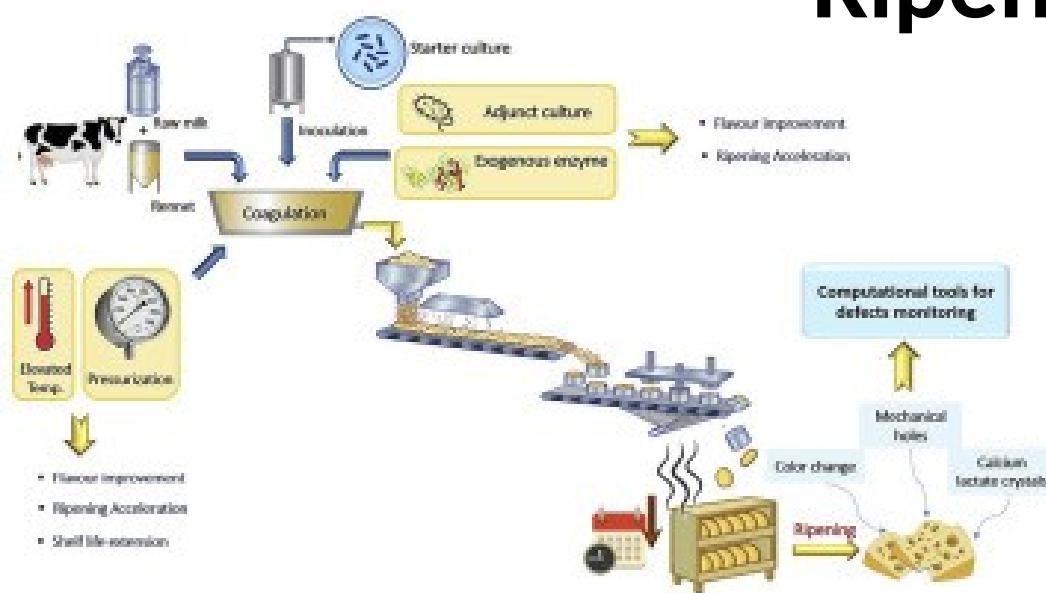


# Biochemical Changes During Cheese Ripening



Computational tools for defects monitoring



Mechanical holes

↓

Color change

↑

Ripening

↓

Cheese

↑

Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> crystals

↓

Color

↑

Flavor

↓

Texture

↑

Time

↑

Temperature

↑

pH

↓

Time

↑

Time

# Biochemical changes during ripening

- Enzymes derived from microorganisms, rennet and milk act mainly on lactose, protein and fat to produce various biochemical changes in cheese during ripening. In primary changes, breakdown of lactose, protein and fat leads to the accumulation of lactic acid, amino acids and fatty acids.
- In case of secondary changes, enzymes primarily from microorganisms act on the primary compounds to generate simpler compounds: i) The secondary reactions from lactate and pyruvate yields propionic acid, citric acid, acetic acid, acetyl methyl carbinol, diacetyl, acetaldehyde, ethanol and other compounds ii) from amino acids are amines, keto acids-aldehydes, methionine etc, iii) from fatty acids like butyric, caproic, caprylic and capric acid are derived acetoacetic acid
- In the degradation process, certain gases are released.  $\text{CO}_2$ ,  $\text{NH}_3$  and  $\text{H}_2\text{S}$  contribute directly to cheese flavor.

