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PROTOCOLS

MICROBIOLOGICAL ANALYSIS IN DAIRY INDUSTRY

ALL PROCEDURES ACCORDING TO ISO REGULATIONS

Inspired by knowledge

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Index

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Dairy industry: present and future

World milk production grew by 1.3% in 2020, amounting to **852 million tons** according to the OECD, being 81% of cow, 15% of buffalo and 4% a sum of goat, sheep and camel sources. For the next 10 years, it is estimated that this market will grow **1.6% annually.** A greater share of fresh dairy products is also anticipated in this market for the next decade, the forerunner of many other products.

This market dimension shows a reality, dairy products and their derivatives are in all refrigerators and pantries in the world, so their control is essential, even vital, to avoid **contamination by pathogens.** *Salmonella, Escherichia coli* O157: H7, *Listeria monocytogenes, Staphylococcus aureus, Yersinia enterocolitica, Bacillus cereus, Clostridium botulinum, Mycobacterium bovis, Brucella abortus* and *Brucella melitensis* are the potential agents of spoilage in this industry and those that should be **absent** in these products.

The absence of these controls has led to outbreaks of diphtheria, polio, typhus or tuberculosis throughout the world in the past. Even with basic pasteurization controls in Europe, between 1 to 6% of food poisoning outbreaks were caused by dairy products between 1993 and 1998 according to the WHO, with ***Salmonella*** and ***S. aureus*** being the most common. Others less common such as *Listeria* or *E. coli* presented a lethality of 16%.

In order to improve and increase the microbial control of the industry, **ISO standards** were established as reference analysis methods for the **quality control** of these products.

PART I:
Initiation and preparation

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Bacterial starter cultures for fermented milk products

Procedure as defined by ISO 27205:2010

Introduction

The preparation of starter cultures is predominantly for lactic acid bacteria, but also includes propionobacteria and bifidobacterial for the manufacture of fermented milk products. Bacteria used in the food industry for its probiotic properties are not contemplated.

ENUMERATION OF LACTIC ACID BACTERIA IN STARTER CULTURES:

- ***Lactobacillus delbrueckii* subsp. *bulgaricus*: MRS Broth method** (ISO 7889).
- ***Lactobacillus acidophilus*: MRS Agar** method with clindamycin and ciprofloxacin (ISO 20128).
- ***Enterococcus faecium*, pediococci and lactobacilli: MRS Agar** following ISO 7889, with a pH in the medium of 6 - 6,4. Incubate for 72h at 37ºC.
- **Lactococci and *Streptocuccus thermophilus*: M17 Agar (CAT. 1318)** (ISO 7889), with a pH of 7,2 for Lactococci (plate at 20ºC for 5 days) and pH 6,8 for *S. thermophilus* (aerobic, plate ate 45ºC for 48h).
- **Citrate-fermenting lactic acid bacteria:** (ISO 17792) The Nickels and Leesment method.
- ***Leuconostoc* spp.:** (ISO 17792) The Nickels and Leesment with vancomycin method.

METHODS FOR ENUMERATION OF *PROPIONOBACTERIUM* SPP. IN STARTER CULTURES

- Modified yeast extract-lactate medium.

METHODS FOR ENUMERATION OF *BIFIDOBACTERIUM* SPP. IN STARTER CULTURES

- By using **TOS Agar (CAT. 2011)** medium containing **Mupirocin (CAT. 6074).**

METHODS FOR DETECTION AND ENUMERATION OF CONTAMINANTS

- **Non-acid lactic bacteria:** ISO 13559.
- **Yeast and moulds:** OSP 6611, ISO 21257.
- ***Enterobacteriaceae*:** ISO 21528.
- **Coagulase-positive Staphylococci:** ISO 6888.
- ***Lysteria monocytogenes*:** ISO 11290. For pathogens test dilute 1g with 25g sterile diluent. Mix and add 225g of convenient strength of broth media.
- ***Salmonella* spp.:** ISO 6785. If analysed in presence of lactic acid bacteria: modify pre-enrichment medium depending on the CFU.
  - Up to 108 CFU: **Buffered Peptone Water (BPW) (CAT. 1402)** + vancomycin 10mg/L.
  - Up to 1011 CFU: **Buffered Peptone Water (BPW) (CAT. 1402)** + vancomycin 10mg/L + malachite green 40mg/L + milk 10g/L.
  - More than 1011 CFU: **Buffered Peptone Water (BPW) (CAT. 1402)** + vancomycin 10mg/L + malachite green 40mg/L + milk 10g/L.

