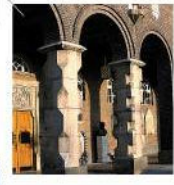


# Food microbiology

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SZÉCHENYI 2020



MAGYARORSZÁG  
KORMÁNYA

Európai Unió  
Európai Szociális  
Alap

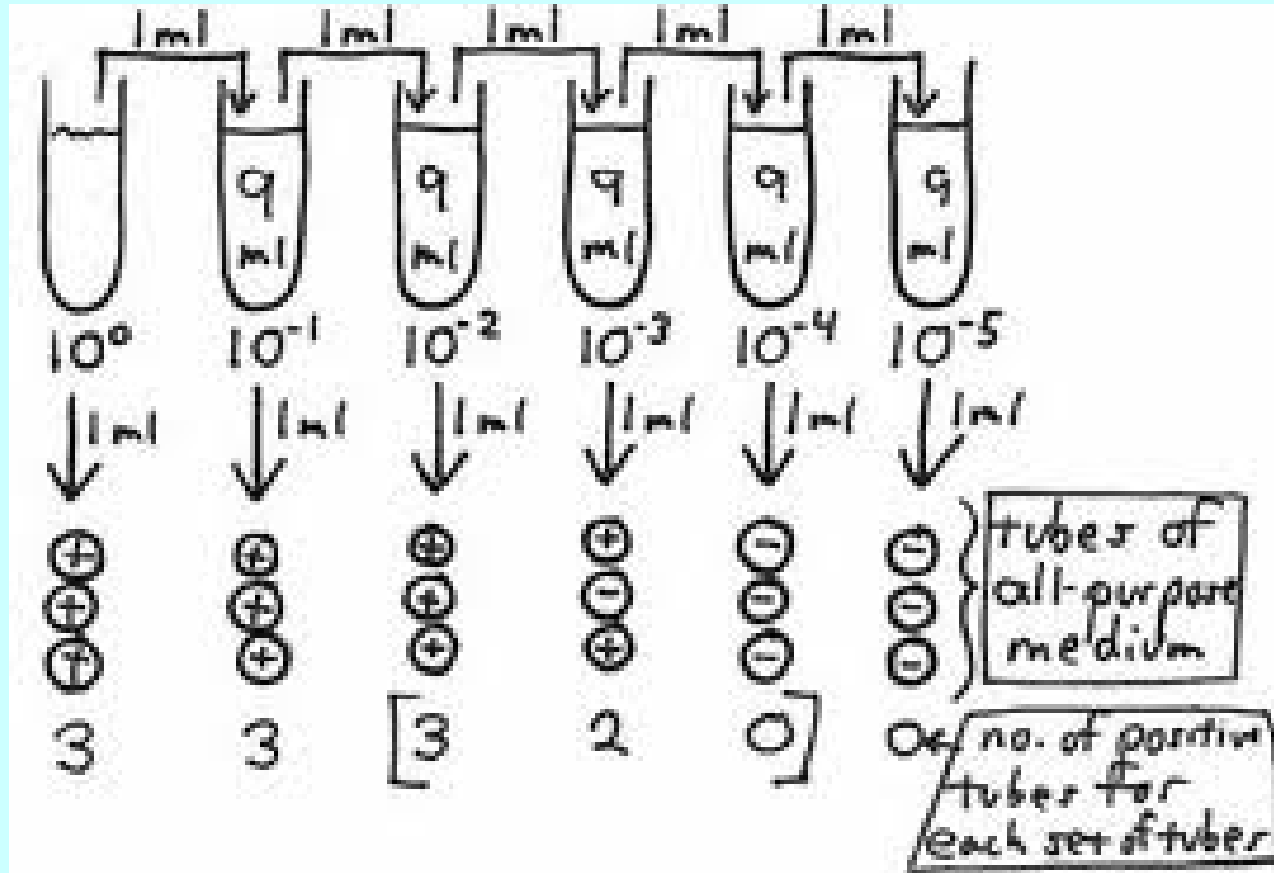


BEFEKTETÉS A JÖVŐBE

# Most probably number (MPN) by Hoskins

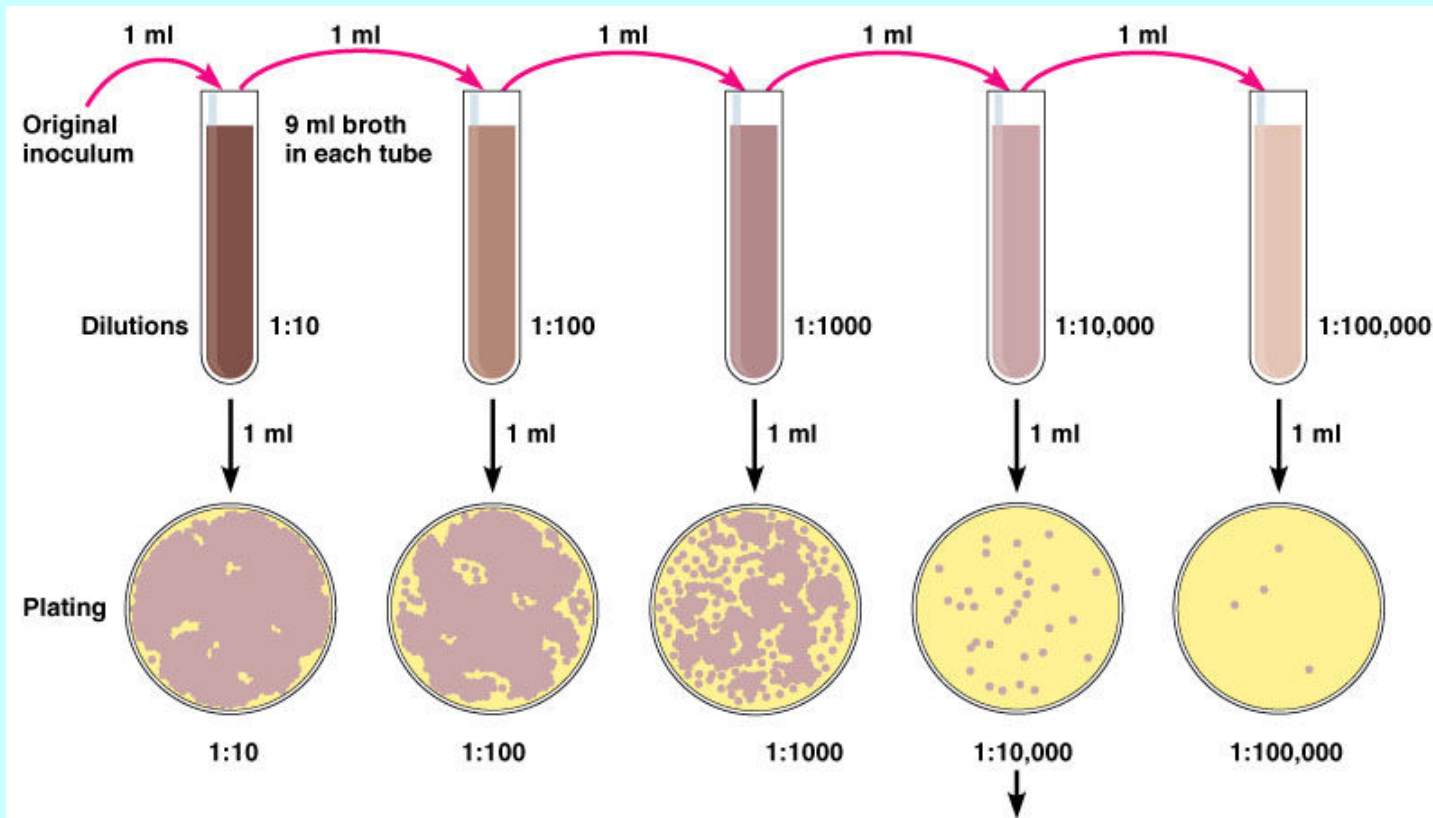
- Make ten-fold dilution series
- Inoculate from all dilutions into a separate tube of an all-purpose broth medium.
- Incubate the tubes
- Observe for the presence or absence of growth.
- Create a key number
- Determine the MPN from the Hoskins table