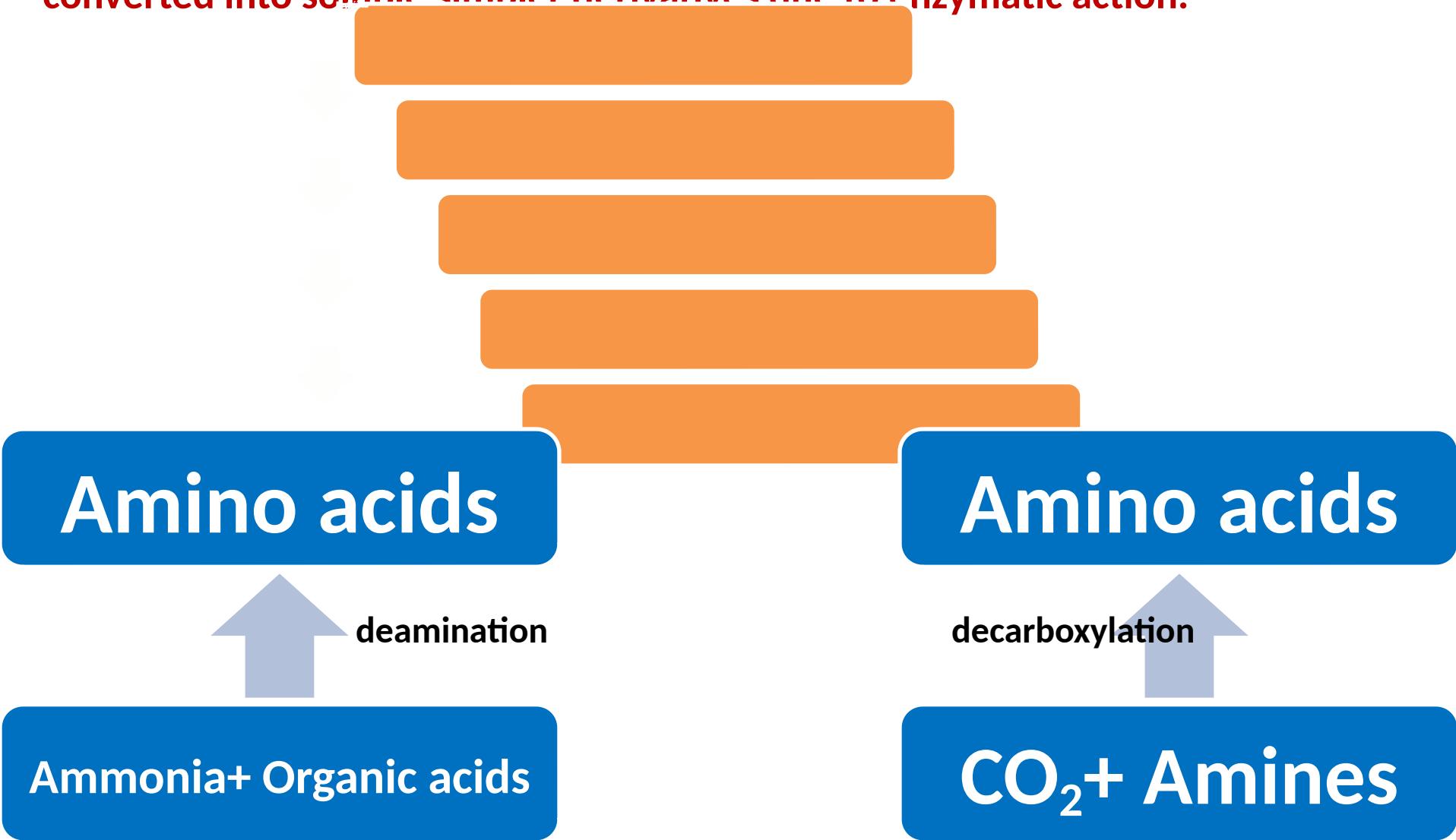


# Lactose metabolism

- Major part of lactose disappears within first few days of ripening in case of hard cheeses but take longer for soft cheeses. Starter bacteria convert lactose to lactic acid, volatile acids, ethanol and other compounds.
- In Emmental cheese, where propionibacteria is incorporated, a part of lactic acid undergoes secondary fermentation to yield propionic acid, acetic acid and  $\text{CO}_2$ .

## Protein breakdown

- Milk protein, a water-insoluble form of nitrogen present in young cheese is converted into soluble simpler derivatives due to enzymatic action.



- The enzymes responsible for protein breakdown are mainly from rennet and microbial sources. Rennin brings about the initial steps of casein degradation into water soluble compounds (mostly proteoses and peptones). Further, steps of breakdown upto amino acids and ammonia level are due to microbial enzymes. In hard cheeses, LAB dispersed evenly in cheese mass bring about the protein breakdown by releasing some extracellular proteinases and subsequently on autolysis some intracellular proteinases, as the ripening progresses. In soft ripening cheeses, protein degradation is chiefly due to extracellular proteinases released by microorganisms.
- In some soft cheeses like Camembert, the characteristic body and texture are a result of extensiv



## Fat hydrolysis

- Only a small fraction of milk fat gets hydrolyzed due to the activity of lipolytic enzymes. This may result into the release of certain products including lower chain volatile fatty acids. These fatty acids are responsible for the development of typical flavour in the cheese. Free fatty acids may interact with ethanol and form esters which may confer typical flavour to cheese.
- The lipolytic enzymes responsible for changes in fat during cheese ripening might come from milk, rennet or microorganisms.
- Rennet has more lipolytic activity if used as rennet paste than when used as rennet extract.
- Molds growing in or on the surface of certain cheese varieties also serve as the source of lipolytic enzymes.
- In blue veined cheese, fatty acids are oxidized by mold enzymes to methyl ketones which are responsible for characteristic flavour to these cheeses.