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Specific rules for milk and milk products preparation

Procedure as defined by ISO 6887-5:2017

Introduction

Milk, being made up of 87% water, is prone to adulteration due to bad farm practices. Moreover, its high nutritive value makes it an ideal medium for the rapid multiplication of bacteria, particularly under an unhygienic production process and storage at ambient temperatures.

We know that, for any manufacturer to provide safe dairy products, good quality raw materials are essential. A milk processor or handler will only be assured of the quality of raw milk if certain basic quality tests are carried out at various stages of milk transportation, from the producer to the processor, and finally to the consumer.

1. Preparations

- Follow ISO 6887-1 for frozen products.
- For dry products mix with diluent (careful of temperature).
- Heterogenic products: homogenize.

2. General approach

Following good laboratory practices and clean procedures, some general observations shall be made:

- For acid products, first neutralize the pH.
- Food products with high fat content shall be diluted to improve emulsion during suspension.

3. Specific procedures

- **3.1. Milk and liquid milk products:** Mix 25 times and dilute 1:10 with **Saline Peptone Water (CAT. 1405)** (general diluent).
- **3.2. Milk powder, milk powder serum, dehydrated milk acid serum, butter powder serum and lactose:** Mix properly. Weigh 10 g and dilute in **general diluent.**
- **3.3. Cheese and cheese derived products:** Weigh 10g and dilute in 90mL of **general diluent.**
- **3.4. Butter:** Heat 10g of sample to melt at 45°C, add 90mL of **general diluent** and mix.

  A portion of 50g is diluted in the same **general diluent** so that proportionally you have 1mL of diluent for 1 g of butter. Heat in water bath at 45°C for no more than 15'.
- **3.5 Ice cream:** Dilute 10g of sample in 90mL of **general diluent** mixed at room temperature.
- **3.6 Custard, cream and deserts:** Add 10g of sample to a flask with crystal beads, dilute and mix at room temperature with 90 mL of **general diluent.**
- **3.7 Fermented milk and sour cream:** mix 10g of sample in 90mL of **general diluent** (pH 7,5).
- **3.8 Infant products based on milk:** Exhaustive mix. Dilute 10g of sample in 90 mL of **general diluent.**

  If the solution is hard to homogenise, add twice the normal amount of **general diluent.**

PART II:
Standard methods for the analysis

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Enumeration of CFU of yeasts and/or molds

Procedure as defined by ISO 6611:2004

Introduction

Growth of yeasts and molds is a common cause of the spoilage of dairy products, causing 5 - 10% of food spoilage. In fermented milks the risk is even higher because these microbes can grow at low pH ranges. Product spoilage leads to the waste of a great amount of food and its consequent economic impact, but, more importantly, they may carry grave consequences in the consumer. Yeasts and molds are not much dangerous by themselves. However, they produce mycotoxins, toxic particles that, in large doses and maintained over time, can lead to serious health problems.

1. Preparations

- Prepare sample following ISO 8261. Duration of the procedure is indicated in ISO 6887-1.

2. Inoculation and incubation

- Transfer to 2 Petri dishes 1mL of sample if liquid, or 1mL of the initial suspension. Repeat being "n" the amount of serial dilutions prepared.
- Transfer 15mL of **YGC Agar (CAT. 1301)** preheated and incubate at 25ºC for 5 days.

3. Interpretation

- Retain those plates with 10 - 150 colonies. In case of overgrown plates, retain a higher dilution even if it has less than 10 colonies.

4. Confirmation at the microscopy

- The identity of any pinpoint or doubtful colonies shall be investigated by microscopy examination.

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Enumeration of characteristic microorganisms in yogurt

Procedure as defined by ISO 7889:2003

Introduction

Yogurt is an essential element in many cultures, and it has been present in history for more than 10.000 years. During all this time, even when people weren't aware of it, microorganisms have been used for its production.