# MPN



# Plate count methods

## Pour plate method

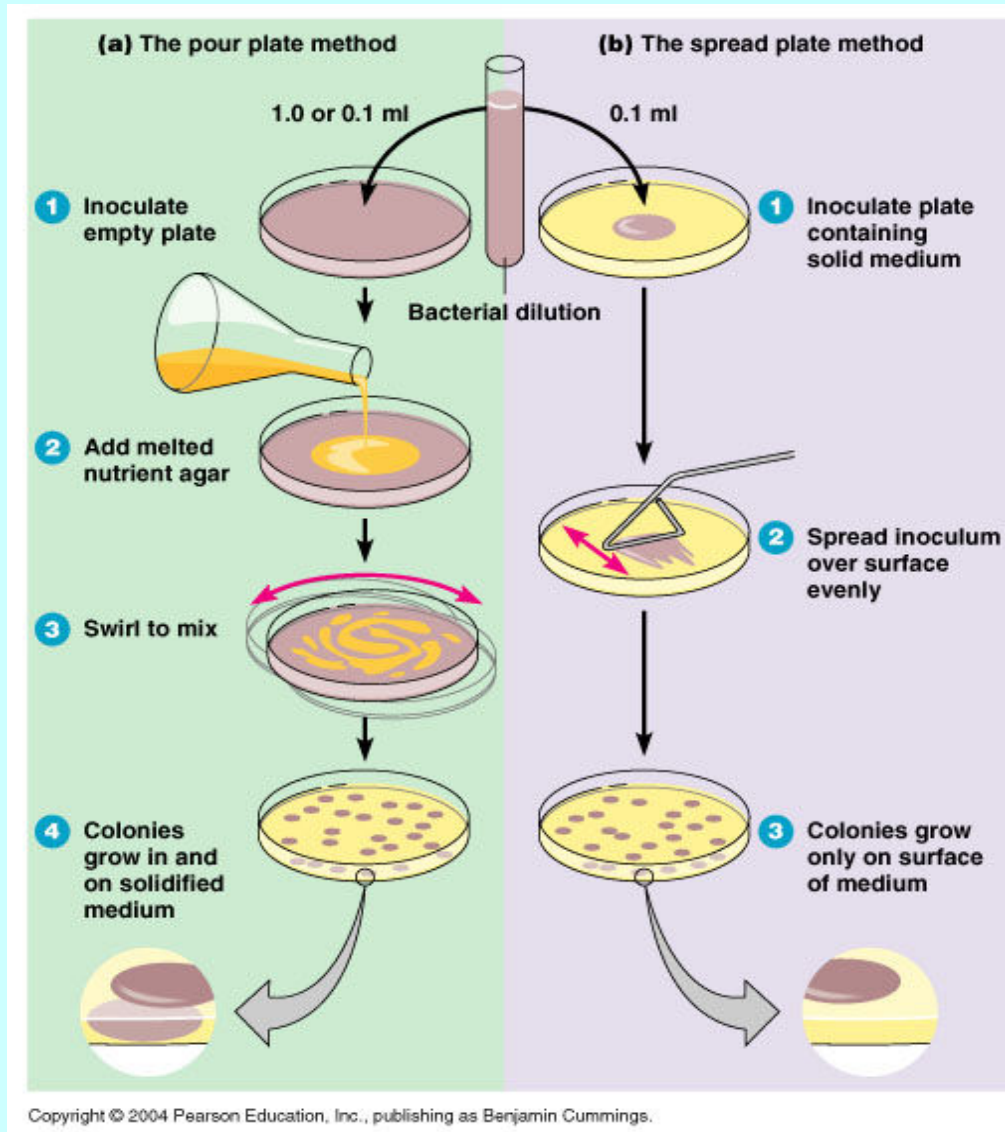


**Calculation: Number of colonies on plate  $\times$  reciprocal of dilution of sample = number of bacteria/ml**  
(For example, if 32 colonies are on a plate of  $1/10,000$  dilution, then the count is  $32 \times 10,000 = 320,000/\text{ml}$  in sample.)

# Pour plate method

- Make ten-fold dilution series
- Add 1 ml cell suspension from all dilutions into sterile petri dishes.
- Pour the appropriate medium into the Petri dish and mix it well with the solution
- Incubate the dishes
- Count the number of colonies (should be between 30 and 300)
- Determine the original cell number by the formula: colony count x dilution

# Plate count methods



# Spread plate method

- Make ten-fold dilution series
- Add 0.1 ml cell suspension from all dilutions to petri dishes containing the appropriate, solidified medium
- Spread the cell suspension over the medium surface with a glass spreader
- Incubate the dishes
- Count the number of colonies (should be between 30 and 300)
- Determine the original cell number by the formula: colony count  $\times 10 \times$  dilution

# Cell number enumeration

- Colony forming unit (CFU)/1ml or 1g
- (From all single cell a colony will be formed)
- MPN: value from the table x dilution
- Pour plate: number of colonies x dilution
- Spread plate: number of colonies x 10 x dilution



# Spread plate method



- MPN is used for the estimation of total germ count, coliform count
- Poured plate is used for the enumeration of yeasts and facultative anaerobes
- Spread plate is used for the enumeration of aerobes and molds

# Optimum temperature

- Optimum temperature for microorganisms is different
  - Psychrophiles (0-25 °C) - can grow in refrigerator
  - Mesophiles (30-37 °C) – most microbes
  - Thermophiles (25-80 °C)
  - Bacterial spores can survive boiling (100 °C)

# Effect of heat treatment

- Mechanism of action of high temperatures:
  - Denaturation of proteins
  - Damage of cell membrane
  - Cell death
- Mechanism of action of low temperatures:
  - Inhibition of key enzymes in cell metabolism
  - Growth reduction effect

