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MASTER DEGREE: FOOD AND BEVERAGE INNOVATION AND MANAGEMENT

CLOTTING PROPERTIES OF *ONOPORDUM TAURICUM* AQUEOUS EXTRACT IN MILK OF DIFFERENT SPECIES

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ACRONIMS AND ABBREVIATIONS

CR	Calf rennet
MCA	Milk clotting activity
MCT	Milk clotting time
RE	Raw extract
RSM	Response surface methodology

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INTRODUCTION AND PURPOSE OF THE THESIS

Plant coagulants hold an important position among various coagulants used in cheese technology. In Mediterranean, Southern European and West African countries some plant extracts have been used as coagulants for centuries in cheesemaking using raw ovine and caprine milks (Ben Amira A. et al., 2017). Various factors such as the high cost and limited availability of rennet, religious concerns, diet habits and ban of recombinant chymosin in some countries, increase in cheese production worldwide, bovine spongyform encephalopathy have led to search for alternative vegetable sources of milk-clotting enzymes (Roseiro et al., 2003).

In Mediterranean regions, crude aqueous extracts with milk clotting properties are traditionally prepared from wild herbaceous plants commonly referred to as "thistles" and scientifically ascribed to different genera within the family of *Asteraceae*, namely *Cynara*, *Silybum*, *Centaurea*, *Carlina*, *Cirsium*, and *Onopordum* (Cardinali, F. et al., 2017).

The selection of a suitable plant coagulant is important to obtain cheese with acceptable quality characteristics.

The extracts from leaves, flowers, latex secretions, fruits, roots and seeds of several plant families (*Cynara spp., Solanum spp., Calotropsis spp., Ficus spp.)* have been recently investigated for their milk-clotting ability (Liburdi et al., 2019; Gutiérrez-Méndez et al., 2019; Nestor et al., 2012; Rajagopalan et al., 2019; Rociò et al., 2020; Rayanatou et al., 2017). Although several plant proteases are able to coagulate milk, some of them have been found to be inappropriate for cheese making due to some drawbacks such as high proteolytic activity resulting in a bitter flavor in the final product and low cheese yield (Mazorra-Manzano Miguel A. et al., 2018; Shah, M.A. et al., 2014).

Nevertheless, *Cynara spp.* flowers extract, containing high concentration of chymosin like proteases (cynarases, cyprosines and cardosines) has been used for years in artisanal cheese manufacturing (Sarmento et al, 2009; Zikiou et al., 2020).

In various studies it has been reported that the extracts from wild cardoon flowers of *Cynara cardunculus L.*, *Cynara humilis L. and Cynara scolymus L.* are highly effective in milk coagulation, expecially ewes' and goat milks.(Barak Abo et al., 2017; Gomes et al., 2019;

Rincón et al., 2017; Esposito et al., 2016; Silva et al., 2000). Spain and Portugal have the great variety and production of raw ewe's and goat's milk cheeses using *Cynara spp.* as a plant coagulant (Serra de Estrela, Serpa, Azeitão, Nisa, Castelo Branco, Évora, Casar de Cáceres, Torta del Casar, Los Pedroches, La Serena, Los Ibores, Flor de Guía). Some of them have been reached a Protected Designation of Origin (PDO) in the European Union (Roseiro et al, 2003; Araújo-Rodrigues et al., 2020).

In addition, the species of *Onopordum acanthium, Onopordum turcicum* and *Silybum marianum* contain aspartic proteases in their flowers with milk-clotting activity (Brutti et al., 2012; Ruscono et al., 2011; Tamer et al., 1994).

In some regions of Italy are used extracts obtained mainly from *Cynara cardunculus* and *Ficus carica*. These are ancient dairy traditions, typical of the Mediterranean basin, which have still been maintained in some Italian regions (Faccia et al, 2007).

Cynara cardunculus L. thistle are most studied, characterized and exploited in cheese making but technological traits of other thistle species have not been fully described yet (Silva et al., 2004). In particular, *Onopordum* spp. have been scarcely studied for their coagulant properties. A partially purified enzyme preparation ("onopordosin") was obtained from *O. acanthium* L. (cotton thistle, Scotch thistle) flowers. The main active component in this extract thistle was an aspartic protease, which is characterized by an isoelectric point of 4.4. The seeds, flowers, and leaves of *O. turcicum* Danin were also found to contain proteolytic enzymes able to coagulate milk (Tamer et al., 1993, 1994), but *Onopordum tauricum* Willd. (Taurian thistle, bull cottonthistle) is still unexplored for this purpose.

No literature data are currently present regarding the proteases of Taurian thistle and their potentiality as milk clotting agents.

The objectives of the thesis are evaluate clotting properties of the aqueous extract from flowers of spontaneously grown *Onopordum tauricum* in milk of different species (ewe, goat, cow) as well as determine optimal conditions for milk clotting by applying the response surface methodology (RSM) approach to study the effect of the independent curdling variables (temperature, pH, amount of enzymatic extract) on the technological performance of the thistle extract. Finally, compare obtained results with the performance of commercially available calf rennet.

Chapter 1
MILK

1.1 Milk definition

By drinking milk, Italian legislation refers to the product obtained by regular, uninterrupted and complete milking of the udder of animals in good health and nutrition (RD 994/29 .art. 15. comma 1).

The term "milk" alone indicates from cow (*Bos Taurus*); for milk of different origin, the origin must be specified (Salvadori del Prato, 2001).

Currently, in accordance with current DPR 54/97, a distinction is made between:

- Raw milk, defined as "the milk produced by secretion of the mammary gland of cows, sheep, goats or buffaloes, not subjected to a temperature above 40 ° C or a treatment having equivalent effect " (art 2. paragraph 1. letter a);
- Milk intended for the manufacture of milk-based products, defined as "the raw milk for processing or liquid or frozen milk made from raw milk, whether or not subjected to a permitted physical treatment, such as a heat treatment or thermization, and whether or not modified in the composition, provided that the modification is limited to the addition or subtraction of its natural constituents "(art. 2. paragraph 1. letter b);
- Heat treated drinking milk, defined as “drinking milk intended for sale to the consumer, subjected to a heat treatment or the pasteurized milk to be sold at the request of the individual user "(art2. paragraph 1. letter c.).

1.2 Milk composition and structure

As it is shown in Fig.1-1 milk can be regarded as a colloidal suspension of fat and casein.