Nowadays we know plenty about which microorganisms are used in this process, the two most important being *L. delbrueckii* subsp. *bulgaricus* and *S. termophilus*. Hence, the need of their microbiological control.

This standard method allows the counting and control of these two microorganisms population in yogurt.

1. Preparation of test portion

- For general requirements follow ISO 8261.
- For non-fruit yogurts: after mixing the sample, weigh 10g of it.
- For fruit yogurts: Blend contents for 1', then weigh 10g.

2. Microscopic examination

- For the determination of proper ranges of dilution. Stain the sample smear with **Methylene Blue (CAT. 5058)** before handed. Estimate the density of cocci and rods.

3. Preparation of primary dilution

- Follow ISO 8261. Maximum weight of sample with diluent shall not surpass 50 g. Blend for 1' and then rinse with diluent to 100 g mass to obtain a 1:10 dilution.
- Serial dilutions as determined in ISO 8261, as well as the duration of the process.

4. Inoculation and incubation

Proceeding with duplicates, transfer 1mL of each dilution to petri dishes:

- For *L. delbrueckii* subsp. *bulgaricus,* pour 15 mL of **Acidified MRS Medium** preheated at 45°C into each Petri dish.
- For *S. termophilus* pour 15 mL of **M17 Agar (CAT. 1318)** preheated at 44 - 47°C.

Mix by rotation and let the mixture solidify.

In the first case, incubate in anaerobic conditions at 37°C for 72h. In the second case, incubate at 37°C for 48h.

5. Colony counting

- Take into account only those plates with 15 - 300 colonies, counting only those with the characteristic features of each microorganism, examining plates under subdued light.

6. Confirmation

Stain selected colonies using **Gram method (CAT. 4600)**:

- Non spore-forming Gram-positive, catalase negative rods for those colonies grown in **Acidified MRS Medium.**
- Gram-positive, catalase negative for chains of cocci or diplococci for those grown **in M17 Agar (CAT. 1318).**

Doubtful strains may be checked following ISO 9232.

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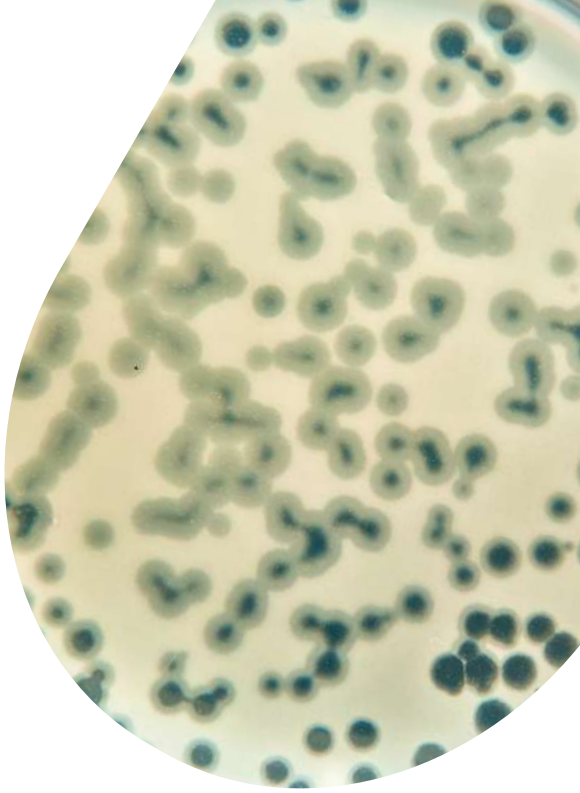

Enumeration of microorganisms in milk

Procedure as defined by ISO 8553:2004

Introduction

Milk can have pathogenic microorganisms such as *Salmonella, Escherichia coli* O157:H7, *Listeria monocytogenes, Staphylococcus aureus, Yersinia enterocolitica, Bacillus cereus, Clostridium botulinum, Mycobacterium bovis, Brucella abortus* and *Brucella melitensis.* These pathogens get into the milk through the mammary gland, even if the animal shows no sign of being infected or ill.