# Types of heat treatment in food industry

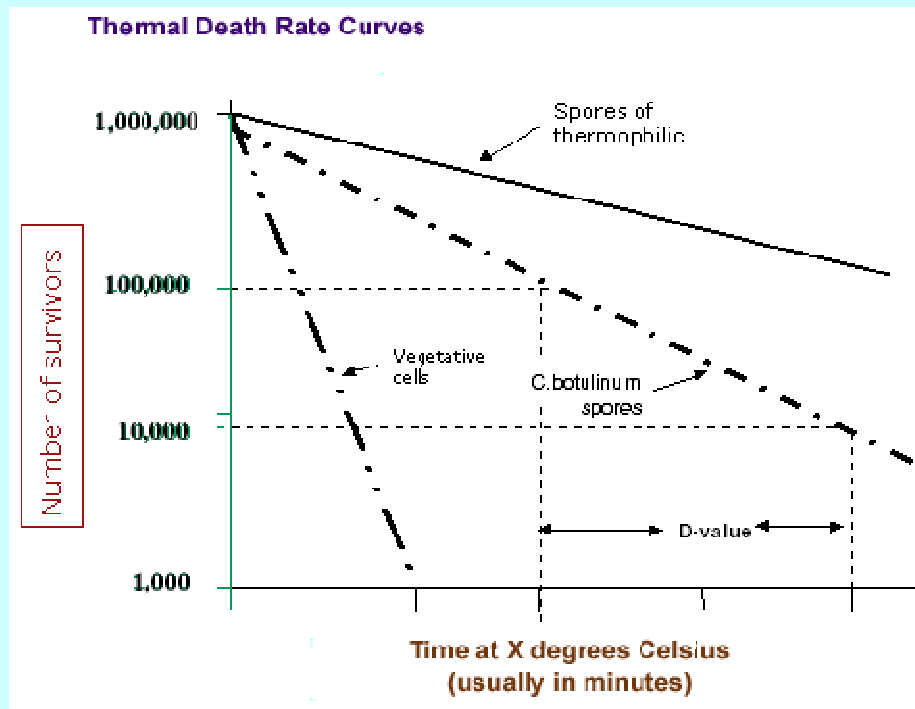
- Pasteurization – kill vegetative forms
  - mild (62-65°C 30 min)
  - fast (72-76 °C 14-15 s)
  - very fast (85 °C 1-2 s)
  - ultra (132 °C 1 s)
- Sterilization (121°C 1,5 atm 20 min) – kill all cells including bacterial endospores

# Investigation on the effect of heat treatment

- Used microorganisms
  - *E. coli* – non spore forming
  - *B. subtilis* – spore forming
- Enumerate the cell count of the bacterial suspensions using the spread plate method
- Treat *E. coli* at 80 °C and take samples at every 5 min. Determine the cell number of the samples using the spread plate method.
- Treat *B. subtilis* at 100 °C and take samples at every 5 min. Determine the cell number of the samples using the spread plate method.

# Investigation on the effect of heat treatment

- Create two tables with the number of survivals
- Create a time-kill curve with your data



# Effect of disinfectants

- Aim: elimination of pathogens in vegetative form

## Disinfectants used in food industry

- Halogenic - Hypochlorous acid (HClO), iodine
- Peroxides -  $H_2O_2$ , Peracetic acid –  $CH_3COOOH$
- Quaternary ammonium compounds



# Investigation on the effect of Hypochlorous acid and a quaternary ammonium compound

- Used microbes
  - *E. coli*
  - *B. cereus*
- Used disinfectants:
  - Hypo (HClO) – 2 %
  - First (quaternary) – 2%
- Solutions to stop the reaction:
  - Na-tiosulfate
  - Tween 40

## Investigation on the effect of Hypochlorous acid and a quaternary ammonium compound

- Add 2-2 ml bacterium suspension to 18 ml disinfectant
- In every 5 min take 1 ml from the solution and add 9 ml stopper solution to it
- Make decimal dilutions from above described samples
- Estimate the number of survivals by the spread plate method

# Effect of UV radiation

- UV cause DNA damage in the cell
- Microorganism have a repair mechanism
- When the time of UV radiation is too long or too intensive the damage will be irreversible

# Effect of UV radiation

- Used microorganism
  - *E. coli*
- Spread 0.1 ml *E. coli* suspensions on TGE mediums.
- Treat the plates under UV lamp for 5, 10, 15, 20 min
- Incubate the plates for 7 days at 37 °C
- Count the survivors on the plates

# Effect of osmolytes on the growth of microbes

- Osmotic pressure of solutions
  - Isotonic – normal material transport through the cell wall
  - Hypertonic – water goes out from the cell, the cell shrinks
  - Hypotonic – water goes into the cell, the cell swells, can burst

# Effect of sucrose on the growth of *yeasts*

- Some yeasts are osmotolerant
- Used yeast: *S. cerevisiae*, *S. diastaticus*, *Z. rouxii*
- Used sucrose solutions: 5, 10, 20; 40 %
- Add 0.1 ml yeast cell suspension to 5 ml sucrose solution
- Incubate the tubes for 7 days at 30 °C
- Count the cells in the tubes by spread plate method

# Effect of salt on the growth of *bacteria*

- Some bacteria are halotolerant
- Used bacteria: *E. coli*, *B. subtilis*, *B. cereus*
- Used salt solutions: 2.5, 5.0, 7.5; 10 %
- Add 0.1 ml bacterium cell suspension to 5 ml salt solution
- Incubate the tubes for 7 days at 37 °C
- Count the cells in the tubes by spread plate method

# Optimal pH for the growth of microorganisms

- pH effect the membrane function
- pH effect also the function of enzymes
- Optimal pH
  - Bacteria: 6.5 – 7.5
  - Fungi: 5-7 (but in some cases can grow below pH 2)



# Effect of pH on the growth of microorganisms

- Used microbes: *E. coli*, *B. subtilis*, *B. cereus*; *S. cerevisiae*
- pH solutions (McIlvain-buffer): 2.2, 4.0, 6.0; 8.0
- Add 0.1 ml microbe cell suspension to 5 ml buffer solution
- Incubate the tubes for 7 days at 30 °C
- Count the cells in the tubes by spread plate method

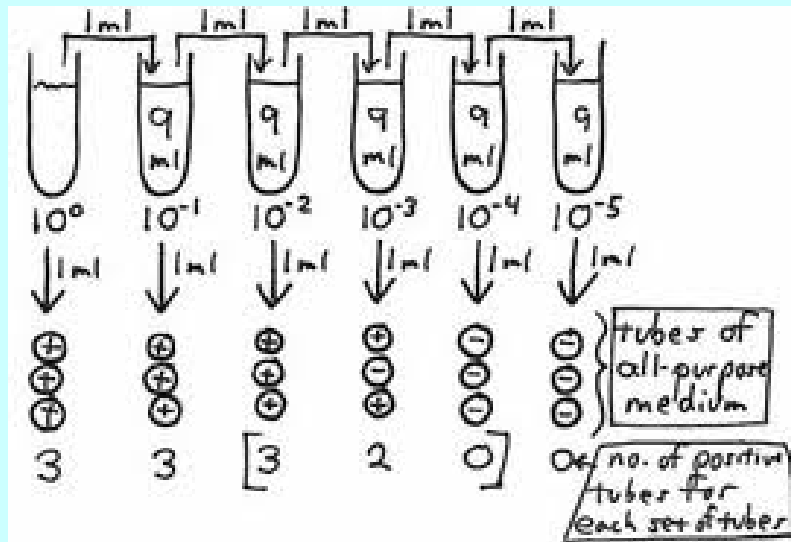
# Total germ count 1

- Weight out aseptically 10 g or 10 ml from the examined food
- Solid food should be masticate
- Add it to 90 ml sterile physiological saline (PS)
- Mix well
- Make a tenfold dilution series (5-6 dilution)

