UNIVERSITY OF LJUBLJANA
BIOTECHNICAL FACULTY
DEPARTMENT OF ANIMAL SCIENCE
CHAIR OF DAIRY SCIENCE

MILK ANALYSIS

INSTRUCTIONS FOR PRACTICUM

NAME

_____

Domžale, 8 - 9 May, 2018

MILK

Milk is a very complex food with over 100.000 different molecular species found. There are many factors that affect the composition of raw milk such as breed, age and physical state of the cow and seasonal variations. Therefore only an approximate milk composition of 87-88 % water and 12-13 % total solids can be given. The total solids consist of approx. 4 % fat and 9 % solids-non-fat (SNF) (proteins, lactose, minerals, vitamins, ...).

MILK QUALITY

Milk quality is composed of:

- Chemical quality
- Microbiological quality
- Physical quality
- Sensory quality

The **chemical quality** of milk describes the content of principal solids of milk such as *fat*, *protein*, *lactose* and *minerals*. These ingredients are called main because they represent major part in the milk. They can be calculated as a percentage of solids, solids-non-fat (SNF) or fat-in-solids (FiS). In this category we can also find minor components such as individual minerals (calcium, phosphorus ...), vitamins, enzymes, and other milk-pollutants such as heavy metals, aflatoxins, pesticides, antibiotics, detergents and disinfectants, whose presence is harmful and therefore illicit.

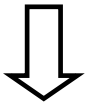
When we speak of **microbiological quality** we distinguish two categories: *total bacterial count* and the presence (we prefer absence ☺) of pathogenic microorganisms. The total bacterial count reflects the level of hygiene of milk production, storage and transport as well as cooling efficiency, while the presence of pathogenic bacteria reflects hygienic and health conditions of milking animals and people who are in contact with milk.

The most important characteristics that determine the **physical quality** are milk *density*, *freezing point*, *titratable acidity* and *pH value*.

Sensory quality is determined by *taste*, *odor*, *color*, *flavor* and *consistency*.

The successful processing of milk with the objective of producing quality products depends not only on the multitude parameters that we have just enumerated, but also on **technological quality** of milk that is described by the composition of milk fat and milk protein, milk enzyme system, groups of microorganisms that compose microbial population of milk, the ratio between the minerals, the amount of citrate, ura, etc... These properties affect the heat stability of milk, coagulation properties of milk, the ability of milk to promote growth and activity of starter lactic acid bacteria, the formation of taste and flavor as well as the stability of the product.

CHEMICAL + MICROBIOLOGICAL +PHYSICAL + SENSORY + TECHNOLOGICAL QUALITY



PROPER MILK QUALITY FOR PROCESSING

According to regulations, milk must meet the following criteria:

1. that is milked for at least 30 days before and no less than 8 days after calving
2. that has a characteristic smell, taste and color
3. that freezing point is not higher than -0.52 ° C, the refractive number not lower than 39
4. that contains at least 3.2 % of milk fat
5. that has a density from 1.028 to 1.034 g/cm^3 at 20 °C
6. that the titratable acidity level does not exceed 7.2 SH (or pH 6.5 to 6.7),
7. that withstand the alizarol test with 71.5 % alcohol
8. that immediately after milking, milk is cooled to less than 8 ° C or transferred to a collection center and then cooled to less than 6 ° C.

In addition to these criteria, the quality of milk also consider:
9. that the content of fat-in-solids (FiS) is at least 27.5%
10. that the content of solids-non-fat (SNF) is at least 8.5%.

Milk payment

The calculated minimum purchase price for cow's milk is usually formed according to fat content, protein content, total bacterial count and somatic cell count.

