram stain procedure
Gram stain

- Dry mount
- Crystal violet 1 min
- Iodine solution 1 min
- Safranin 30 sec
- Rinse with water
- Ethyl alcohol 30 sec

Gram +

Gram -

The gram-positive cell wall
- Peptidoglycan
- Plasma membrane

The gram-negative cell wall
- Outer membrane
- Peptidoglycan
- Periplasmic space
Direct microscopic examination of microorganisms

- Direct microscopic clump count (DMCC)
  - Measure total bacterial cells (living and death cells)
  - Commonly use in enumerating bacteria in milk or liquid/semi-liquid foods
  - Direct count from micro meter slide

- Count bacterial cells
  Number of M/O in the filed $\times$ MF

$$MF \text{ (microscopic factor)} = \frac{(x)(y)}{\pi r^2} = \frac{10,000}{\pi r^2}$$
Direct microscopic examination of microorganisms

- Direct microscopic clump count (DMCC)
  - Advantages
    - Rapid
    - Require minimum equipment (not expensive)
    - Prepare more than one samples, read later
    - Different morphology and Gram types can be identified
  - Disadvantages
    - Only for samples which contain large number of M/O
    - Precision (Small volume of sample is examined)
    - Debris or food particles may interfere visualization
    - Analyst fatigue
Indirect methods

- Allow bacteria to grow and measure results under the condition established
- Reflection (number of M/O) based on growth and activity of microorganisms in CFU (Colony Forming Unit) (CFU/g or ml)

- Aerobic plate count (APC)
- Standard plate count (SPC)
- Plate loop count (PLC)
- Spiral plate count (SPLC)
- Membrane filtration (MF)
- Most probable number (MPN)
- Dye reduction test
Indirect methods

- Aerobic plate count (APC) (AOAC)
- Standard plate count (SPC) (APHA)
  - Estimate the actual number of living M/O in a sample by assumption that microbial cells mix with agar medium will grow to from visible separated colonies
  - Report the result as Colony Forming Unit (CFU/ml or g)
    - Methods: Pour plate method or spread plate method
    - Medium: Plate count agar
    - Incubation: AOAC 35 °C for 48 hrs
      - APHA apply to dairy product
Dilution techniques

Original sample
9 ml H₂O (1/10 dilution)
9 ml H₂O (1/100 dilution)
9 ml H₂O (1/1,000 dilution)
9 ml H₂O (1/10,000 dilution)

Mix with warm agar and pour.
Spread plate count

Pour plate count

Incubation

Count plate between 25-250 CFU
Indirect methods

- Plate loop count (PLC)
  - Modified for diary product
  - Use volumetrically loop for transfer sample
  - No dilution required
  - Sterile by washing with diluting agent

Advantages: process many sample
Disadvantage: less precise (small volume)
Indirect methods

- Spiral plate count (SPLC)
  - No dilution required
  - Prepare agar plate ➔ rotate plate ➔ gradually deposit sample
  - Count selected area ➔ 25-250 CFU

Automated tool for depositing sample
**Indirect methods**

- **Spiral plate count (SPLC)**
  - **Advantage**
    - Automatic
    - No dilution required
    - Safe time
  - **Disadvantage**
    - Need equipment
    - Expensive
**Indirect methods**

- **Membrane filtration (MF)**
  - Suitable for liquid or semi-liquid samples (eg. Water)
  - Commonly use for Coliform and Staphylococcus spp
  - Filter M/O size more than 0.45 μm
  - Plate filter on agar medium

- **Advantages**
  - useful in small number of M/O in sample (<25 CFU/ml)

- **Disadvantages**
  - Not apply for large volume of sample
Indirect methods

Most probable number (MPN)

- Statistic approach to quantitate the numbers of bacteria which utilize a multiple dilution to estimate population of microorganisms in foods
- Use to estimate the number of M/O (less accurate than plate)
- Examine large amount of sample due to replication
- The growth of M/O in the medium turbidity/change in color of medium

Advantages
- Use to detect the small number of M/O in sample
- Examine more sample size

Disadvantages
- Less precise
- Time consuming
**Most Probable Number (MPN)**

Positive: Gas production
Change of medium color

<table>
<thead>
<tr>
<th>10^1</th>
<th>10^2</th>
<th>10^3</th>
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<tbody>
<tr>
<td><img src="image" alt="Images of test tubes" /></td>
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</tr>
</tbody>
</table>