# Total germ count 2

- Inoculate 1 ml from all dilution into 3-3 tubes containing TGE broth
- Incubate the tubes (24 h, 37 °C)
- Observe for the presence or absence of growth – turbidity, color change.
- Create a key number: take into account the last dilution where all tubes were positive then the subsequent 2 dilutions and write the number of positive tubes

# Total germ count 3



- Key number is: 320
- From the Hoskins table you can find the MPN value: 0,93
- Multiply this value with the dilution factor of the first member of the key number:
- $0,93 \times 10^2$  CFU/g

# Determination of aerobic spore forming bacteria 1

- Members of the genus *Bacillus* can be found in this group *B. subtilis*, *B. cereus*, *B. megaterium* causing food spoilage and poisoning
- At 80 °C vegetative cells die but the spores survive
- After incubation the spores will germinate and all cells in the broth comes from the germinated spores

# Determination of aerobic spore forming bacteria 2

- Weight out aseptically 10 g or 10 ml from the examined food
- Solid food should be masticate
- Add it to 90 ml sterile physiological saline (PS)
- Mix well
- Make a tenfold dilution series (5-6 dilution)

# Determination of aerobic spore forming bacteria 3

- Inoculate 1 ml from the first 3 dilutions into TGE broth
- Heat the tubes at 80 °C for 20 minutes
- Incubate the tubes (24 h, 37 °C)
- Observe for the presence or absence of growth - turbidity.
- Create a key number as described above

# Determination of anaerobic spore forming, H<sub>2</sub>S producing bacteria 1

- Members of Clostridium genus can be found in this group
- From sulfite they can form H<sub>2</sub>S and if Fe-citrate is present in the broth FeS will be formed
- FeS gives black color to the broth
- Anaerobic environment is created by adding an oil layer on the surface of the broth
- RCM broth is used containing cystein (sulfur containing amino acid) and Fe-citrate



# Determination of anaerobic spore forming, H<sub>2</sub>S producing bacteria 2

- Weight out aseptically 10 g or 10 ml from the examined food
- Solid food should be masticate
- Add it to 90 ml sterile physiological saline (PS)
- Mix well
- Make a tenfold dilution series (5-6 dilution)

# Determination of anaerobic spore forming, H<sub>2</sub>S producing bacteria 2

- Inoculate from the first two dilutions to RCM broth
- Heat the tubes at 80 °C for 20 minutes
- Close the tubes with a paraffin layer
- Incubate the tubes (24 h, 37 °C)
- Observe for the presence or absence of growth – turbidity and/or black coloration.
- Positive: turbidity – non H<sub>2</sub>S forming anaerobes; black color - H<sub>2</sub>S forming anaerobes

# Determination of yeasts and molds

- Weight out aseptically 10 g or 10 ml from the examined food
- Solid food should be masticate
- Add it to 90 ml sterile physiological saline (PS)
- Mix well
- Make a tenfold dilution series (3 dilution)

# Determination of yeasts

## Pour plate method

- Yeasts are able to grow under semi-anaerobic conditions.
- Use malate extract (ME) agar with antibiotic to prevent the growth of bacteria.
- Add 1 ml from each dilution to sterile Petri dishes.
- Pour lukewarm (40 °C) ME agar on the inoculum (about 15 ml) and mix it carefully by swirling.
- After the agar is solidified invert the Petri dish and incubate it for 24 h at 30 °C.

# Determination of molds

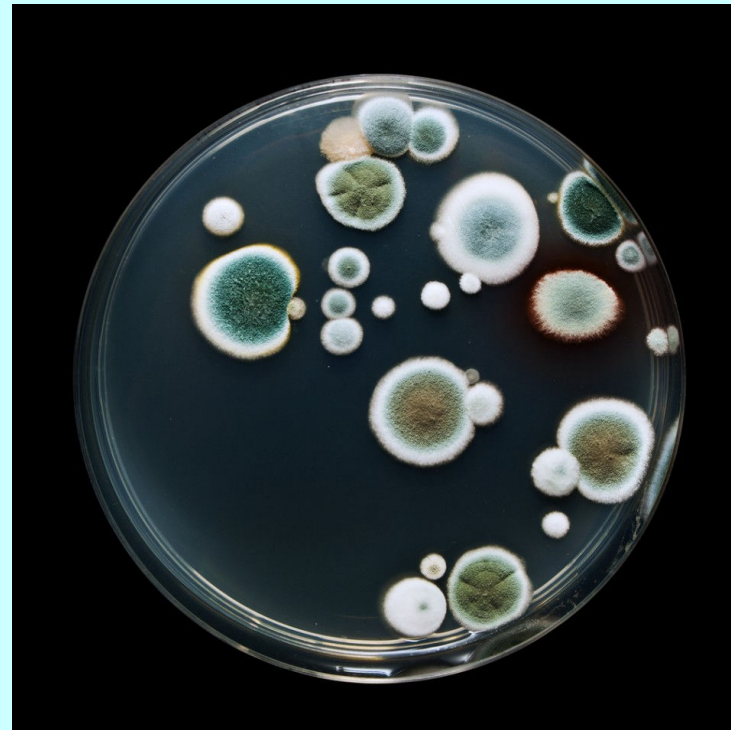
## Spread plate method

- Molds are strictly aerobes. Can grow only on the surface of a medium.
- Use malate extract (ME) agar with antibiotic to prevent the growth of bacteria.
- Drop 0.1 ml suspension from all dilution on prepared ME medium and spread it evenly on the surface of the agar.
- Incubate for 48-72 h at 30 °C.

# Determination of cell count

- Count plates with 30 – 300 colonies
- Yeasts: colony number  $\times$  dilution factor
- Molds: colony number  $\times 10 \times$  dilution factor

# Yeasts and molds counting



# Determination of Salmonella

- In the Salmonella genus Gram negative rod shaped bacteria can be found. There are two species *S. enterica* and *S. bongori*, 6 subspecies and a high number of serovars.
- Salmonella species are pathogens causing food poisoning.
- Determination of Salmonella is the duty of official laboratories.
- In the European Union no *Salmonella* is allowed in 25 g food.