Depending on the content of **total bacterial count** in milk, milk **used to be** classified in four classes:

Quality class	**Total bacterial count (CFU/ml)**
E (extra) quality	Up to 50.000
1. quality class	50.001 - 100.000
2. quality class	100.001 - 400.000
3. quality class	400.001 - 800.000

Nowadays, regarding the EU regulations, basic milk hygiene requirement in the EU should follow:

Drugs/ml	<0.004µg
Somatic cells/ml	≤400 000
Bacteria (CFU)/ml	≤100 000

Somatic cell count (**SCC**) is an indicator of the quality of milk. *White blood* cells (leukocytes) constitute the majority of somatic cells in question. The number of somatic cells increases in response to pathogenic bacteria like *Staphylococcus aureus*, a cause of mastitis (inflammation of udder). General agreement rests on the values of less than 100.000 cells/ml for uninfected cows and greater than 250.000 (but not exceeding 400.000 cells/ml) for cows infected with significant pathogens (sub-clinical mastitis). The somatic cell count in the milk also increases after calving when colostrum is produced.

Milk-Specific (Recknagel) Phenomenon
Milk density **increases** slowly after milking (by up to 0.001 g/cm3) and reaches a constant value between 3-6 hours after milking. Therefore, milk density is never measured immediately after milking due to milk stabilization. This phenomenon was discovered by Recknagel. The density increase is due to slow solidification of milk fat and hydration of milk proteins and, to a lesser extent, to escaping of air bubbles.

Preservation of milk samples

Preservatives should normally **not be added** to milk samples intended for

- microbiological examination
- sensory examination
- freezing point determination
- testing the presence of antibacterial substances

but **may be added** to milk samples intended for automated analysis such as

- determining the main milk components with Milkoscan
- somatic cell count analysis with Fossomatic
- total bacterial count analysis with BactoScan

MILK DENSITY

Density (ρ) is the mass of a particular volume of material, expressed in kg/m^3 or g/ml. Since density is highly dependent on temperature, we measure it at 20 °C. The density of milk is also used in equations for calculating content of solids in milk. $\rho^{20\ °C}$ of cows' milk is on average 1030 kg/m^3, **spanning between 1028 and 1034 kg/m^3**, but depends on the composition and density of individual components of the milk at 20 ° C as follows:
- Water 998 kg/m^3
- Fat 918 kg/m^3
- Protein 1400 kg/m^3
- Lactose 1780 kg/m^3
- other substances (mostly minerals) 1850 kg/m^3

Milk density is measured with **LACTODENSIMETER**.

Correction factor
it the T of milk sample is not exactly 20 °C, the measured density can be corrected with factor of ±0.00025/°C. If the T of milk sample is higher than 20 °C, for each degree we add correction factor, but if the T is lower than 20 °C we subtract the correction factor. For example:
$\rho^{20} = \rho^{25} + 5 \times 0{,}00025$
$\rho^{20} = \rho^{17} - 3 \times 0{,}00025$

LACTODENSIMETER

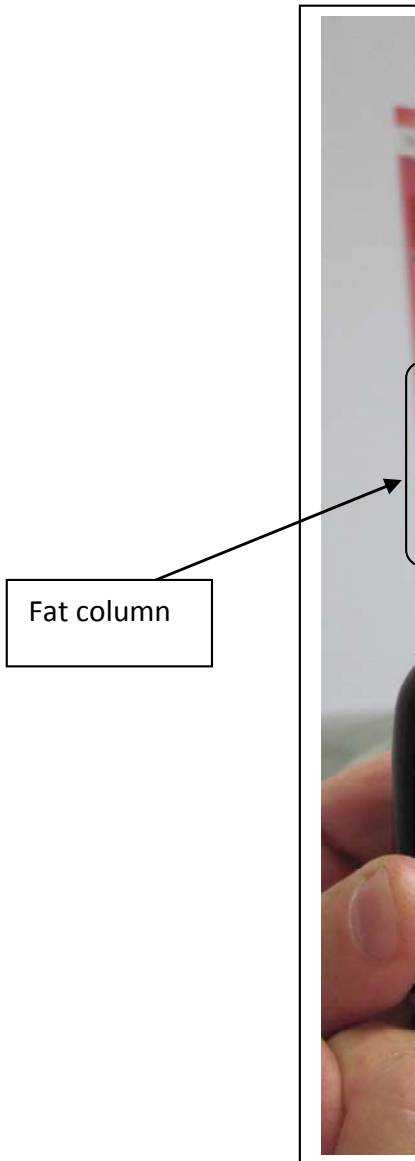
MILK FAT CONTENT

Performed by the routine **Gerber method**
Chemicals used: H_2SO_4, amyl alcohol
We perform analyze in BUTYROMETER.