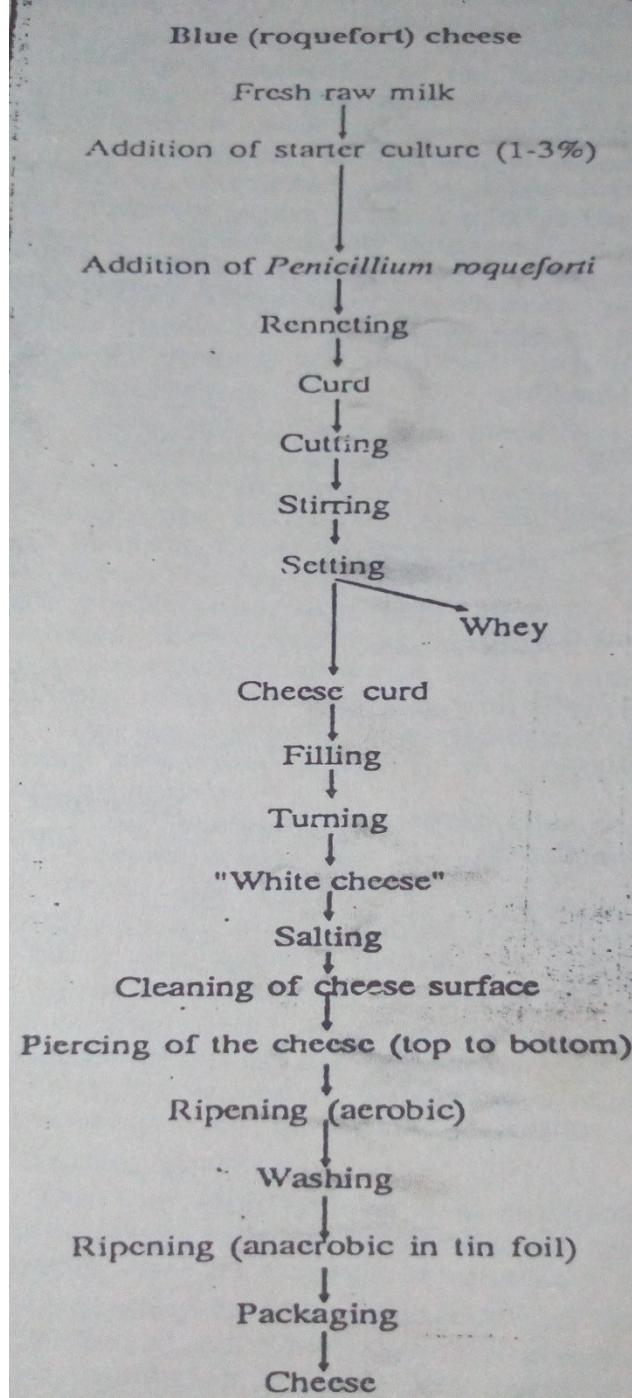
## Citrate metabolism

- Citrate metabolism by certain bacteria called citric acid fermenters (CAF) leads to production of diacetyl, a potent flavour compound. Although diacetyl plays a major role in imparting flavour to high moisture soft cheeses consumed fresh, it also contribute in giving flavour to curd cheeses.
- Besides producing diacetyl, CAF also produce considerable amounts of carbon dioxide from citrate leading to gas defects in cured cheeses. Gas spaces are however, desirable in Blue cheeses. Hence, it is desirable to incorporate CAF in their conventional starters during the manufacture of such cheeses.

# Internally Ripened Blue Mold Cheese

- Roquefort, Stilton, and Gorgonzola are examples of cheeses in which development of flavor is dominated by metabolism of *Penicillium roqueforti* or *P. glaucum*. These molds grow throughout cheese (internally ripened) and are able to grow in the low-oxygen, high-salt conditions that are typical of these cheeses.
- To facilitate exchange of air with CO<sub>2</sub> produced in cheese (via mold metabolism), cheese is manufactured to produce an open texture and is pierced or punched with large-bore needles.
- Blue cheese is usually made from a blend of heat-treated (raw) or pasteurized skim milk and homogenized cream.
- Pasteurization destroys most of the milk lipase, which is believed to aid in ripening of cheese and kills most nonstarter bacteria, especially lactobacilli, which might play an important role in overall development of characteristic flavor.
- The starter is *L. lactis*, and citrate-metabolizing strains (*L. Lactis* subsp. *lactis* biovar *diacetylactis* and *Leuconostoc* sp.) are sometimes added. Carbon dioxide produced through metabolism of citric acid expands mechanical openings in cheese, which in turn allows for more intrusive growth of mold.

- Free fatty acids released through lipolysis and via oxidative decarboxylation are converted to methyl ketones. Of particular importance in blue-veined cheeses are 2-heptanone and 2-nonenone responsible for distinctive blue cheese flavor. Secondary alcohols, methyl and ethyl esters derived from fatty acid metabolism and proteolysis, are essential for the well-balanced and distinctive flavor of blue veined cheeses.
- During initial salting and ripening of blue cheese, there is a conspicuous lack of visible growth of mold and yeasts on the surface. The low pH and high salt content keep the level of these microorganisms in check. Some manufacturers also use a hot brine treatment ( $72^{\circ}\text{C}$ ) to kill microorganisms at the cheese surface. However, as cheese matures, salt diffuses in and the pH rapidly increases (5.8 up to 6.5). Yeasts and molds metabolize lactic acid and hydrolyze protein, releasing ammonia and amino acids. Both metabolic activities result in pH increase.