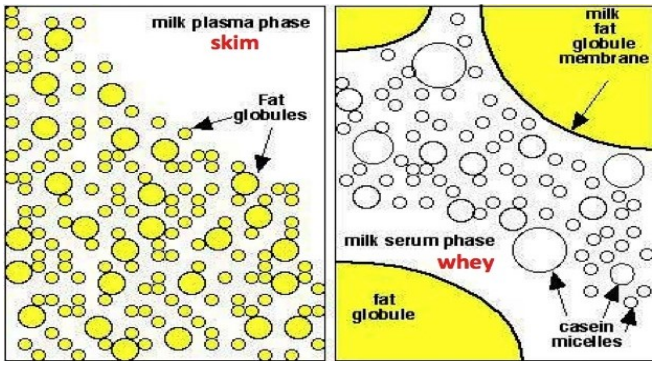


Figure 1-1:Milk structure (Walstra et al., 1979)

The chemical composition of milk depends on various factors: species, breed, age of the animal, feeding, health condition, environmental factors and lactation period (Table 1-1).

In general, the main constituents of milk are water, lactose, protein (casein and whey protein), lipids, minerals, salts, vitamins, enzymes (Haung et al., 2007).

While milk of all species has similar overall characteristics and classes of constituents, these differ in specific terms both qualitatively (i.e. exact nature of constituents) and quantitatively (i.e the amount of each constituent / liter).

Table 1-1: Average chemical composition of milk of different species of mammals (for 100 g of fresh milk)(Haenlein, 2006; 2010)

Milk type	Protein, %	Casein, %	Fat, %	Lactose, %	Ash, %	Water, %	Total solids, %
Cow	3.3	2.8	3.9	4.7	0,7	87.4	12.6
Goat	3.3	2.7	4.5	4.6	0.6	87.0	13.0
Sheep	5.6	3.9	7.5	4.4	0.9	81.6	18.4

The casein fraction coexists with the insoluble minerals as a calcium phosphate-casein complex. The water and its soluble constituents (lactose, native whey proteins, some minerals, citric acid and minor components) are referred to as serum (Law and Tamin, 2011). Figure 1-2 shows the main milk constituents.

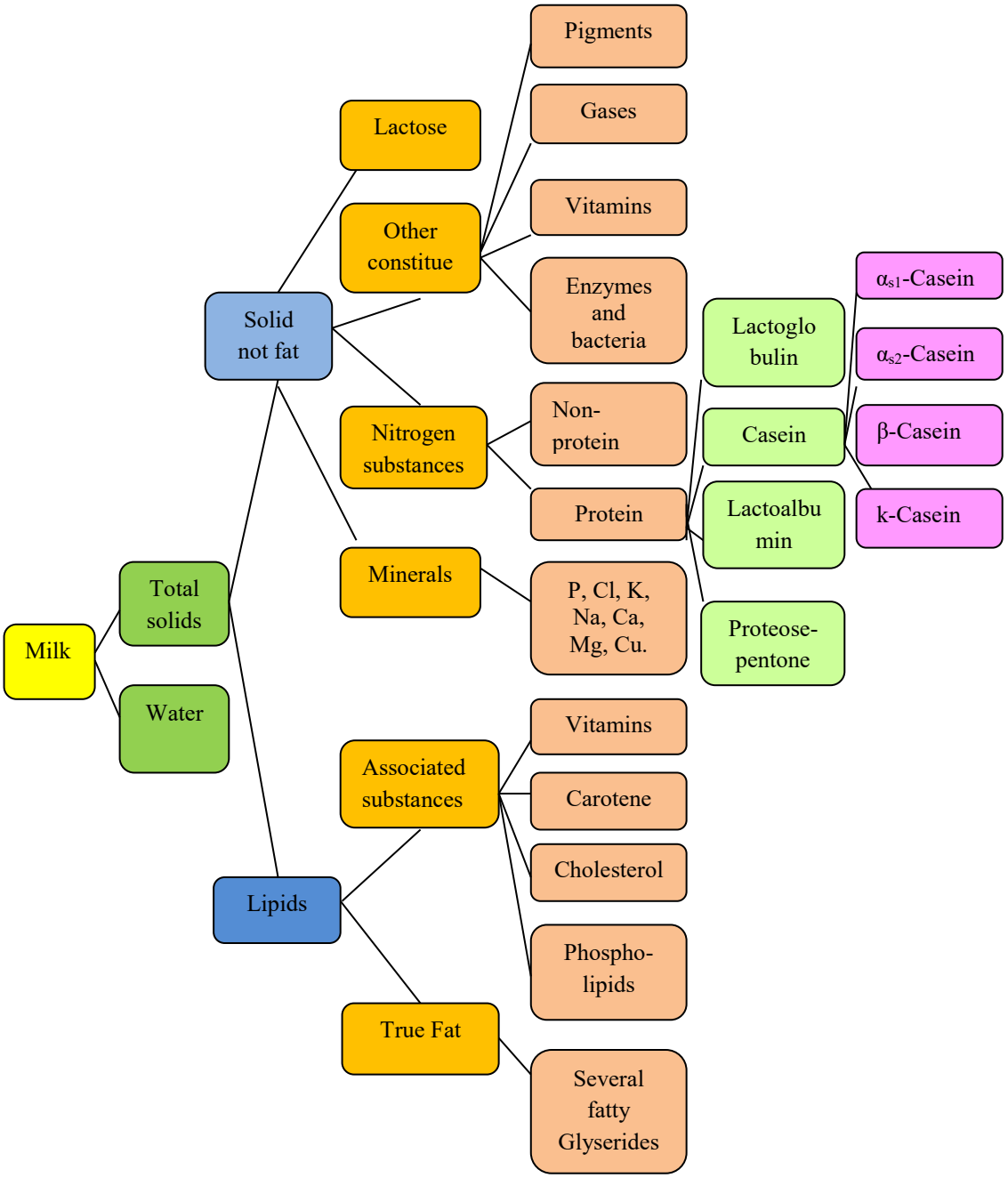


Figure 1-1: Milk constituents (Ramesh et al., 2008)

1.2.1 Water

Water is the major component of milk, representing 87% of the total composition in cow milk. The other components (13 %) are suspended or dissolved in this medium. A small

amount of water is bound to the milk protein and some hydrated to the lactose and salts giving milk a water activity (aw) of 0.993.
The water content in milk is also expressed by the cryoscopic index or freezing point; cryoscopic index values above -0.510 C indicate watering of the milk itself (Salvadori del Prato, 2001).