Principle of the method:
Separation of milk fat in butyrometer by centrifugation after degradation of protein coat around milk fat globules with sulfuric acid (H_2SO_4), the separation is easier after the addition of small amounts of amyl alcohol. **Butyrometers are graduated for direct reading of fat content.**

Protocol:
In butyrometer, we add 10 ml of H_2SO_4, than 10.75 or 11 ml of milk and finally 1 ml of amyl alcohol. We mix, centrifuge and read the fat content.
The role of sulphuric acid (H_2SO_4) is to destroy the thin membrane that surrounds the fat globules which enables easier separation of milk fat, while amyl alcohol lowers the surface tension and facilitate direct reading of the fat content from the measurement scale of the butyrometer.

According to the regulation:
raw milk must contain at least 3,2 % fat



MILK FRESHNESS

determination of acidity

- where we determine the "acidity" of milk by:
a) titratable acidity: from **6,0** to **7,5 SH**
b) pH measurement: from **6,6** to **6,8**

- or by two rapid (qualitative) tests
a) alcohol test: corresponding white color with lack of precipitates
b) alizarol test: corresponding brick-red color with lack of

A) TITRATABLE ACIDITY – Soxhlet Henkel (SH) method

Quantitative analysis

Objective

The **titratable acidity** according to Soxhlet-Henkel (SH) is the volume of NaOH solution (0,25 M) which is used up in the titration of **100 mL of a milk**. The titration is performed to a certain standard colour shade using phenolphthalein as indicator that changes color from colorless to faint pink.

The acidity in milk indicates the consumption of NaOH necessary to shift the pH-value from 6,6±0,1 (corresponding to fresh milk) to a pH-value of 8,2-8,4 (phenolphthalein).

Method

Preparation of standard solution

Pipette 50 ml of milk into plastic container and mix with exactly 1 ml of cobalt sulphate solution ($CoSO_4$). The standard faint pink color is developed and it holds for maximum 3 hours.

Determination of the titratable acidity

50 ml of the milk pipette into a plastic container and add 2 ml of phenolphthalein (2%). Then you titrate, while continuously swirling, with 0,25 M NaOH until you reach the color of standard solution. You should not need more than one minute for the titration.

RESULT

$SH = V_{0,25M\ NaOH} \times 2$

Fresh milk: titratable acidity up to 7,2 SH (according to regulation)

B) ALCOHOL AND ALIZAROL TEST

Qualitative analysis

Objective of the ALCOHOL test

The alcohol test is used on fresh milk to indicate whether it will coagulate on processing. Normal fresh milk does not react to the addition of alcohol. As soon as the microorganisms ferment milk sugar (lactose) to lactic acid and milk acidity increases to about 9.0 SH, such milk precipitates when the equal amount (alcohol single test) of alcohol is added. When double amount of alcohol is added (alcohol double test), milk

precipitates already at lower acidity such as 8,0-8,5 SH. This test serves as a quick test to assess the quality of milk.

Reagent: 75% Et-OH

Method
- mix equal volumes of milk and 75% alcohol in a test tube. The best amount is 2 ml of each
- after intensive mixing examine the tube to determine whether the milk has coagulated: if it has, fine particles of curd will be visible

Interpretation of results

SINGLE TEST	DOUBLE TEST
milk : alcohol = 1:1	milk : alcohol = 1:2
Fine particles of curd are visible at acidity higher than 9 SH	Fine particles of curd are visible at acidity higher than 8 SH

Objective of the ALIZAROL test (colorimetric determination of pH)

The alizarol test, due to its simplicity as well as its suitability to point out more than one defect of milk is a practical and well-suited procedure to test raw milk for acceptability, either on the farm or from the tanker at the dairy plant entrance. The main objective of the test is to indicate milk in which an unaccepted level of acidity has developed.

The stability of the protein complex in milk is destabilized by acid and therefore a positive alizarol test is also an indication of the heat stability of milk (i.e. the resistance of the protein complex against the denaturation by heat). The test is therefore also popular where the heat stability of milk proteins is of importance during processing, e.g. during the production of milk powder or UHT milk.