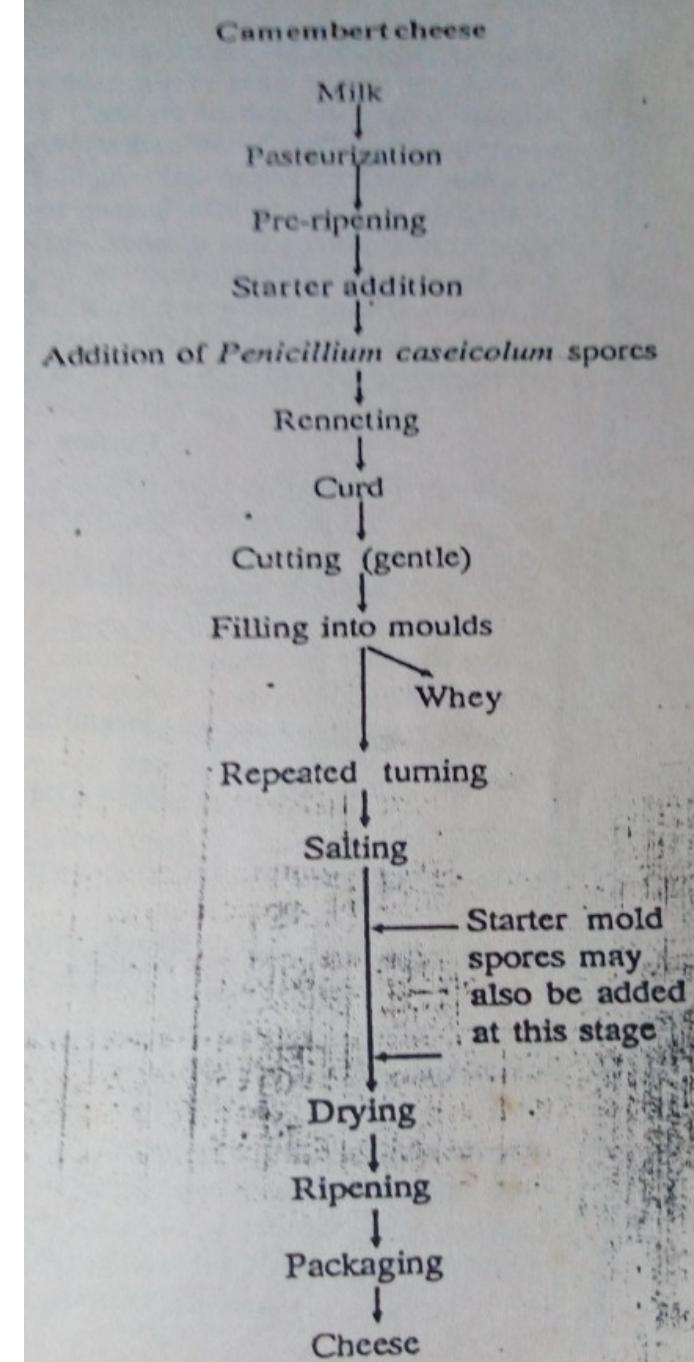


# Mold Surface Ripened Cheese

- Cheese is removed from forms and brined or dry-salted (salt rubbed or sprinkled on the surface). After salt is absorbed (1 day), spores of *P. camemberti* are sprayed onto the surface. Spores may also be added directly to the milk.
- Cheese is not pierced as in blue cheese, so mold does not grow in the interior of the cheese unless there is an area of unfused curd (mechanical openings or holes).
- Cheese is transferred to shelves in rooms of high relative humidity (90-95%) at 10°C. It is placed on mats or perforated sheets to allow air contact with as much surface area as possible. This permits growth of the mold evenly over the entire surface area of the cheese.
- Cheese is also turned regularly to expose bottom areas and keep soft cheese from being imprinted with the perforated mats or sticking to them.
- After approximately 2 weeks in ripening rooms, mold has developed sufficiently, and cheese is wrapped for sale. It is then stored at a low temperature (4-7°C) for further ripening (2-4 weeks)

- Before growth of *P. camemberti*, cheese is firm, crumbly, and acid. As mold grows, it metabolizes lactic acid and hydrolyzes protein. Just beneath the surface growth, cheese is very soft, creamy, and appears slightly translucent and more yellowish than the interior portion of the cheese. As ripening continues, the interior becomes progressively softer and creamier just as at the surface. This progression is referred to as ripening from the outside to the inside. The change in the body of cheese results, from migration of ammonia from the inside to the outside of the cheese. Migration of ammonia from the outside to the inside of the cheese raises the pH of cheese from 4.8 to 6.5. This alters hydration of casein with the net result of an increase in fluidity of cheese. With an increase in pH, naturally occurring milk proteinase, plasmin (not active at low pH), hydrolyzes protein, further softening cheese.

- Metabolism of *P. camemberti* results in hydrolysis of milkfat (lipolysis) and subsequent oxidative decarboxylation of free fatty acids to methyl ketones. Of particular importance to the flavor of Camembert are 1-octen-3-ol, 1, 5-octadien-3-ol, and 2-methylisoborneol.