They are the main reason raw milk goes through different sterilization processes as UHT, usual sterilization or pasteurization. The ingestion raw milk has a considerably higher chance of provoking adverse effects on the consumer than drinking pre-treated milk.

This standard method specifies a technique for the enumeration of microorganisms in raw milk by using the plate-loop technique at 30°C.

1. Preparations

- Warm samples at 15 - 20°C and shake.

2. Inoculation and incubation

- Collect 0,001mL of the sample in Petri dishes and wash them with 1mL of sterile diluent repeat as many times as needed. Sterilize loop after every 20 uses.
- Add 10 - 12mL of **PCA Milk Agar (CAT. 1033)** to each Petri dish, and incubate at 30°C for 72h.

3. Counting colonies

- Count manually. Only plates with 10 - 300 colonies are to be considered.

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Identification of tipical microorganisms (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*)

Procedure as defined by ISO 9232:2003

Introduction

Lactobacillus bulgaricus and Streptococcus thermophilus have been used since the old Tracia for the fermentation of sheep milk into cheese and yogurt in 6000 - 7000 BCE.

Nowadays, these microorganisms are used for the same process, therefore its identification and confirmation are crucial for the correct development of the process in the industry. To do so, this ISO norm establishes reference protocols and resources to reach the goal.

1. Isolation

- Select colonies for isolation from plates seeded as indicated in ISO 7889, using the stipulated broths to obtain pure cultures after incubation. Incubate at 37°C for 24h.

2. Identification and confirmation

2.1 Identification of *L. delbrueckii* subsp. *bulgaricus*. Use skimmed milk and MRS broth (CAT. 1215) prior to any test.

- **Morphology:** incubate at 37°C for 24h in skimmed milk, stain with **Methylene Blue (CAT. 5058)**, then check.
- **Growth in broth at 15°C and 45°C:** use a drop of inoculated **MRS Broth**. To incubate at both temperatures for 7 days.
- **Fermentation of sugars:** follow instructions of fermentation kit.
- **Catalase reaction:** mix equal volumes of **MRS Broth.** Incubated at 37°C for 18 - 24h with 1,5% hydrogen peroxide. Observe possible bubble formation at room temperature for 20 minutes.
- **Production of CO_2:** inoculate 10 mL of culture medium with 0,1mL **MRS Broth** of the test strain and incubate at 37°C for 24h with the tube covered with an agar layer.
- **Lactic acid enantiomers:** Subculture twice, check purity with **Methylene Blue (CAT. 5058)** and then subculture a third time. After testing for purity at the microscope check enantiomers Check enantiomers.

2.2 Identification of *S. termophilus.*

- **Morphology:** incubate at 37°C of 24h in litmus milk. Then stain smears of cultures with **Methylene Blue (CAT. 5058)** for 10'.
- **Catalase reaction:** mix equal volumes of **MRS Broth.** Incubate at 37°C for 18 - 24h with 1,5% hydrogen peroxide. Observe possible bubble formation at room temperature for 20 minutes.
- **Growth in litmus milks at 10°C and 45°C:** test tubes with litmus milks shall be added a drop of **M17 Broth.** Incubate for 7 days at both temperatures.
- **Growth in presence of sodium chloride:** inoculate test tubes with one drop of **M17 Broth.** Pre-incubated overnight at 37°C. Incubate at 37°C for 7 days.
- **Action in litmus milk:** litmus milk acidified by *S. termophilus* turns into pink.

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Enumeration of *Pseudomonas* spp. in milk and milk products

Procedure as defined by ISO 11059:2009

Introduction

Pseudomonas contamination usually means defects in appearance, taste, texture, and odor, which can sometimes lead to loss of trust in the brand.

They can easily appear after the fermentation and the calcic lactate production, as the pH rises enough for *Pseudomonas* to grow. Their presence usually is detectable because of the bitter taste and rancid odor.

For these reasons they must be detected, and the products contaminated discarded.

1. Test portion, initial suspension and dilutions

- Prepare them in accordance with ISO 6887-5.

2. Inoculation and incubation

- Culture in **PPA plates** the proper serial dilutions.
- Pipette 0.1mL of sample to each and incubate at 25°C for 48h.