1.2.2 Lactose

Lactose is a sugar found only in milk. Lactose content of milk varies between 3,6 and 5,5.In addition to lactose, milk contains small amounts of glucose, galactose and other saccharides.
Lactose is inversely proportional to concentration of fat and casein, as it actively contributes to osmotic pressure milk: the synthesis of lactose in the breast, osmotically draws water from the blood and increases the volume of secreted milk, influencing the concentration of other elements and keeping the water: lactose ratio constant.
In cheese making most of the lactose remains dissolved in the whey (from which it can be prepared commercially) and the remaining in the curd. For this reason, cheese that is prepared from the curd is low in carbohydrates.
Lactose plays an important role in the transformation of milk as it is the substrate where lactic bacteria grow and therefore, through different types of fermentation, influences the characteristics of the final product.
These fermentations are of great interest to the dairy industry and three are particularly taken into consideration:

- *lactic fermentation* by bacteria belonging to the *Streptococcus* and *Lactobacillus* genus important for lowering the pH during the cheesemaking process;
- *the propionic one*, implemented by the genus *Propion bacterium*, which determines the formation of the typical holes in cheeses such as Asiago Pressato PDO;
- *the butyric*, caused by clostridia which is responsible for the late swelling, undesirable in all productions.

When lactose is attacked by lactic acid bacteria contained enzyme lactase, which splits lactose molecules into glucose and galactose which is consequently transformed into lactic acid (Salvadori del Prato, 2001).

1.2.3 Lipids

Lipids are found in milk organized in the form of emulsion globules, tiny spheres in which a lipoprotein membrane encloses the triglycerides, and are present in quantities equal to approximately 3.5-4%.

Milk lipids are made up 98-99% of triglycerides, while the remainder is made up of monoglycerides, diglycerides, phospholipids, sterols (in particular cholesterol) and other lipid molecules.

The fatty acids that make up milk fat are mainly saturated (61%). Many are short-chain like butyric acid, caprinic and caprilic acid. The most abundant fatty acids are palmitic, oleic, stearic and myristic acids.

It is primarily responsible of the flavor and aroma typical for the milk of the different species. During the cheese-making process, the fat is almost entirely incorporated in the curd network and therefore positively influences the cheese yield (Salvadori del Prato, 2001).

1.2.4 Milk Proteins

Milk proteins represent about 3-3.8 % and are made up of two large fractions: casein (about 78%) and whey proteins (about 18%).Figure1-3 shows the distribution of protein fractions in milk (Fox and McSweeney, 1998).

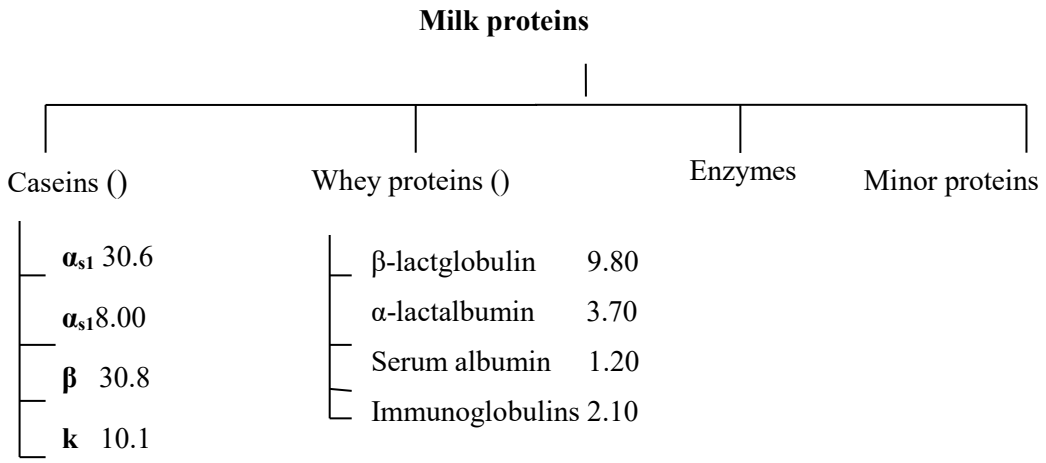


Figure 1-2: Distribution of protein fractions in bovine milk (% of total protein w/w)

Milk also contains different enzymes coming from several sources: the native milk and bacteria that are added intentionally for fermentation. The former are normal constituents of milk and are called original enzymes. The latter, bacterial enzymes vary in type and abundance according to the nature and size of the bacterial population. Several enzymes in milk are utilized for quality testing and control. Among the most important ones are peroxidase, catalase, phosphatase and lipase (Farkye, 2003).

Lipases are enzymes that degrade fats. The major lipase in milk is lipoprotein lipase. It is mainly associated with the protein micelle. Agitation during processing may bring the lipase into contact with the milk fat resulting in fat degradation and off-flavors. Pasteurization will inactivate the lipase in milk and increase shelf-life (Bylund, 2003).

Proteases are enzymes that degrade proteins. The major protease in milk is plasmin, which is part of complex system. Some proteases are inactivated by heat and others not. Protein degradation can be undesirable and result in bitter off-flavors, or it may provide a desirable texture of cheese during ripening (Bylund, 2003).

Alkaline phosphatase is a heat sensitive enzyme in milk that is used as indicator of pasteurization. If milk is properly pasteurized, alkaline phosphatase is inactivated.

Lactoperoxidase is one of the most heat-stable enzymes found in milk. Both lysozyme and lactoperoxidase (when combined with hydrogen peroxide and thiocyanate) present antibacterial properties (Bylund, 2003).

1.2.4.1 Caseins

The major proteins in milk are the caseins and it is precisely this characteristic that makes them suitable for cheese making.

The caseins are a group of phosphor proteins in milk. They are conjugated proteins containing phosphoric acid as the prosthetic group. Acidification of raw skimmed milk to pH 4.6 at 20^{o}C will coagulate this fraction. The casein proteins include four groups; α_{s1}-caseins, α_{s2}-caseins, ß- caseins, and k-caseins. The composition of the major caseins in the micelles are α_1(38%), α_2(10%), ß (36%) and k (13%).