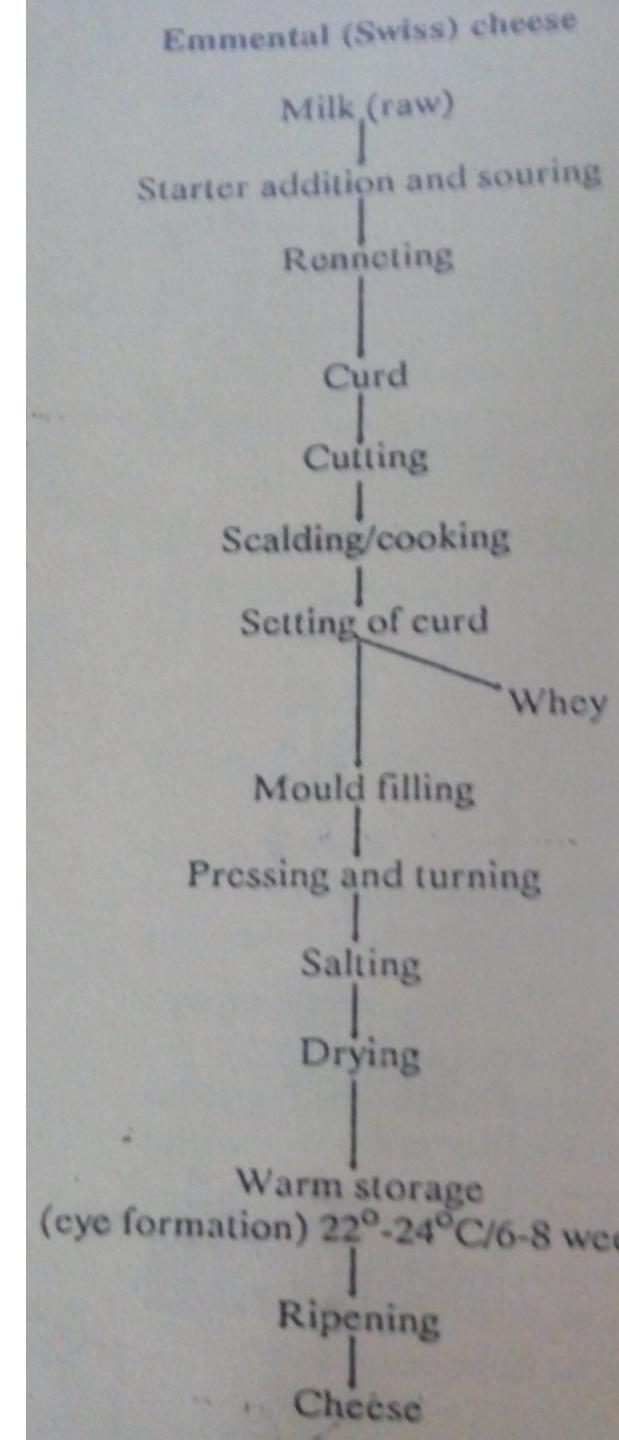


## Hard Cheese-With eyes

- In Swiss-type cheeses, gas (CO<sub>2</sub>) is formed by *P. fruedenreichii* subsp. *shermanii* through metabolism of lactic acid. Eyes develop through formation of CO<sub>2</sub> by metabolism of specific secondary bacteria.
- Starters for Swiss and baby Swiss cheese are predominantly *S. thermophilus* with small amounts of *L. delbrueckii* subsp. *bulgaricus*, *L. helveticus*, and *L. lactis*.
- In the traditional method of Swiss cheese manufacture, all curd from the cheese vat (a round kettle) is enclosed in a cheese cloth, lifted into the cheese form, whey is manually pushed out of the curd mass, and cheese is pressed. Because serum is at a high pH during pressing, less calcium phosphate is dissolved in whey as compared to cheeses of a more acidic nature.
- Phosphate acts as a buffer and helps keep pH of curd from getting too low. Low pH (5.1) inhibits growth of *Propionibacterium* sp. and is involved in development of a short, inelastic body in cheese. After pressing, the curd mass is brine salted.

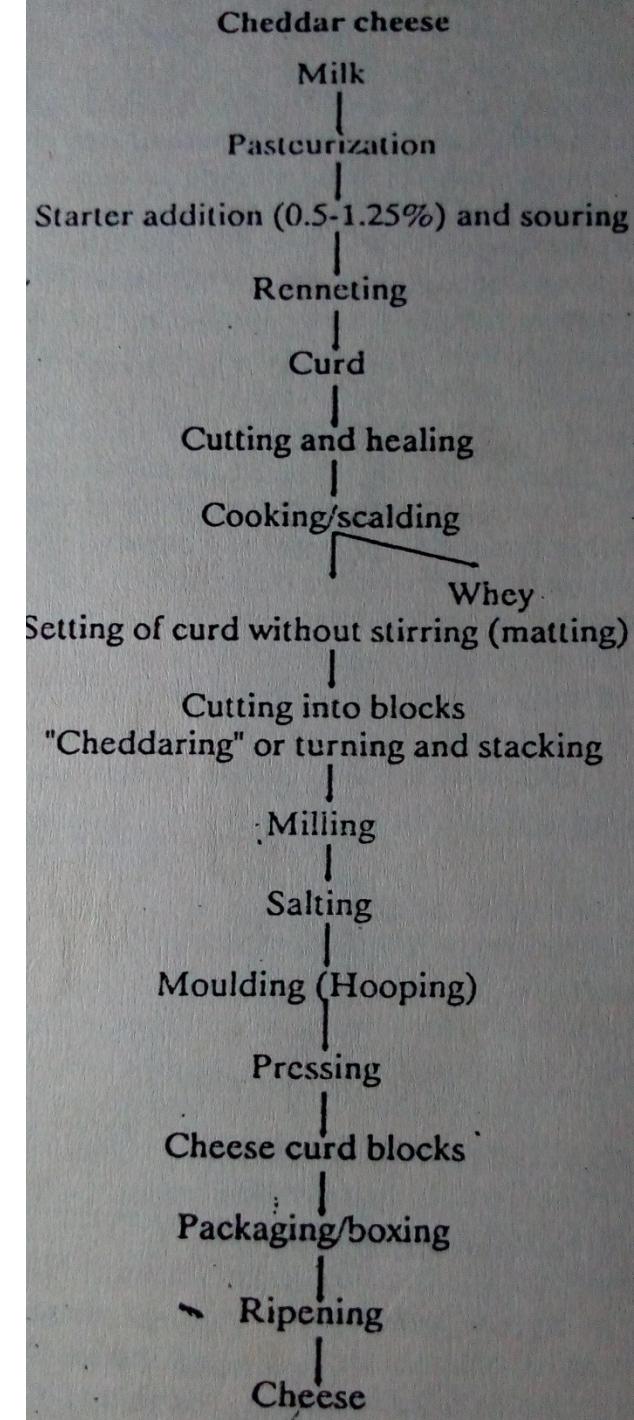
- To ensure curd fusion and complete sugar metabolism, cheese is held at 7°C (prewarm room) for several days. Cheese is then placed in a warm room (20-26°C for Swiss)