By adding the pH-indicator alizarine to the solution an indication of whether the milk is abnormally sour or alkaline can be obtained.

Alcohol precipitates proteins, while **alizarin** (color indicator) changes color due to the level of milk acidity (as a result of concentration of hydrogen ions). Color is compared to

the color scale where the individual colors (brownish/purple - normal, yellow - sour, purple - alkaline, pink - sweet coagulation) are declared to the certain pH value.

Reagent: saturated solution of alizarine in 70% Et-OH = alizarol

Objective of the test
The stability of the protein dispersion in milk is maintained by hydration (combining with water) and by the negative electrical charges on the protein particles. If either of these two factors undergoes a change, the proteins will flocculate. Alcohol is a dehydrator and therefore destablises the protein. If the protein is already slightly unstable due to souring of the milk, dehydration with alcohol will lead to the precipitation of the protein in the form of flakes.

Method
- mix equal volumes of milk and alizarol in a test tube. The best amount is 2 ml
- after intensive mixing **examine** the **tube content** for **color** and **flocculation**

Interpretation of results

TYPE OF MILK	pH	FLOCCULATION PROPERTIES	COLOR
Fresh milk	6.60 – 6.45	None	Brownish-purple
Slightly sour	6.30 – 6.50	Possibly small flakes	Brownish-pink
Sour	6.00 – 6.20	Small flakes	Brownish-pink
Very Sour	<6.00	Big flakes	Yellow
Sweet coagulation	6.60 – 6.75	Big flakes	Brownish-purple
Mastitis	6.80 +	Small flakes	Violet
Added alkaline	6.80 +	None	Violet

Factors affecting the test
- acidity: the presence of lactic acid is the most important cause of a positive alizarol test. At the higher acid level (lower pH) the protein suspension in milk will be less stable with the result that protein flocculation (coagulation) will occur when acid milk is mixed with alcohol.
- mastitis: mastitic milk differs from normal milk as regards, amongst others, the protein and mineral composition. These differences cause the casein (protein) to flocculate more readily in mastitic milk. As the pH of mastitic milk is normally higher than that of normal milk the alizarine colour will turn violet if such milk is tested.
- sweet curdling: certain contaminating bacteria are capable of producing rennin like enzymes, which increase the viscosity of the milk and even flocculate casein in the absence of acid. This phenomenon is known as sweet curdling. Such milk can be identified by inoculating aseptically a small quantity there-off into sterilised milk and

incubating the inoculated milk at room temperature for a day or more on which the viscosity of the milk will

increase at the normal pH of fresh milk. Large numbers of these bacteria e,g. 800,000 /ml milk was found to create a positive reaction in previously sterilised milk.
- mineral imbalance: too high or to low a concentration of minerals such as calcium, phosphate and citrate will increase the ease with which casein will flocculate in the presence of alcohol. This aspect is complex and not well define. It is well known that the addition of very small amounts of calcium and magnesium will normally result in a positive test while the addition of phosphate and citrate salts may alleviate the situation. In more extreme cases excess phosphate and citrate may however be the cause of a positive test.
- stage of lactation: the chemical composition of early and late lactation milk differs from that of normal milk in the sense that it is characterised e,g. by higher levels of albumin, globulin and chloride. Due to these differences the protein suspension in such milk is usually very unstable against alcohol. Milk is normally very unstable to alcohol in early lactation, thereafter gradually becoming more stable and remaining at a fairly constant level of stability which is specific for each cow. Towards the end of lactation milks from some cows become more stable but a decrease in stability is more common.

PROTEIN CONTENT IN MILK

Formaldehyde titration (method described by Schulz)

Objective
Neutral aqueous solutions of amino acids in the presence of neutral formaldehyde solution react acid. When formaldehyde is added to milk, amino groups of proteins are bound to formaldehyde and the acidic groups are released, which can be determined by titration as amount of milk protein.

METHOD
Preparation of standard solution
25 ml of the milk pipette into a plastic container, add 0.5 ml of cobalt sulphate solution ($CoSO_4$) and 1 ml of potassium oxalate. The standard faint pink colour is developed and it holds for maximum 3 hours.