# Determination of Salmonella

## Step 1

- Sometimes the number of salmonella is very low. Before determination we have to make an enrichment step.
- Weight out aseptically 25 g or 25 ml from the examined food.
- Add it to 225 ml pre-enrichment broth (Trypticasein Soy Broth (TSB)). This broth is not selective, it is used for the recovery of injured cells.
- Incubate for 24 h at 37 °C.

# Determination of Salmonella

## Step 2

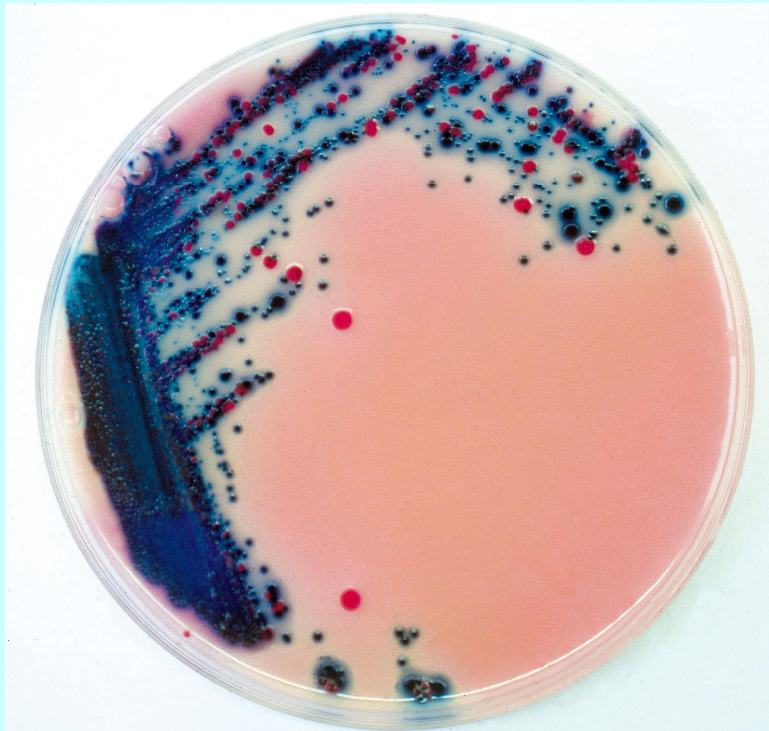
- Selective enrichment of *Salmonella* species in Rappaport Vassiliadis Broth.
- Malachite green and Mg chloride inhibit the growth of other bacteria (*Shigella*).
- Add 10 ml from the TSB broth to 90 ml Rappaport Vassiliadis Broth.
- Incubate for 24 h at 37 °C.

# Determination of Salmonella

## Step 3

- Selective determination of salmonella species on Rambach Agar.
- This selective medium is inhibitory for many microorganisms other than *Salmonella*.
- Streak a loop from the enrichment broth on Rambach Agar.
- Incubate for 24 h at 37 °C.
- Salmonella colonies are red, coliforms form blue and other bacteria colorless colonies.

# Rambach Agar



Red colonies:  
Salmonella spp.

Blue colonies: *E. coli*

Author: CHROMagar, France

# Verification of the presence of Salmonella on TSI Agar

- Triple Sugar Iron (TSI) Agar is used.
- Contain: 1% lactose, 1% sucrose, 0.1% glucose; phenol red as pH indicator and compounds for the production of hydrogen sulfid.
- An agar slant is made with aerobic and anaerobic areas.
- Positive colonies from Rambach agar are picked up on a needle and then TSI slant is inoculated by first stabbing the butt down to the bottom, and then streaking the surface of the slant.

# TSI Agar – Interpretation of the results

- Many enterobacteria ferment glucose with the production of acids which will change the color of the medium. However, glucose will be used up soon and the organisms in the presence of oxygen are forced to catabolize peptones and amino acids for their energy supply. Alkaline end-products from these substances will revert the pH of the slant to an alkaline pH and thus change the color of the agar slant back to red (after 18-24 hours).

# TSI Agar – Interpretation of the results

- Organisms such as *Salmonella* spp. or *Shigella* spp. and other organisms which attack glucose but do not ferment lactose or sucrose will produce an alkaline slant and acid butt in TSI slants in 18 to 24 hours. If the glucose is metabolized to CO<sub>2</sub>, the gas will be seen as bubbles or cracks in the agar butt. If hydrogen sulfide is formed during growth, a gray or black streak of iron sulfide is seen.
- Organisms which attack lactose and/or sucrose, such as *Escherichia*, will produce acid slants and acid butts usually with the formation of gas.

# TSI Agar – Interpretation of the results

Agar Butt

Yellow color	Glucose fermentation
Red color	No glucose fermentation
Black color	H <sub>2</sub> S production
Bubbles or cracks	Gas production during glucose, lactose fermentation



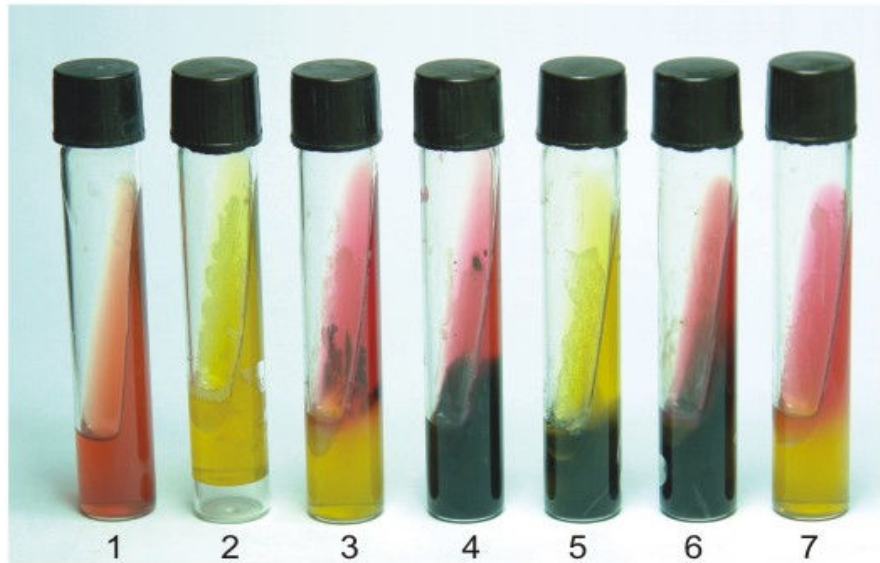
# TSI Agar – Interpretation of the results

Agar surface

Yellow	Lactose and/or sucrose fermentation
Red	No lactose and/or sucrose fermentation

Salmonella positive: agar butt: yellow with black, gas formation, red surface.