3:3:2
11x 10^2 MPN/ml

3:2:2
compare to MPN table

<table>
<thead>
<tr>
<th>No. of Tubes Positive in</th>
<th>MPN</th>
<th>No. of Tubes Positive in</th>
<th>MPN</th>
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</thead>
<tbody>
<tr>
<td>Series A</td>
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<td>Series C</td>
<td>Series D</td>
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<tr>
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<td>0.11</td>
</tr>
<tr>
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<td>2</td>
<td>1</td>
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</tr>
<tr>
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<td>2</td>
<td>2</td>
<td>0.20</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td>0.24</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0.16</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>1</td>
<td>0.20</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>2</td>
<td>0.24</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>3</td>
<td>0.29</td>
</tr>
</tbody>
</table>
Indirect methods

- Dye reduction test
  - Estimation of viable microorganism that posses reducing capacities
  - More number of microorganisms Less time for bacteria to change the color of indicator dye
  - Methylene blue reduction test
  - Resazurin test
    - Raw milk quality
Potential error in measurement methods

- Biological systems
- Sample & sampling techniques
- Personnel
- Glass and plastic ware
- Water & dilution blank
- Media
- Sterilization
- Culture techniques
- Incubation
- Counting & calculation
Modified and rapid methods

A procedure that gives reasonable satisfactory results that can be applied to prevent or confirm problems from microorganisms or microbial toxins

- More rapid and reasonably well correlated with the regular (conventional) methods
- Need these methods
  - to clear product for distribution
  - to verify corrective procedure
  - to intervene or stop distribution to consumer in an outbreak
Modified and rapid methods

- Immunoassay
  - ELISA
    - Listeria-Tek Listeria monocytogenes
    - TECRA Bacillus diarrheal toxin
  - Immunodiffusion
    - Microslide technique

- ATP-Bioluminescence technique
  - Bactofoss

- DNA hybridization
  - Gene-track

- Polymerase Chain Reaction
Modified and rapid methods

- Immunoassay
  - ELISA
    - Listeria-Tek: Listeria monocytogenes
    - TECRA: Bacillus diarrheal toxin
  - Rapid, sensitive, specific screening test

Interpretation:
- Using microtiter plate reader (ELISA reader)
- Intensity of color related to numbers of M/O or toxins
Modified and rapid methods

- Immunodiffusion
  - Microslide technique
  - Confirm test for enterotoxin
  - Require toxin 50 ng/ml
  - Require long incubation time (>2 days)

Interpretation:
- Control toxin give precipitate line
- Positive sample will give precipitate line (Join line of identity)
- Negative, if no line of identity or extend line of identity

Microslide coated with agar

1. Specific antisera
2. Test sample
3. Reference enterotoxin
4. Test sample
5. Reference enterotoxin
Modified and rapid methods

- ATP-Bioluminescence technique
  - Specific reaction of ATP (in living cells) and enzyme complex (Luciferin/luciferase)
  - Release fluorescence light depend on amount of ATP
  - Calculate the number of cells based on amount of ATP
  - Rapid (1-2 min)
  - Use in dairy product for estimating total bacteria count

Bacto Foss

1. Sample intake
2. Filtration
3. Lysing of somatic cells
4. Washing
5. Extraction of ATP
6. Measure fluorescent light
7. Calculate number of bacterial cells
Modified and rapid methods

- DNA hybridization
  - Target DNA (bacterial DNA) is attached to membrane filter (nitrocellulose filter)
  - DNA probe (known oligonucleotides) bind to target DNA
  - Detect by radioisotope or colorimetric techniques
  - Comercially available
    - Gene trak
    - Salmonella typhimurium
    - Listeria monocytogenes
    - Escherichia coli
Sample lysis

Hybridization

Hybridization capture

Enzyme label

Detection
Modified and rapid methods

- Petriflim test kit
  - Test kit for the enumeration of E. coli O157:H7 and coliform
  - Rapid (1 hr after routine E. coli test)
  - Use violet red bile nutrients, beta-glucoronidase indicator to identify E. coli, Tetrazolium indicator dye to enhance visualization of other gram negative bacteria
  - Coliforms ferment lactose and produce gas red colony with gas bubble
  - E. coli react with indicator to produce blue colony with blue precipitation
Polymerase Chain Reaction (PCR)

- Detect target DNA of bacteria of interest
- The number of specific DNA sequence in the sample
  primers + PCR reaction \[ \rightarrow \] Many copy of DNA sequence
- Very sensitive method (can detect 50-100 ng of bacterial DNA)
- Use for the detection of several bacterial species in foods