Determination of protein content with titration, using 1/7 M NaOH solution

Step 1: pipette 25 ml of milk into plastic container, add 0,25 ml of phenolphthalein and 1 ml of potassium oxalate. After, neutralize mixture with 0.25 M NaOH solution to pink color (resembling to the color of standard solution).

Step 2: Then add 5 ml of neutral formaldehyde solution and after 2 minutes again titrate with 1/7 M NaOH until you reach the colour of standard solution.

You should not need more than one minute for the titration.

The consumption of 1/7 M NaOH solution in the second titration corresponds to the protein content of milk.

RESULT

1 ml of 1/7 M NaOH = 1 % of protein

Result: % protein=$V_{0,143\ NaOH}$ needed for titrating milk sample

Normally in milk: 3.2-3.5 % protein

HEAT TREATMENT OF MILK

enzymatic tests

Milk is pasteurized or sterilized in order to protect consumers from illnesses that could be result of potentially pathogenic microorganisms present in raw milk. Temperatures of pasteurization destroy agents of tuberculosis, brucellosis, mouth disease, *E. coli* and other pathogenic microorganisms. It may happen that pasteurization is not successfully accomplished. Since the methods to detect presence or absence of pathogenic micro-organisms are difficult and time-consuming, other indicator methods are used to determine whether the milk has been properly heat treated. This allows milk enzymes, peroxidase and phosphatase. Peroxidase is inactivated at high pasteurization and sterilization temperatures, while alkaline phosphatase is already inactivated in all types of pasteurization. In milk, which has been properly pasteurized, enzymes are not active.

Alkaline Phosphatase (ALP) test

Alkaline phosphatase (ALP) is an enzyme naturally present in raw milk. At temperatures above 60 °C is gradually inactivated and at the proper pasteurization is completely inactivated (denatured). Proving ALP activity is based on the ability of an enzyme that catalyzes the hydrolysis of di-Na-para-nitrophenylphosphate (phosphate ester). The addition of di-Na-para-nitrophenylphosphate to milk, it is degraded by phosphatase induces its hydrolysis in disodium phosphate and phenol, which is yellow in color.

Reagents:
- buffer solution: 3.5 g of $NaCO_3$, and 1.5 g of $NaHCO_3$ per 1000 ml of water
- 0.15 g of di-Na-para-nitrophenylphosphate per 100 ml of buffer solution

Method:
Pipette 5 ml of buffer solution of di-Na-para-nitrophenylphosphate in a test tube, add 1 ml of milk sample, stir and incubate at 37 ° C (optimum T for ALP activity) for 30 minutes. If the heat treatment of milk was inadequate, ALP is still active and liberates phenol from a disodium phenyl phosphate substrate. **Liberated phenol colors milk sample with intensive yellow color.** If the heat treatment of milk was adequate, ALP was inactivated leading to no liberation of phenol and no change in color.

Peroxidase test

The enzyme peroxidase is also naturally present in raw milk. It is inactivated during thermal treatment of milk at 70 °C in 150 minutes, at 75° C in 2.5 minutes or at 80 °C in 2.5 seconds.

Peroxidase enzyme catalyzes the oxidation of various organic substances with hydrogen peroxide ($H_2O_2 \rightarrow H_2O + ½\ O_2 + 21.5$ kcal). To detect the reaction, milk sample is mixed with H_2O_2 and substance (1,4-phenylendiamine) which bound released oxygen and turns blue. In milk that has been properly heat treated peroxidase is inactive and milk remains white.

Reagents:
- 2% aqueous solution of 1,4-phenylenediamine ($C_6H_8N_2$)
- hydrogen peroxide: 9 ml of 30% H_2O_2 diluted with water to 100 ml

Method:
Pipette 5 ml of milk sample in a test tube, add 2 drops of 2% 1,4-phenylenediamine and 1 drop of 3% of H_2O_2. Mix well and after 30 seconds observe the change in color.