# Bacteria on TSI agar slants



## Triple Sugar Iron Agar (M021)

1. Control
2. *Escherichia coli* ATCC 25922
3. *Salmonella Typhi* ATCC 6539
4. *Proteus vulgaris* ATCC 13315
5. *Citrobacter freundii* ATCC 8090
6. *Salmonella Typhimurium* ATCC 14028
7. *Shigella flexneri* ATCC 12022



From left to right: Control; *P. aeruginosa*, *E. coli*, *Salmonella Typhimurium*, *Shigella flexneri*

# TSI reactions

Microorganism	Agar butt	Agar surface	Formation of H <sub>2</sub> S
<i>S. Paratyphi A</i>	Y+G	R	+
<i>S. Typhimurium</i>	Y+G	R	+
<i>S. Paratyphi B</i>	Y+G	R	+
<i>S. Enteritidis</i>	Y+G	R	+
<i>Sh. dysenteriae</i>	Y	R	+
<i>Ent. aerogenes</i>	Y+G	Y	-
<i>E. coli</i>	Y+G	Y	-
<i>Citrobacter spp.</i>	Y+G	Y	+
<i>Klebsiella spp.</i>	Y+G	Y	-
<i>Pr. vulgaris</i>	Y+G	Y	+
<i>K. pneumoniae</i>	Y/Y+G	R	-
<i>Ps. aeruginosa</i>	R	R	-

Y= yellow color (acid production)

Y+G= yellow color +gas production

R= red color, no change in color (no acid production) or alkaline pH revert yellow color back to red

+ = H<sub>2</sub>S production, black color

- = no H<sub>2</sub>S production

# Determination of Enterobacteriaceae 1

- Gram negative bacteria, members of the *Salmonella*, *Proteus*, *Klebsiella*, *Serratia*, *Citrobacter*, *Escherichia*, *Enterobacter*, *Shigella* can be found here
- Enterobacteria Enrichment (EE) broth is used in the first step
- Brilliant green and ox-bile inhibit the growth of Gram-positive bacteria

# Determination of Enterobacteriaceae 2

- Weight out aseptically 10 g or 10 ml from the examined food
- Solid food should be masticate
- Add it to 90 ml sterile physiological saline (PS)
- Mix well
- Make a tenfold dilution series (5-6 dilution)

# Determination of Enterobacteriaceae 3

- Inoculate 1 ml from all dilution to 3-3 tubes containing EE broth
- Incubate the tubes (24 h, 37 °C)
- Observe for the presence or absence of growth - turbidity
- Create a key number as described above
- Based on the MPN table add the cfu/g or ml

# Determination and identification of glucose fermenting Enterobacteriaceae

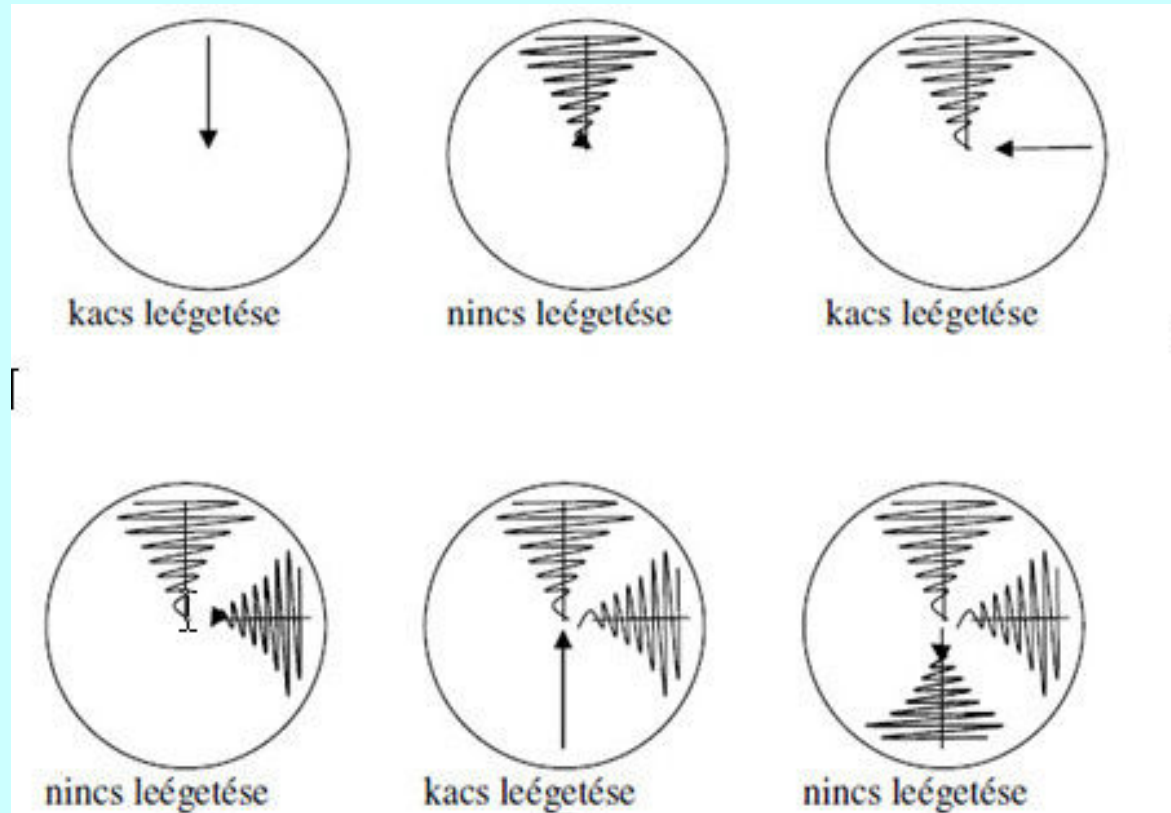
- Use VRBG Agar
- VRBG = Violet Red Bile Glucose agar will exclude lactose fermenting coliform bacteria.
- Bile salts and crystal violet inhibit the growth of Gram-positive cocci. Glucose fermenters produce red colonies with red-purple halos in the presence of Neutral Red, the pH indicator.

# Determination of glucose fermenting Enterobacteriaceae

- Streak from the positive tubes a loopful inoculum on VRBG agar
- Incubate the plates for 24 h at 37 °C
- Pink colonies with a reddish halo verify the presence of glucose fermenting Enterobacters.