Caseins are present in the form of large colloidal aggregates, called casein micelles, held together by hydrogen bridges and by the bonds that form between the ionic calcium and the phosphate groups of these proteins.

Its precipitation can be obtained by acidification (through the action of bacteria or the addition of acids) which by lowering the pH shift the balance of calcium and phosphorus and bringing the pH closer to the isoelectric point (pH 4.6) of the casein and destabilize it, or it

can be obtained enzymatically by adding rennet, which removes a carbohydrate part which by its hydrophilicity helps the casein to remain dispersed in water.

The α- and ß- caseins are calcium sensitive or insoluble, whereas k-casein is soluble in the presence of calcium. k-casein has a stabilizing effect on the casein micelle, permitting the existence of the colloidal dispersion and preventing the other caseins from precipitating. Therefore if k-casein is proteolysed by the action of the enzyme rennin, it results in destabilization of the caseinate complex, thus forming an insoluble part, the para-caseinate, and a soluble part, the whey proteose. As a result of the destabilization of the k-casein, the milk clots and a gel is formed (Bobe et al., 1998).

According to Potocnic et al. (2011) some differences in casein fractions between cow, goat and ewe milk were observed and it is shown in Table 1-2.

Table 1-2:Differences in casein fractions between cow, goat and ewe milk (Potocnic et al., 2011)

Fraction	Cow	Sheep	Goat
Casein g/Kg	25.1	43	24
$\alpha_{s1,s2}$ –casein %	48.5	50.23	21.2-32
β-casein %	35.7	39.95	48-60
k-casein %	12.6	9.82	12-20
Micelle size, nm	182	210	260

1.2.4.2 Whey proteins

Whey proteins represent about 16-18% of the total proteins in cow's milk and are proteins with lower molecular weight that heat easily denatures. They can be divided into three main groups:

- albumin: α-lactoalbumine, β-lactoglobulin, serum albumin;
- globulin: e.g. immonuglobuline;
- protein-peptones: e.g. σ-proteose.

α-lactalbumin intervenes in the synthesis of lactose, β-lactoglobulin is instead characterized by the richness of sulfur amino acids. Immonuglobulins are important for immunization. The σ - proteose is, unlike the previous ones, a thermostable whey protein, and is responsible for the formation of the so-called "milk skin", a thin lipoprotein film that is formed during boiling, when the σ-proteose rises to the surface and dehydrates (Farrell H.M., 2004; Ng-Kwai-Hang, K. F., 2011).

Chapter 2
CHEESE

2.1 Definition and classification of Italian Cheeses

According the legislative point of view (r.d.l. No 2033 of 15/10/1925) “cheese is the food product obtained from whole milk, partially skimmed or skimmed, or from cream, following acidic or rennet coagulation, also by using of ferments and sodium chloride”.
Commonly means as cheese the milk derivative obtained by precipitating casein.
The cheeses can be classified according to a series of parameters, which combine in various ways.
These parameters refer to:
- type of milk used;
- fat content;
- consistency, in relation to the water content;
- technology used for the production and processing temperature of the curd;
- seasoning period;
- denomination.

1) Classification by the type of milk used:
- vaccines;
- pecorino;
- buffaloes;
- goats.

2) Based on the fat content, expressed on the dry substance, it is possible to identify (Law n. 142/1992):

- *fatty cheeses*, whose fat content is greater than 35% of the dry substance (Robiola, Gorgonzola, Taleggio, Bitto, Fontina, Montasio, Bra, Raschera, Grana Padano, Parmesan Reggiano, Pecorino, etc.);
- *light cheeses*, when the fat content varies between 20 and 35% of the dry matter;

- *low-fat cheeses*, prepared with skim milk, with a fat content of less than 20% of the dry substance.

3) Based on the consistency, linked to the percentage of water contained:

- *soft cheeses*, when the water content is greater than 45% (e.g. Robiola,Quartirolo, Stracchino, Crescente, Mozzarella, Burrata, Gorgonzola, Caprini, Casatella, Squacquerone, ...). They can be with crust (like Taleggio) and without crust (like Pannerone);
- *semi-hard cheeses*, when the water content is between 35 and 45% (e.g.Ragusano, Asiago, Bitto, Fontina, Bra, Castelmagno, Italico, ...);
- *hard cheeses*, when the quantity of water is less than 35% (e.g. Grana Padano, Parmigiano Reggiano, Pecorino Romano, Montasio, Pecorino Sardo, Fiore Sardo).

4) Based on the technology used and the curd processing temperature:

- *raw cheeses*, when, during processing, the curd does not undergo any heating beyond the coagulation temperature (eg Robiola, Mozzarella, Crescente, Gorgonzola);
- *semi-cooked cheeses*, when the heating of the curd does not exceed 48 ° C (e.g. Asiago, Fontina, Italico);
- *cooked cheeses*, if obtained by heating the curd above 48 ° C (e.g. Grana Padano, Parmigiano Reggiano, Montasio, Bitto);
- *pasta filata cheeses*, if characterized by a spinning of the curd in hot water at 70-90 ° C (e.g. Mozzarella, Fiordilatte, Caciocavallo, Provolone, Ragusano);
- *blue cheeses*, when molds are deliberately inoculated in the milk which will develop inside the cheese paste contributing to the maturation of this with specific enzymatic activities (e.g. Gorgonzola, Castelmagno).

5)Based on the seasoning period:

- *fresh cheeses*, which, obtained by acidic or rennet coagulation and not subjected to seasoning, do not have crust or surface microflora and must be consumed within a few days of production (e.g. Mozzarella, Fiordilatte, Crescente, Casatella);
- *matured cheeses with short maturation*, whose maturation does not exceed 30 days (eg.Taleggio, Murazzano, Bra, Lombard Quartirolo, Asiago, Monte Veronese, Casciotta d'Urbino);
- *medium-ripened aged cheeses,* whose aging does not exceed 6 months (eg.Fontina, Castelmagno, Raschera, Toma Piemontese, Valtellina Casera, Provolone Valpadana, Caciocavallo Silano, Canestrato Pugliese, Pecorino Siciliano, Pecorino sardo, Bitto);

- *slow matured aged cheeses*, from 6 months of aging onwards (e.g..Grana Padano, Parmigiano Reggiano, Fiore sardo).