Interpretation

occurrence of indigo-blue color	raw milk
occurrence of a gray-blue color	milk has been heated to a temperature 79-80 ° C
no change in color (white color)	milk has been heated above 80 ° C

MILK SOLIDS

- direct method: drying of milk sample at 102 °C up to the constant weight
- indirect method: calculating by the use of Fleishmann equation

Fleishmann equation:

$$\%\ \text{solids} = 1{,}2 * \%\ \text{m} + 2{,}665 * \frac{100 * \rho^{20} - 100}{\rho^{20}}$$

% m – fat (by Gerber method)
ρ^{20} – density at 20 °C (by lactodensimeter)

Calculations:

SNF (Solids-non-fat): %S - %F =

(fat-in-solids) $\mathrm{FiS} = \frac{\%F}{\%S} \times 100\% =$

According to regulation, in milk must be:
- content of fat in solids at least 27,5 %,
- content of solids non fat at least 8,5 %.

PRODUCTION OF FERMENTED MILK PRODUCT – natural cup set yoghurt

Milk products prepared by lactic acid fermentation (e.g. yoghurt) or a combination of this and yeast fermentation (e.g. Kefir) are called fermented or cultured milks.

Fermented milk is the collective name for products such as yoghurt, ymer, kefir, cultured buttermilk, filmjölk (Scandinavian sour milk), cultured cream and koumiss (a product based on mares' milk). The generic name of fermented milk is derived from the fact that the milk for the product is inoculated with a starter culture which converts part of the lactose to lactic acid. Dependent on the type of lactic acid bacteria used carbon dioxide, acetic acid, diacetyl, acetaldehyde and several other substances are formed in the conversion process, and these give the products their characteristic fresh taste and aroma. The microorganisms used in the production of kefir and koumiss also produce ethyl alcohol.

YOGHURT

Yoghurt is the best known of all fermented milk products, and the most popular worldwide.
The consistency, flavour and aroma vary from one district to another. In some areas, yoghurt is produced in the form of a highly viscous liquid, while in other countries it is in the form of a softer gel. Yoghurt is also produced in frozen form as a dessert, or as a drink. The flavour and aroma of yoghurt differ from those of other acidified products, and the volatile aromatic substances include small quantities of acetic acid and acetaldehyde.

The typical yoghurt starter culture is composed *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*.

- Set type: incubated and cooled in the package, Figure A
- Stirred type: incubated in tanks and cooled before packing, Figure B

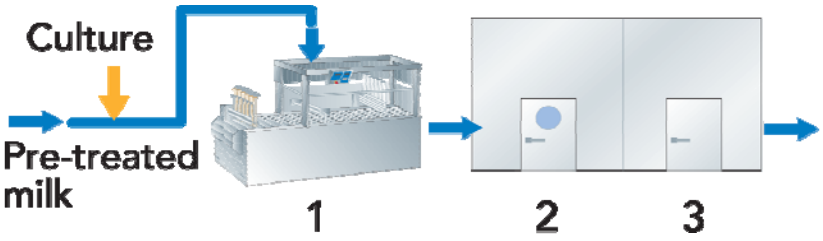


Figure A. Set yoghurt.