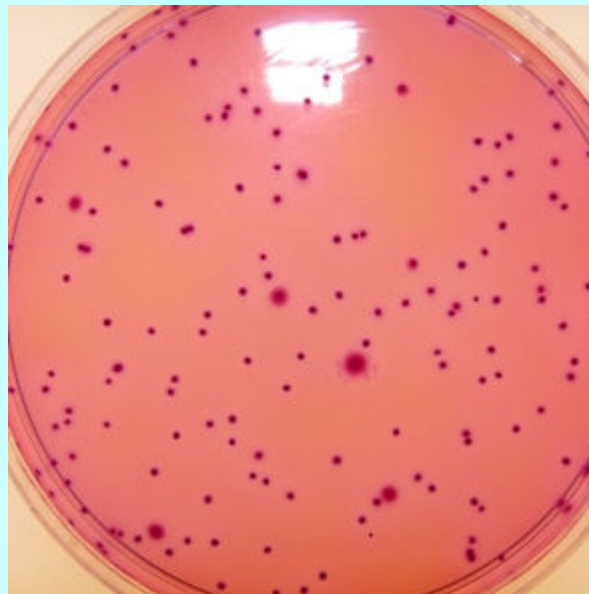
# Streaking plate technique



# Streaking plate technique

1. Streak a line with the loop containing the inoculum at the top end of the agar plate until 1/3 of the plate is covered
2. Sterilize the loop in the flame, cool it and move it in a zig-zag horizontal pattern over the straight line.
3. Rotate the plate about 60 degrees and streak a new line
4. Sterilize the loop again and repeat step 2
5. Rotate the plate about 60 degrees and streak a new line
6. Sterilize the loop again and repeat step 2
7. At the end you will get individual colonies

# *E. coli* on VRBG Agar



# Determination of coliforms 1

- The coliform group includes aerobic and facultative anaerobic, Gram-negative, non-spore forming bacilli that ferment lactose and form acid and gas at 35°C within 48 hours.
- Brilliant Green Bile Broth (BBL) is used to confirm the presence of coliforms.
- Oxbile and Brilliant Green inhibit Gram-positive bacteria and many Gram-negative bacteria, other than coliforms. During lactose fermentation gas production occur. This is detected by Durham tubes.

## Determination of coliforms 2

- Weight out aseptically 10 g or 10 ml from the examined food
- Solid food should be masticate
- Add it to 90 ml sterile physiological saline (PS)
- Mix well
- Make a tenfold dilution series (5-6 dilution)

# Determination of coliforms 3

- Add Durham tubes to the BBL broth.
- Inoculate 1 ml from all dilution to 3-3 tubes containing BBL broth
- Incubate the tubes (24 h, 37 °C)
- Observe for the presence or absence of coliforms – turbidity with bubble formation.
- Create a key number from the positive tubes as described above
- Enumerate cell count from the MPN table

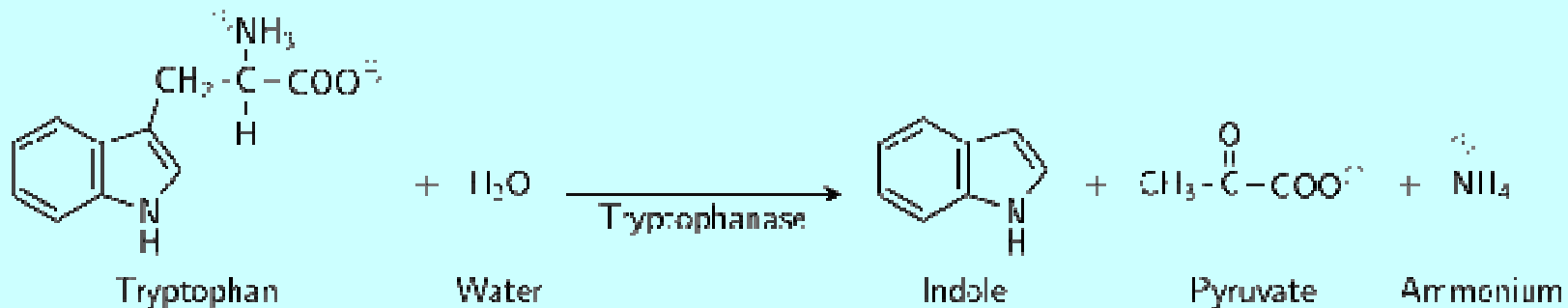
# Brilliant Green Bile Broth



- From left to right:
  - Uninoculated BBL broth
  - Non-coliform bacterium
  - Coliform bacterium

# Verification of the presence of *E. coli*

- Indole is generated by reductive deamination from the amino acid tryptophan. *E. coli* is able to produce indole.





# Verification of the presence of *E. coli*

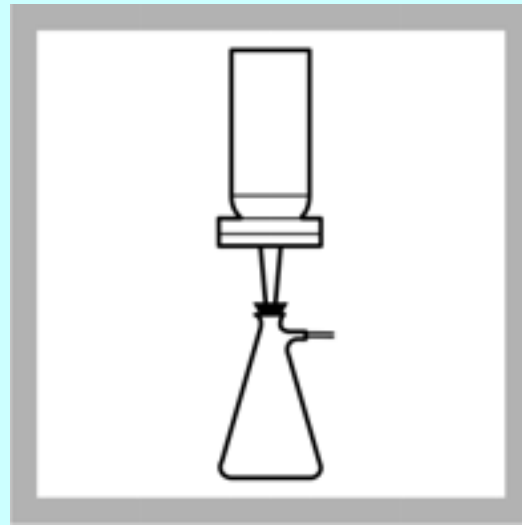
- From the coliform positive tubes add 1 ml to tryptophan containing broth.
- Incubate for 24 h at 44 °C.
- Add 5 drops of Kovac's reagent to the culture broth.
- Positive result: red/pink layer at the top of the tube.

# Positive indole test



# Determination of coliforms from water 1

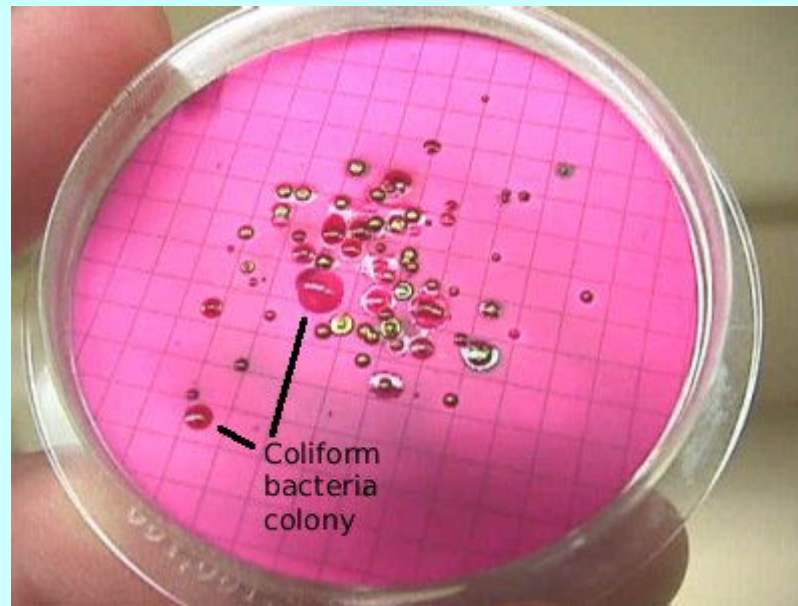
- Membrane Filtration (MF) method is used
- Filter 100 ml sample through a membrane filter with pore size of 0.45  $\mu\text{m}$ .



# Determination of coliforms from water 2

- Place the filter on Chromocult Coliform Agar
- Incubate for 24 h at 37 °C
- Count the colonies with the different colors
- Blue colonies: *E. coli*, pink colonies: coliforms
- Using other chromogenic media you can get different colony colors.