6) By denomination:

- *Protected Designation of Origin (PDO) cheeses*: they are "cheeses produced in geographically delimited areas, observing loyal and constant local uses and whose product characteristics derive mainly from the conditions of the production environment" (Chapter II, art. 5.). This denomination is now sanctioned and protected at EU level by RegulationEUNo.1151/2012)
- *Protected Geographical Indication (PGI) cheeses*: these are "cheeses produced on the national territory, observing fair and constant uses, whose product characteristics derive from particular characteristics of the raw materials or production technique" (Chapter II, art. 5.). Even this denomination is now sanctioned and protected at EU level by Regulation (EU) No. 1151/2012;
- *"Traditional specialty guaranteed" (TSG) cheeses*: these are cheeses whose specificity consists in respecting a detailed traditional production method, while there is no link with a geographical area: they can therefore be produced throughout the national territory. They are protected by Regulation (EU) No. 1151/2012. In Italy the only example is the Mozzarella TSG.
- *traditional cheeses*: there are over 450 so called 'regional' cheeses, e.g.:, Fossa cheese, Burrata, Cacio Marcetto, Bagòss, Piacentinu di Enna, Casieddu di Moliterno, Casolet, Val Camonica, Dobbiaco, Paglierina Rifreddo, Tosèla del Primiero, Formaio Embriago,Morlacco del Grappa, etc.

2.2 Cheese making process

Cheese is a very varied group of dairy products, produced in a great range of flavors and forms throughout the world.

Cheese making could be considered as a process concentrating milk components, in particular fat and protein contents, which are determinant factors of cheese yield. Its manufacture essentially involves coagulation of cheese milk, dehydration of the gel to form curd and treatment of the curd (e.g. stirring, cheddaring, texturization, salting, moulding and pressing). The moulded curd may be consumed fresh (shortly after manufacture, for example within 1 week) or matured to form a ripened cheese. Cheese manufacturing represents complex chemical and physical phenomena.

Cheese making involves a number of main stages (Fifure 2-1) which are common to most types of cheese (Almena-Aliste and Mietton, 2014; Law et al., 2006; Everett, 2017; Fox et al., 2015).

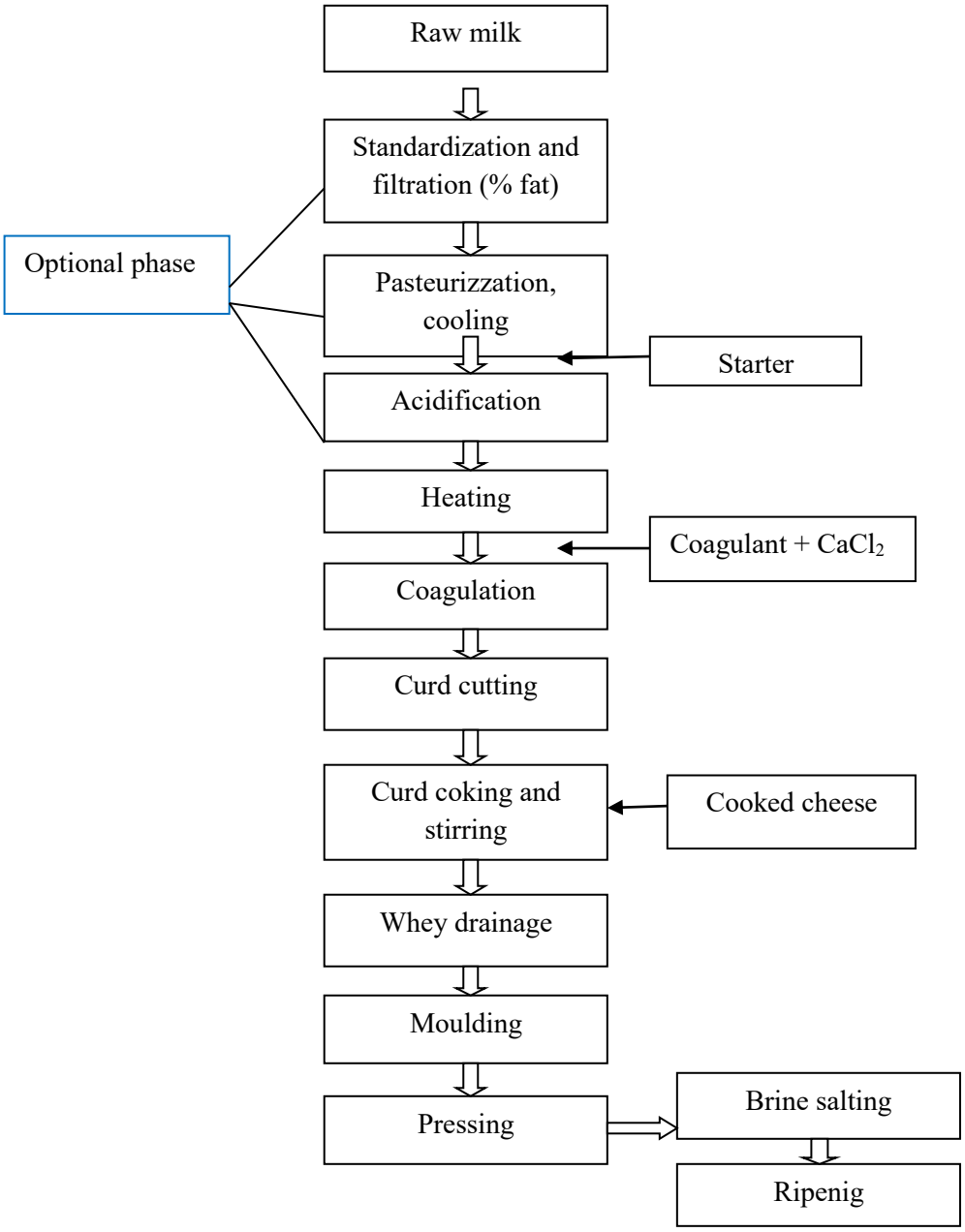


Figure 2-1:Overview of cheese making operations

Cheese can be obtained from milks of different species (ewe, goat, cow, buffalo) and the type of milk influences the chemical composition and organoleptic characteristics.
The manufacturing of cheese is essentially a concentration process, where milk fat and casein are concentrated approximately ten-fold, while the whey proteins, lactose and water soluble salts are removed with the whey.