1. Cup filler
2. Incubation room
3. Rapid cooling

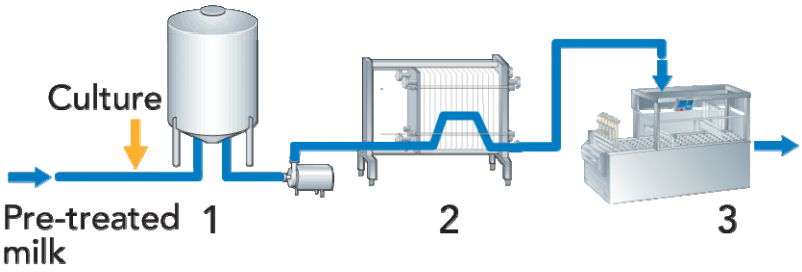
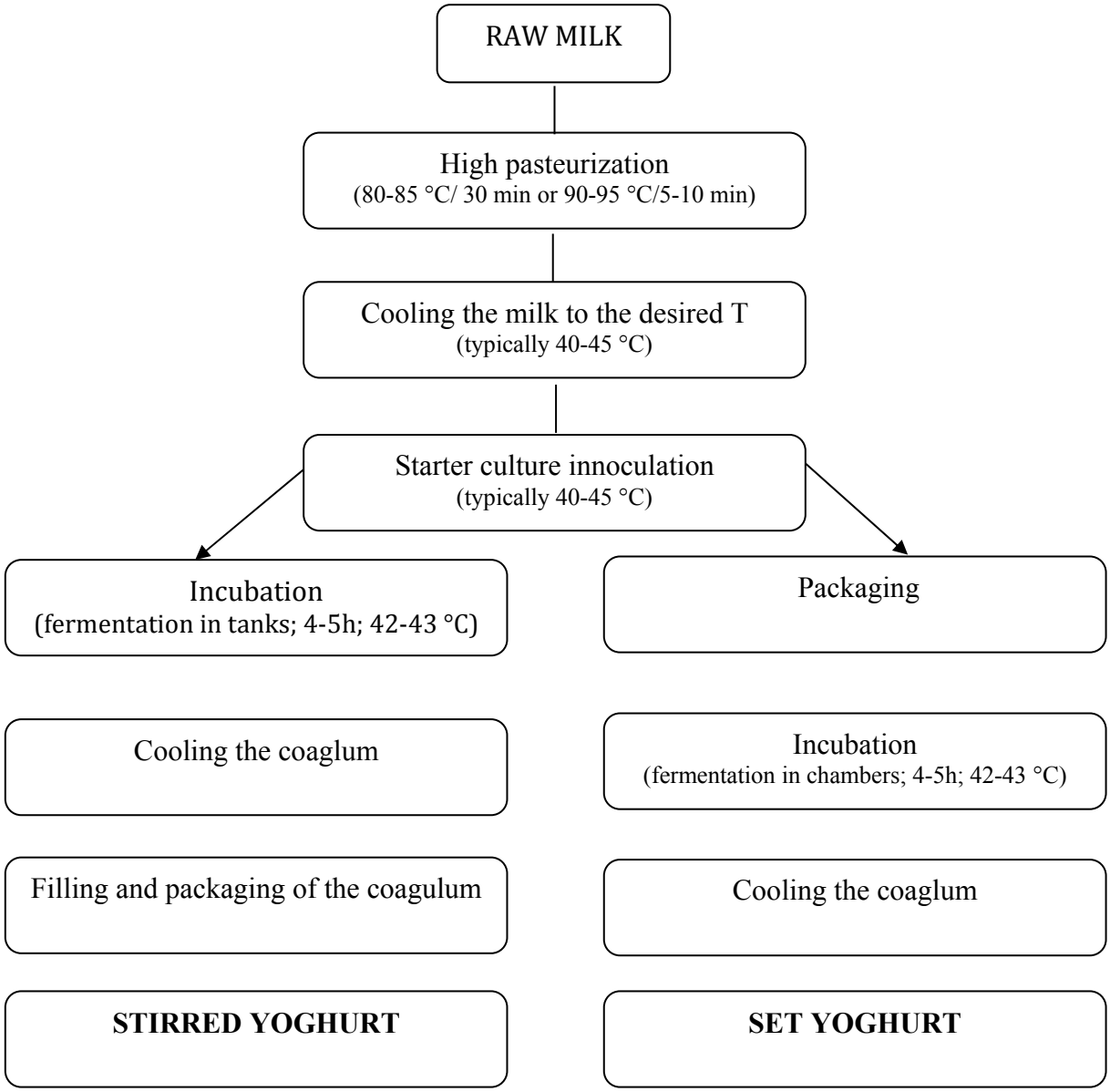


Figure B. Stirred yoghurt.

1. Incubation tank
2. Cooler
3. Cup filler


RAW MILK
High pasteurization
(80-85 °C/ 30 min or 90-95 °C/5-10 min)
Cooling the milk to the desired T
(typically 40-45 °C)
Starter culture innoculation
(typically 40-45 °C)
Incubation
(fermentation in tanks; 4-5h; 42-43 °C)
Packaging
Cooling the coaglum
Incubation
(fermentation in chambers; 4-5h; 42-43 °C)
Filling and packaging of the coagulum
Cooling the coaglum
STIRRED YOGHURT
SET YOGHURT