# Determination of coliforms from water



# Determination of Enterococci 1

- Gram positive cocci, *Enterococcus faecalis* and *Enterococcus faecium* are indicators of fecal contamination
- Enterococcus selective broth is used in the first step
- Sodium azide inhibit the growth of other bacteria

# Determination of Enterococci 2

- Weight out aseptically 10 g or 10 ml from the examined food
- Solid food should be masticate
- Add it to 90 ml sterile physiological saline (PS)
- Mix well
- Make a tenfold dilution series (5-6 dilution)

# Determination of Enterococci 3

- Inoculate 1ml from all dilution to 3-3 tubes containing Enterococcus selective broth
- Incubate the tubes (24 h, 37 °C)
- Observe for the presence or absence of growth - turbidity
- Create a key number as described above
- Enumerate cell count from the MPN table



# Verification of the presence of Enterococci on Chromocult Enterococci Agar

- Growth of Enterococci is stimulated. They cleave the chromogenic substrates in the medium resulting in red colonies.
- Sodium azide and ox bile inhibit most accompanying microbial flora.
- Non-Enterococci produce colourless, blue/violet or turquoise colonies.

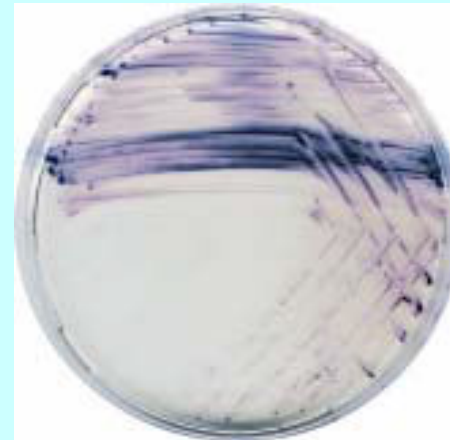
# Verification of the presence of Enterococci

- Streak from the positive tubes a loopful inoculum on Chromocult Enterococci agar
- Incubate the plates for 24 or 48 h at 37 °C
- Red colonies verify the presence of Enterococci.

# Chromocult Enterococci Agar



*Aerococcus faecalis*



*Aerococcus viridans*

Determination of  
*Staphylococcus aureus*

# Determination of *Staphylococcus aureus*

- *Staphylococcus aureus* is a gram positive coccus. The coagulase positive strains causing hemolysis are considered to be human pathogens.
- The methycillin resistant *S. aureus* (MRSA) can cause even death.

# Baird Parker agar

- Baird Parker Agar is used for the isolation and differentiation of coagulase-positive staphylococci in food.
- The medium contains lithium and tellurite, which inhibit most of the contaminating microflora, while glycine and pyruvate enhance Staphylococci growth.
- .

# Baird Parker agar

- Staphylococci can reduce tellurite to telluride, which results in grey to black coloration of the colonies.
- With the addition of egg yolk, the medium becomes yellow slightly opaque. A clear halo develops around colonies from coagulase positive *Staphylococcus aureus*

# Determination of *Staphylococcus aureus* 1

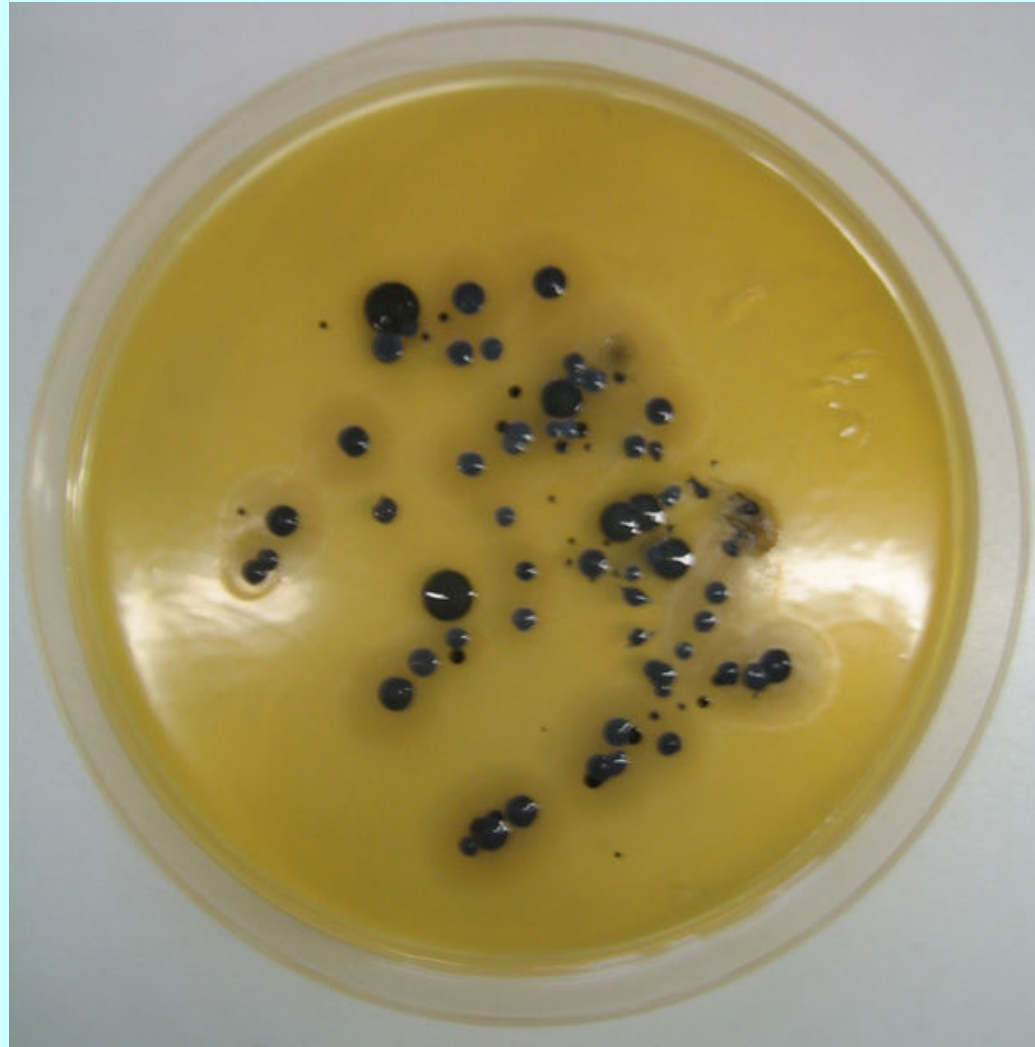
- Weight out aseptically 10 g or 10 ml from the examined food
- Solid food should be masticate
- Add it to 90 ml sterile physiological saline (PS)
- Mix well
- Make a tenfold dilution series (3 dilution)



# Determination of *Staphylococcus aureus* 2

- Inoculate Baird Parker Agar with 0.1 ml suspension from all dilutions
- Spread the inoculum over the medium
- Incubate for 24 h at 37 °C.
- Count the black colonies with a clear halo around.
- $\text{CFU/g} = \text{colony count} \times 10 \times \text{dilution factor}$

# *S. aureus* on Baird Parker Agar



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