- In Swiss-type cheeses, gas ( $\text{CO}_2$ ) is formed by *P. fruedenreichii* subsp. *shermanii* through metabolism of lactic acid. Eyes develop through formation of  $\text{CO}_2$  by metabolism of specific secondary bacteria.



## Hard Cheese-Without eyes

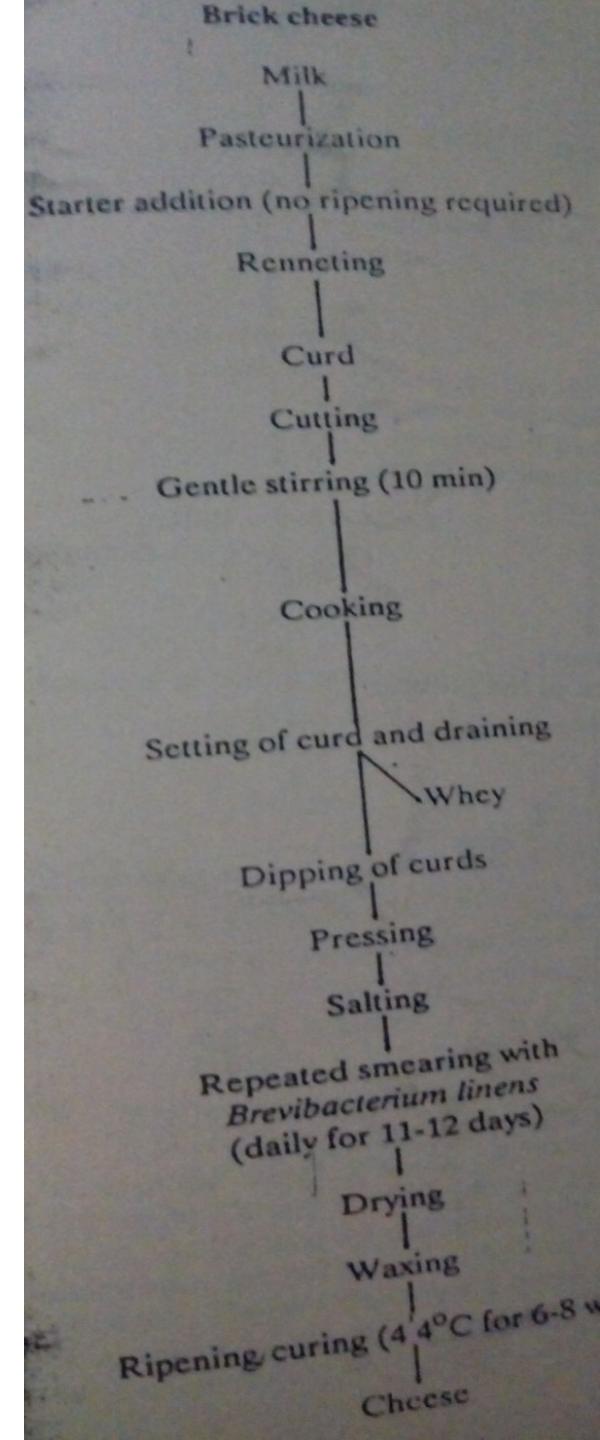
- Pasteurized or heat-treated (67–70°C for 20 s) whole milk is used. *Lactococcus* spp. is the starter, with *L. lactis* subsp. *cremoris* being preferred for longhold cheese.
- Once the coagulum is cut, curd is heated to 38–39°C. After proper stir-out, whey is completely drained and curd is either continuously stirred or allowed to mat. Stirred curd is preferred if the cheese will be used for process cheese. Cheddared curd is preferable for table cheese. Cheddared curd cheese is thought to develop a better flavor and has a smoother body than stirred curd cheese. When the curd reaches the desired pH, curd is salted. In Cheddared curd, the matted curd is cut into large pieces, which are periodically turned (Cheddared) in the vat until the proper pH is reached (pH 5.4–5.5). The slabs of curd are then cut into finger-sized pieces (milled), salted, put into forms, and pressed. Cheeses are generally stored at 7–9°C. There is considerable variation in details of Cheddar cheese manufacture resulting from mechanization of the process, size of vats and rate of acid development by starter. The pH of cheese at 1 week is generally 4.95–5.1.