3. Counting and selection

- Counting and selection of colonies from the plates with 150 or less UFC, selecting 5 colonies from each plate.

4. Confirmation

First, a subculture in **Nutrient Agar (CAT. 1060)** shall be done. Incubate plates at 25°C for 24 - 48h.

- Oxidase reaction: **+**, incubate for 5 - 30s in presence of **Oxidase Reagent (CAT. 6007).**
- Fermentation of glucose: **-**, incubate at 25°C for 24h in **BCP Glucose Agar (CAT. 1320).**

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Enumeration of presumptive *E. Coli* in milk and milk products

Procedure as defined by ISO 11866-1: 2005

Introduction

Escherichia coli is a group of microorganisms with a great diversity and only a few are pathogenic when ingested. However, its is still one of the most common species associated to toxiinfections, included milk and milk products. Generally, the consequence of their infection is the unspecific enterocolitis. It is more commonly found in raw milk, but it can survive certain processes and post-process conditions, so its detection is necessary and mandatory.

1. Preparation

- According to the method given in ISO 8261.

2. Initial suspension

- Test portion must be prepared, and dilutions made following ISO 8261.

3. Inoculation

- Three tubes of double strength modified **Lauryl Sulfate Medium**, transfer to each 10 mL of sample if liquid or 10mL of primary dilution.
- Then transfer to 3 tubes with enrichment medium 1mL of sample if liquid or 1mL of primary dilution in the case of other products. (Repeat if necessary).
- Mix inoculums with medium.

4. Incubation

- Incubate tubes at 30ºC for 24h.

5. Confirmation

- Add 0,5mL of **Kovac's reagent (CAT. 5205)** and mix for 1'.
- Gas formation and fluorescence shall be checked.

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Enumeration of presumptive *E. Coli.* Plate count at 44°C

Procedure as defined by ISO 11866-2:2005

Introduction

This method is preferred for those samples in which, comparatively, there are large numbers of presumptive *E. coli*: more than 100 CFUs per gram or 10 CFUs per milliliter. Caution must be kept in the results interpretation as some pathogenic *E. coli* strains do not grow at 44°C.

1. Sample preparations and dilutions

- According to the method given in ISO 8261.

2. Resuscitation

- Place cellulose acetate membranes on **MMG Agar.** Pour on them different dilutions for the sample. Incubate at 32°C for 4h.

3. Incubation in selective medium

- Transfer membranes to the **TBA Medium (CAT. 1013)**.
- Incubate at 44°C for 24h.

4. Detection and enumeration

- Pipette 2mL of **Kovac's Reagent (CAT. 5205)** over the membranes. Then, dip the membranes in the same reagent.
- Remove excess of reagent. For a permanent record, place the membrane under UV light for 30'.

5. Confirmation

- Add 0,5mL of **Kovac's Reagent (CAT. 5205)** and mix for 1'.
- Gas formation and fluorescence shall be checked.

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Enumeration of contaminating microorganisms. Colony count technique at 30°C

Procedure as defined by ISO 13559:2002

Introduction

These microorganisms suppose a grave potential danger to human health. *Staphylococcus aureus, Salmonella* spp., *Listeria monocytogenes, Escherichia coli* O157:H7 and *Campylobacter* spp. are the most common pathogens associated to raw milk consumption.

1. Preparation of test portion and initial suspension

- For the general requirements follow ISO 6887-1, for specific ones follow ISO 8261, as well for dilutions.

2. Inoculation

- Inoculation in a general culture media for general requirements (ISO 6887-1) or specific ones like butter, fresh cheese, or fermented milk (ISO 8261).
- Incubate at 30°C for 72 ± 2h in **Gelysate Agar (CAT. 1187).**

3. Colony count

- Count colonies with typical characteristics of contaminating microorganisms.