2.3 Milk treatment prior to cheese making

The suitability of milk as a raw material for cheese production depends largely on conditions at the dairy farm (e.g. feeding animals on badly prepared silage can adversely affect the quality of several varieties of cheese) and milk pretreatment at the cheese manufacturing plant (e.g. cold storage and pasteurization) (Bylund, 2003).

2.3.1. Filtration

Raw milk as produced on the farm and transported to the processing plant generally contains varying amounts of visible, invisible impurities. This foreign matter includes straw and hair pieces, dust particles, leukocytes (somatic cells), insects, etc. If not effectively removed, such extraneous insoluble matter can result in deposits in milk handling equipment such as cooler, and, more importantly, cause unsightly appearance. Relatively large pieces of such material are usually removed by straining (passing the milk through a fine metal-gauge strainer or metallic sieve on the farm, at the processing plant). This steps of aesthetic improvement of product are particularly useful for overcoming the problem of sediments in fluid milk (Bylund, 2003).

2.3.2 Cold storage

If the milk is not used for cheese making immediately after reception, is should be cooled down and stored at 5-8 ºC. At low temperatures, the renneting ability of milk is slightly reduced. The reduction in renneting ability is greatest for milk, which has been pasteurized prior to being stored. When milk is stored at low temperatures, part of the β-casein and calcium ions are released from the casein particles (Bylund, 2003).

The use of refrigerated milk could resulted in a weaker curd, lower curd yield and greater losses of fat and curd fines into the whey than the use of milk stored at 10-20 ºC. Although raw milk is still used in both commercial and farmhouse cheese making. However, in most cases, the cold milk is heat treated before processing (Grandinson, 1986).

2.3.3 Standardization

Standardization is the process of changing the solids composition of milk from what is received from the producer. In market milk industry, this normally involves removing some fat by natural creaming or centrifugation, by adding skim milk or cream (fat).
Milk standardization gives the cheese maker the ability to manipulate the composition of the final cheese by controlling the composition of the starting milk in order to meet the legal definition of the specific variety and to improve yields (Fox and McSweeney, 1998).

2.3.4 Thermization and Pasteurization

Thermization refers to the heat treatment of milk at sub-pasteurization temperatures (typically 50–70°C for 5–30s) on reception at the dairy to reduce the viable bacterial load in the milk and minimize changes in quality and processability prior to conversion into the final product. This greatly reduces the development/occurrence of bacterial-associated enzymatic activities in the milk during subsequent cold storage, as reflected by lower levels of peptides and free fatty acids in the stored milk. Consequently, thermization generally improves the yield and quality of cheeses prepared from milks that have been cold stored. Thermization does not fully inactivate all spoilage and pathogenic bacteria. To achieve this, a more intensive pasteurization step is typically applied at a later stage in the process. (Rukke et al., 2011).
Milk intended for the production of soft cheeses is pasteurized at 72 ºC for 15 seconds to destroy pathogenic microbes, reduce the total microbial load and other undesirable microorganisms, such as yeasts and coliforms, that may alter the cheese characteristics by producing carbon dioxide and undesirable proteolysis. Pasteurization also inactivates some enzymes, reverses shifts in the mineral balance of milk induced by cold storage, and influences the microflora of non-starter lactic acid bacteria in the final cheese. In medium and long ripened cheeses, milk is generally processed, as the development of pathogenic microorganisms will be hindered by the dairy microflora and by the ripening conditions.

Milk is then cooled after pasteurization to 32°C to bring it to the temperature needed for the starter bacteria to grow.
However, spore-forming microorganisms in the spore state survive pasteurization and can cause serious problems during the ripening process. One example is *Clostridium tyrobutyricum*, which forms butyric acid and large volumes of hydrogen gas by fermenting lactic acid. The butyric acid has an unsavory taste, and the gas destroys the texture of the cheese completely.
More intense heat treatment would reduce this particular risk but would also seriously impair the general cheesemaking properties of the milk as it results in significant denaturation of whey proteins and their resulting incorporation into cheese curd, with significant effects on the renneting ability of milk: the coagulation takes longer, the coagulum becomes weaker, and the whey exudation is slower (Kelly et al., 2008).

2.3.5 Starter-culture addition

Starter cultures are used in the cheese making process to assist coagulation by lowering the pH prior to rennet addition. Starter is normally added to the milk at renneting temperature. The metabolism of starter cultures contribute to the desirable flavor compounds, help prevent the growth of spoilage organisms and pathogens and promotes syneresis (extraction or expulsion of a liquid from a gel).
This process is usually accomplished by the addition of lactic acid bacteria that convert lactose to lactic acid.
Lactic acid bacteria can be added to milk in the boiler in the form of natural lactic cultures (*Streptococcus thermophilus, Lactobacillus bulgaricus)* or selected lactic cultures.
Most varieties of cheese cannot be made without the addition of a "starter" which is a culture of carefully selected lactic acid-producing bacteria (Salvadori del Prato, 2001).

2.4 Conversion of milk to cheese curd

2.4.1 Heating

Milk is heated up to the coagulation temperature, depending on the type of cheese to be produced but in any case always higher than 15 ° C and below 35 ° C, owing to create the

ideal conditions for the enzyme and hence the coagulant activity and the proteolytic activity (Law et al., 2011).

2.4.2 Calcium chloride addition ($CaCl_2$)

Calcium plays an essential role in the coagulation of milk by rennet and in the subsequent processing of the clot. In a typical cheese production plant, $CaCl_2$ is added before the addition of the coagulant, without affecting final cheese quality. Addition of $CaCl_2$to cheese milk can alleviate cold-storage and heat-induced impairments of clotting and curd firmness by improving poor coagulating milk (Law et al., 2011).

A low concentration of Ca ions in the cheese milk causes a soft coagulum. This results in heavy losses of finess (casein) and fat, as well as poor syneresis during cheesemaking. Between 5-20 g of $CaCl_2$ per 100 kg of milk is normally enough to achieve a constant coagulation time and result in sufficient firmness of the coagulum. By adding more $CaCl_2$ the amount of rennet used can be reduced, as the $CaCl_2$ supports the action of rennet. However, excessive addition of $CaCl_2$ may make the coagulum so hard that it is difficult to cut (McSweeney, 2007).