# Semi-hard (Surface Ripened Cheese)

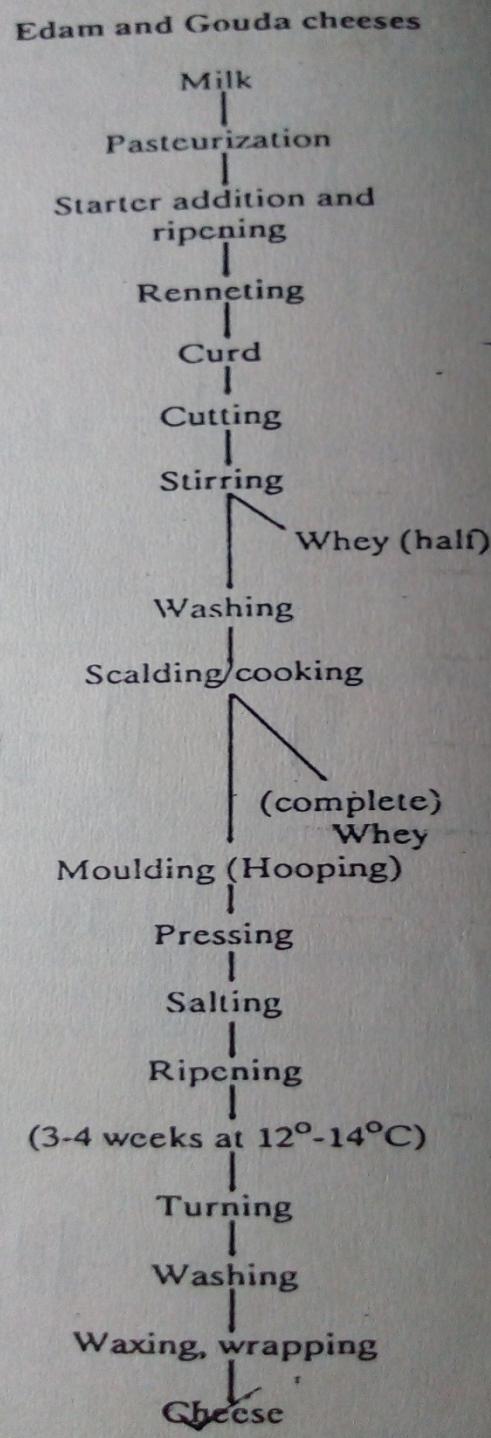
- Brick cheese are known for their highly malodorous, they smell in a pleasant sort of way. The strong odors arise from putrefaction of protein, which releases ammonia and sulfide compounds ( $H_2S$  and methyl mercaptan).
- These cheeses are made from whole milk with *Lactococcus* spp. starters.
- After cutting the coagulum, a portion of whey is removed (25–50% of milk weight) and replaced with hot water. This raises the temperature and removes lactose from curd, which prevents cheese from becoming too acidic, a development that could delay ripening. After 30–60 min, whey is drained while curd is stirred. If an open-bodied cheese is desired, all or most whey is removed. Curd is put into forms and may or may not be pressed. Once pressed, cheese is brined, ‘smeared,’ and placed in a high-humidity room (90–95% relative humidity) at 13–15°C. Cheese is slightly acid after brining (pH 5.2–5.4). The pH depends on amount of whey removed and water added during manufacture. Once brined, cheese is inoculated with the smear. The smear is a mixture of several microorganisms, most importantly yeasts, micrococci, *Arthrobacter* and *B. linens*, which develop as a layer on a cheese surface as it ripens.

- Smearing is done by scraping the smear layer from an already ripened cheese into a brine solution and then rubbing fresh cheeses with the smear-containing brine. Microorganisms in the smear may be purchased separately, mixed, and the cheeses inoculated. *Arthrobacter* and *B. linens* give the smear a red-orange color. Yeasts (*D. hansenii*, *Candida spp.*, *G. candidum*, and *Y. lipolytica*) metabolize lactic acid and the pH of the cheese increases.
- The pH must be greater than 5.5 for *B. linens* to grow. Yeasts also synthesize vitamins (pantothenic acid, niacin, and riboflavin), which may be essential for *B. linens* to grow. A symbiotic relationship thus exists between growth of yeasts and *B. linens*.



# Semi-hard (Internally Ripened Cheese)

- In Gouda and Edam cheeses, CO<sub>2</sub> is formed from metabolism of citric acid by *Leuconostoc* spp. and *L. lactis* subsp. *lactis* biovar *diacetylactis*.
- *Lc. Lactis* is used as starter for Gouda and Edam cheeses. Gouda and Edam are manufactured similarly, but Edam is lower in moisture and fat content.
- Gouda cheese may be ripened for extended periods at the warm room temperature. Because citric acid is limiting in cheese, there is no fear of excessive gas being formed by the added lactococci or *Leuconostoc*. As cheese ages, casein is hydrolyzed (proteolysis) by residual coagulant, nonstarter bacteria, and plasmin (native milk proteinase). Proteolysis eventually destroys elastic properties of the casein network. Thus, if gas is formed in cheese after much proteolysis has occurred, slits are formed.



# Rennet

- Milk coagulation is a basic step in cheese manufacturing.
- Calf rennet, the conventional milk clotting enzyme obtained from the fourth stomach of suckling calves was the most widely used coagulant in cheese making all over the world to manufacture most of the cheese varieties.
- The worldwide reduced supply of calf rennet and the increase in cheese production and consumption have stimulated research for milk-clotting enzymes from alternative sources to be used as calf rennet substitutes.
- Further, if calf rennet is used as a coagulant for cheese making, the product has to carry a tag of non -vegetarian which may lead to non-consumption of cheese by vegetarian population of the world.