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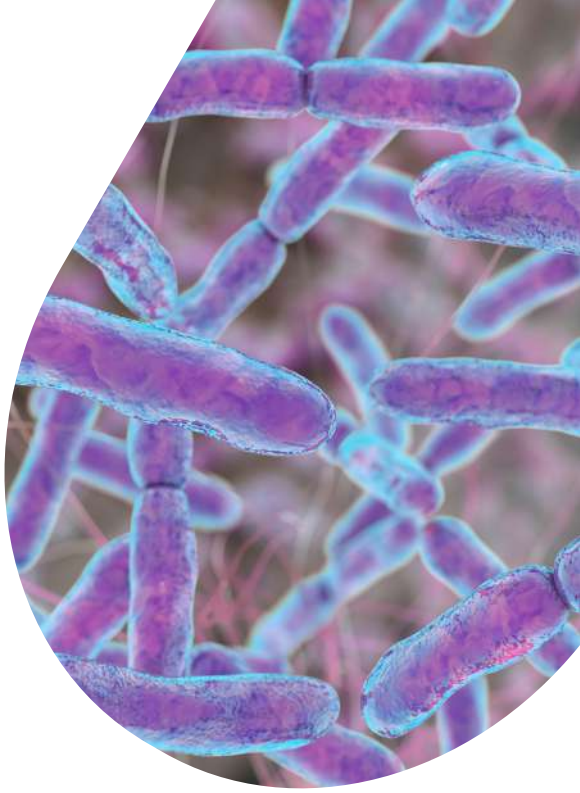
Enumeration of presumptive bifidobacteria



Procedure as defined by ISO 29981:2010

Introduction

Bifidobacterium is an important probiotic agent for many milk products, as they offer numerous health benefits: lowering of serum cholesterol levels, enhancing immune function, or reducing lactose intolerance. Generally used a component of the starter culture intended to produce fermented beverages, cottage cheeses, ice creams, and frozen desserts.

1. Preparation of test portion

1.1 Dried milk products.

Weigh 90 g of **Saline Peptone Water (CAT. 1405)** or **Ringer 1/4 Solution (CAT. 4101)** in each bottle and heat in bath at 45°C. Then add 10g of test sample. Heat for 5' and and cool while shaking for 2'.

1.2 Yogurt like products (ISO 6887-5).

At RT weight 90 g of **diluent**, pour in closed bottles and mix. Add 10 g of test samples and mix again.

2. Microscopic examination

Microscopic examination to determine proper dilutions to be used. Alternatively, use phase microscopy.

3. Preparation of decimal dilutions

They shall be done 1:10 in sterile diluent. Mix before and after. Four steps of dilution recommended.

4. Inoculation and incubation

1mL per dilution into Petri dishes with **TOS Propionate Agar Base (CAT. 2011)** supplemented with **MUP (CAT. 6074)**, 2 replicates each. Add 12 - 15mL of medium and mix. Cultivate at 37°C for 72h.

5. Colony counting

The bifidobacterial colonies are those with whitish colour. Count those plates with countable amount of colonies.

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Enumeration of the specially thermoresistant spores of thermophilic bacteria in dried milk

Procedure as defined by ISO 27265:2009

Introduction

This method is limited to dehydrated milk products. The absence of spores in them is essential, so the final product has no potential poisoning agents. Spore forming microorganisms can adapt themselves to survive UHT processes by forming spores, growing and contaminating the final product once conditions are less aggressive.

Spores can survive processes under 140°C, like those between 90°C and 121°C. Once these products are left to a room temperature, those spores can develop full microorganisms as *Clostridium* or *Cronobacter.*

The infection of *Cronobacter* in infants is not frequent, but is very dangerous, even lethal (septicemia or meningitis). They are most found in dried milk for babies.

1. Preparation

- Prepare the primary dilution according to ISO 6887-5.

2. Sample heat treatment

After diluting sample 1:10, transfer 10mL to different tubes. Place them in boiling water bath. Record times for:

- Sample reaches 100°C. Close steam vent.
- Pilot reaches 105,5°C (max. 6').
- Maintain a constant temperature of 106°C for 30' since reaching the second step.
- Turn off heat source. When pilot tube is under 100°C release pressure in all vessels and transfer rack to a bath at 15 - 25°C.

3. Inoculation and incubation

- Transfer 1mL of primary dilution, secondary dilution and pilot to 3 petri dishes each with **BCP Count Skim Milk Agar with 0,2% mass starch.** Now pour 15mL of media in each. Mix and cultivate at 55°C for 48h.

4. Counting colonies

- Count the plates under subdued light.

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