2.4.3 Coagulant addition

Calf rennet is the coagulant traditionally used for coagulation of milk. The term “rennet” is given to the coagulant extracted from the abomasum of young ruminants (calves, lambs or suckling kids) slaughtered before weaning. It contains two active fractions: chymosin (95% of the enzyme activity) and pepsin. The first is very coagulating while the latter ones strongly proteolytic.

Furthermore, although rennet is used most often, other coagulants are available from animal, vegetable or microbial sources. The type of coagulant used depends on the desired characteristics of cheese.

2.4.4 Coagulation

Coagulation refers as destabilization of the casein micelles, which flocculate and aggregate to form a gel enclosing the soluble milk components. It can be caused by

acidification, by the action of an enzyme or by a combination of these two (Troch et al., 2017).

A. Acid coagulation

In acid coagulation of milk, casein micelle properties are altered by a lowered milk pH without the presence of rennet. Acid coagulation can be achieved by the addition of an organic acid such as citric, acetic or tartaric acid, high-acid whey or through adding starter cultures. Acid coagulation precipitates caseins at their isoelectric point (pH 4,6). When the milk reaches the pH value of 4,6 the micelles lose their negative charge and start to interact with each other allowing the formation of aggregates (Fox & McSweeney, 1998).

B. Enzyme-induced coagulation

Enzymatic coagulation of milk is the modification of casein micelles via limited hydrolysis of casein by rennet, followed by calcium-induced micelle aggregation.

Generally, coagulation is divided into three underlying steps with different mechanisms (Fox &McSweeney, 2004):

- *enzymatic phase* in which the κ-casein is degraded by chymosin;
- *non-enzymatic phase* that corresponds to the formation of a gel through the aggregation of degraded micelles.
- *devolopement of the three dimensional gel network (firmness).*

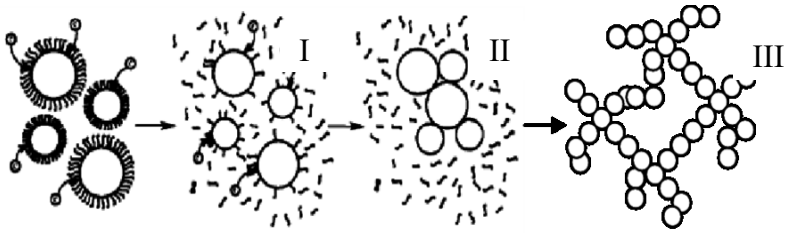


Figure 2-2:Illustration of the rennet coagulation process. (I) k-casein removal by chymosin (II) para-CN aggregation and (III) gel network formation (McSweeney, 2007).

During the enzymatic phase, chymosin causes the hydrolysis of the C-terminal part of κ-casein by cleavage at the Phe_{105}-Met_{106} bond. This hydrolysis produces para-κ-casein (the N-terminal part of κ-casein), which remains attached to the micelles and glycomacropeptide (the C-terminal portion) that will be released into the whey. The release of the hydrophilic glycomacropeptide results in destabilization of the micelles which become less negatively charged and more hydrophobic.

After losing its water-soluble tail, k-CN can no longer keep the casein particles separated, so they begin to form a gel. The clusters continue to grow until they form a continuous, three

dimentional network followed by further solidification through calcium cross-linking and traps water inside, forming a gel (Walstra et al., 2006).
The third stage refers to an ongoing firmness in the development of the gel network. The gel is unstable, and following the contraction of the micelles, it expels the liquid phase out of the curd. This phenomenon, called syneresis, separates the curd, containing casein and fat, from the whey, containing lactose, minerals and soluble proteins.
Coagulation is enhanced by decreasing pH, increasing calcium concentration and temperature (no aggregation below 20 ºC) (Troch et al., 2017).

2.4.5 Curd cutting

The cutting of the curd has the purpose of inducing and regulating the separation of the whey. When the gel formed by the action of the rennet has become sufficiently resistant, a series of successive cuts of the entire coagulated mass are made, first in strips, then in coarse cubes and, finally, in smaller and smaller granules. The size of the granules is typical of each process, small (rice grain) for hard cheeses to be aged, larger (walnut / hazelnut) for fresh and soft cheeses (Benett and Johnston, 2004).

2.4.6 Curd processing (cooking, drainage)

The typical cooking phase of some hard or semi-hard cheeses involves heating the curd, maintaining curd stirring, at a temperature of about 44-46 ºC for semi-cooked cheeses, 54-56 ºC for those cooked with the purpose of further purging and facilitate their aggregation. Raising the temperature will increase the firmness of the curds by enhancing syneresis and also enhance fermentation process by the starter bacteria.
The cooked curd (curd grains) must be separated from the whey, which is accomplished by draining the whey from the bath through a sieve-like strainer (Law et al., 2011).

2.4.7 Curd molding and pressing

The principal purpose of molding is to allow the curd to form a continuous mass; matting of high-moisture curds occurs readily under their own weight but pressing is required for low-moisture cheese.

Pressed cheese are submitted to a pressing system after have been molded with the purpose of assist final whey expulsion, provide texture, shape the cheese, and provide a crust to the cheeses with long ripening periods (Fox et al., 2000).

2.4.8 Salting

Salt in cheese serves two major roles — namely, it acts as a preservative and contributes directly to flavor and quality. The n preservative action of NaCl is due to its depressing effect on the water activity of the cheese. Moreover, salt increases the osmotic pressure of the aqueous phase of foods, causing dehydration of bacterial cells, killing them or, at least, preventing their growth. NaCl contributes directly to saltiness in cheese, a flavor that is generally highly appreciated. It contributes indirectly to flavor of cheese by its controlling influence on microbial and enzymatic activities which, in turn, influence lactose metabolism, cheese pH, degradation of fats and casein, and the formation of flavor compounds, such as peptides, free amino acids, and free fatty acids. In addition to these functions, salt exerts a number of important effects on cheese. Salt, together with pH and calcium level, has a large effect on the extent of para-casein hydration or aggregation, which in turn affects the water binding capacity of the casein matrix, its tendency to synerese, its rheological and textural characteristics, and its cooking properties (McSweeney, 2007).The cheeses can be salted in three ways: dry, in brine, in pasta.