## Desirable Characteristics of Rennet Substitutes

- ✓ The ratio of milk clotting activity to proteolytic activity should be high. This means that the milk should clot without much proteolysis i.e. breakdown of proteins to peptides. This prevents excessive nonspecific proteolysis during manufacture and hence protects against a weak gel structure, high losses of protein and fat in the whey, and reduced yields of cheese solids. Moreover, it avoids excessive proteolysis during maturation and thus ensures the correct balance of peptides of different molecular weights and hence desirable flavor, body, and functional characteristics in the ripened cheese.
- ✓ It should be thermally stable (comparable to calf rennet) at pH and temperatures used during cheese making. This influences the level of residual rennet in cheese and hence influences the biochemical changes that take place during ripening.
- ✓ It should have low thermal stability at temperatures of whey processing otherwise it may hinder the utilization of cheese whey in various products.
- ✓ It should impart desired flavor, body, and texture characteristics to the finished cheese.

**Milk-clotting Enzymes from Plants:** Enzymes from many plant sources may be used as clotting enzymes in cheese making but most of the plant proteases are strongly proteolytic and cause extensive digestion of the curd, resulting in reduced yields, bitter flavors and pasty -bodied cheese.

**Papain:** The latex of the plant *Carica papaya* yields papain and several other proteases. It has powerful milk clotting activity but it is also highly proteolytic.

**Ficin:** Ficin is present in the latex of several species of the genus *Ficus* (fig) such as *Ficus glomerata*, *Ficus religiosa* and *Ficus carica* but the best source is *Ficus carica*. Cheese made with ficin develops bitter flavor which decreases in intensity during curing.

**Others:** Bromelain from pineapple has also been considered as a possible substitute for calf rennet. An enzyme extracted from *Withania coagulans* also used in the manufacture of Surti and Cheddar cheese. Extracts from the flower petals of *Cynara cardunculus* used for the manufacture of Serra cheese from sheep's milk by Portuguese farmers.

## Microbial Rennet

- ✓ A large number of microorganisms are known to produce milk clotting enzymes. The possibility of a few of the microorganisms proving successful as rennet substitutes may appear from the fact that they play an important part during the manufacture of cheese.
- ✓ Microbial enzymes are known to exhibit considerable variations in the range of activity, substrate specificity and mode of action. Even more important is the fact that they can be produced economically on any desired scale.
- ✓ Many hundreds of bacterial and fungal cultures have been investigated for milk clotting enzymes and their proteolytic abilities.
- ✓ Milk clotting enzymes from bacteria like *Streptococcus liquefaciens*, *Micrococcus caseolyticus*, *Bacillus cereus*, *B. polymyxa*, *B. mesentericus*, *B. coagulans* and *B. subtilis* have been used as coagulating enzymes. Milk clotting enzymes from fungi are also used during cheese making. Some of the fungi producing such enzymes are *Aspergillus nidulans* and *Cladosporium herbarum*.

## Micoorganisms used in commercial production of microbial rennet

Name	Source of Enzyme
Hannilase	<i>Mucor miehei</i>
Rennilase	<i>Mucor miehei</i>
Fromase	<i>Mucor miehei</i>
Miki	<i>Mucor miehei</i>
Marzyme	<i>Mucor miehei</i>
Novadel	<i>Mucor pusillus</i>
Noury	<i>Mucor pusillus</i>
Meito	<i>Mucor pusillus</i>
Emporase	<i>Mucor pusillus</i>
Suparen	<i>Endothia parasitica</i>
Mikrozyme	<i>Bacillus subtilis</i>
Sure curd	<i>Endothia parasitica</i>

- The protease of the *Mucor miehei* coagulants degrade casein fairly rapidly in the pH range 5.5 -7.0; but inspite of this, the incidence of bitter cheese using this enzyme is low. The enzyme is very sensitive to temperatures in the region 37-45°C and is destroyed at 70°C. This enzyme is used successfully for many types of cheese.
- *Mucor pusillus* extract is also used as microbial rennet. It is highly proteolytic than calf rennet or the *Mucor miehei* extract. It tends to give hard curds because of its high proteolytic activity and the curd tends to lose fat into the whey, thereby giving lower yield as compared to others. Thus, this enzyme is usually used in combination with other enzymes.

## Recombinant chymosin

- ✓ Many rennet substitutes are used for cheese making but many of these proteolytic enzymes from microbial or plant origin cause flavor, texture and yield changes in certain types of cheese that are different than those produced by calf rennet. Further, they are not suitable for long ripening cheeses as they have a different range of non-specific activities than chymosin and do not produce the correct flavors on prolonged ripening.
- ✓ Recombinant rennet/chymosin can be prepared by gene transfer technology in which the bovine chymosin is cloned in a suitable production strain and the enzyme is produced by fermentation.
- ✓ This enzyme can be isolated and used in cheese making as a coagulant having properties similar to that of calf rennet.
- ✓ *E. coli*, *Kluyveromyces lactis* and *A. niger* var. *awamori* have been successfully used for production of calf rennet.
- ✓ In addition to the benefit that such chymosin can be produced in large-scale fermentors at low cost, recombinant and highly pure chymosin also has some other advantages such as specific, low proteolytic activity, predictable coagulation behavior and vegetarian approval.