- ***Dry salting:*** it is carried out using course salt which is rubbed on the surfaces and on the side of the cheeses to be treated. The salt dissolves and penetrates the form through the surface; the operation is repeated several times, depending on the size of the shape andeach time it is accompanied by a turning of the shape: it is often manual, but it can also be mechanical.
- ***Brine salting:*** it is carried out by immersing the cheese in concentrated solutions of sodium chloride, kept at relatively low temperatures, 4-5 ° C for soft cheeses and between 10 and 18 ° C for the others, in order to quickly cool the cheese and reduce the metabolic activity of the lactic flora. The concentration of the brines varies between 15% and 26% by weight of NaCl. Higher concentrations are preferred for hard cheeses and lower concentrations for soft cheeses.
- ***Salting in paste:*** it is carried out by adding the salt directly to the coarsely divided curd, before putting it into shape (Bylund, 2003).

2.4.9 Ripening

Ripening refers to the biochemical, microbiological, structural, physical and sensory changes that occur during storage post manufacture and transform the fresh curd to a cheese with the desired characteristics. Ripening is a slow phase, crucial for the development of aroma and flavor, brought about by the action of the many enzymes released by lactic acid bacteria.

Ripening of cheese takes place usually in ripening rooms where temperature, humidity and other factors must be controlled differently for each type of cheese.

The biochemical and physical changes are induced by the microbiological and enzymatic heritage of the cheese derived from milk, by the lactic cultures used for acidification, by the type and quantity of rennet used and by the addition of any specific enzymes (Fox et al., 1993).

The ripening of cheese involves following major biochemical events:

1. *Glycolysis*: Lactose is metabolized to lactic acid, which may then be catabolized to form acetic and propionic acids, carbon dioxide, esters and alcohol by the enzymes of the microorganisms in the milk, including the added starter.
2. *Lipolysis*: The lipids are broken down to form free fatty acids, that may then be catabolized to form ketones, lactones and esters by natural milk enzymes and those that are added to create the flavor in particular cheese varieties.
3. *Proteolysis*. Protein transformation is the most important phenomenon of the maturation process. Proteolysis influences the consistency and flavor of the cheese by breaking the integrity of the reticular structure assumed by casein and producing minor peptides, peptones, amino acids, organic acids and ammonia. The ratio between soluble nitrogen and total nitrogen is used as an indicator of the level of maturation. Protein degradation is generated by the action of enzymes that come from the coagulants used, lactic bacteria and molds, as well as from proteolytic enzymes naturally present in milk, including in particular, plasmin.
4. *Reduction of water* by evaporation, proportionally to the maturation time. Its reduction results in the concentration of salts and all soluble components and significantly affects the vitality of the microflora.

2.5 Factors influencing the coagulation process of milk

Milk coagulation factors can be divided in several groups as follows:

a) Chemical compositional factors, which directly affect milk coagulation (e.g., fat/protein ratio, colloidal calcium phosphate content of milk, casein concentration, etc.). Compositional factors are widely influenced by a large number of animal-related, physiological and environmental factors, which indirectly affect milk coagulation (e.g., animal nutrition, genetic variance, seasonal effect, lactation stage, lactation number, milking frequency, subclinical mastitis, etc.);
b) milk pre-treatment factors, which modify the existing chemical composition of milk (e.g., refrigeration, pasteurization and homogenization, etc.)
c) technological factors, which affect coagulation directly during the process (pH, coagulation temperature, calcium concentration, enzyme concentration and type, protein content).

The determination of the milk coagulation properties is extremely important, since raw milk from cows, ewes or goats (of the same or different breeds) has different coagulation capacities, due to their different chemical composition.

2.5.1 Type of milk (raw and pasteurized)

Raw milk cheeses are known to have more intense and strong flavor and different texture due to natural microbiota and enzymes. Nevertheless, there are concerns about safety of these products.

It has been recognized for a long time that heat treatment of milk modifies several of its physicochemical properties and impairs its rennetability (Montilla et al., 1995).

Heating milk at temperatures greater than 70 ºC results in thermal denaturation of whey protein which may reach 30% when a pasteurization at 75 ºC for 6 min is applied, and 75-90% in the case of indirect UHT treatments (Rayanal et al., 1998). Heat treatments at temperatures above 90 ºC for more than 10 min can completely inhibit gelation. The main contributing factor to this effect of heating on gelation properties is the fact that the denatured whey proteins attach to the casein micelles by forming complexes with the k-casein via disulfide bonds. These bond avoid the casein micelles to interact strongly between each other (Fox et al., 2000).

It is well established that milk which had been heated at temperatures above 70 ºC has a longer coagulation time and forms a weaker curd than the original unheated milk.
However, other physico-chemical modifications induced by heating, such as changes in milk salts equilibria, micelle size and hydration, might also interfere with the renneting process, especially during the aggregation and gelification phases.
In addition, heating has a marked effect on the milk salts equilibrium and their interaction with casein. Heating leads to a decrease in diffusible calcium and inorganic phosphate, due to precipitation of calcium phosphate, which may be cause an extension on cooling . There is also some evidence that, when severe heat treatments are applied, irreversible changes of the salt system occur due to a modification of the nature of colloidal calcium phosphate. This implies an alteration of calcium exchangeability and micelles integrity after heating (Montilla et al., 1995; Raynal et al., 1998).

2.5.2 pH of milk

The pH of the milk strongly influences the rennet-induced coagulation of milk. The effect of pH is mainly on the first (enzymatic) stage of coagulation. As the pH of the milk decreases, the enzyme moves closer to its optimum pH, speeding up the reaction. Optimum pH for the action of chymosin in milk is 6.0, but the optimum pH is lower for isolated caseins or synthetic peptides. A reduction in milk pH to a value in the range 6.6 -6.0 results in a reduction in the rennet coagulation time of bovine, ovine, and caprine milk (Pellegrini et al, 1997; Castillo et al., 2000) (due to reduced electrostatic repulsion) and a faster rate of increase in gel firmness.
According to Law and Tamine (2010) the pH has a large effect on coagulation and the properties of the curd, as a reduction in pH will speed up the rate of k-CN hydrolysis and the subsequent aggregation of casein micelles.

2.5.3 Coagulation Temperature

The coagulation of renneted micelles is very temperature-dependent. Temperature has a larger effect on the aggregation phase than on the enzymatic phase.
The optimal condition for curd formation in milk with chymosin is 40-45 ºC, but this temperature is not suitable for cheese making. Rennet coagulation for cheese making generally occurs at 32-35 ºC for proper firmness. At lower temperature rennet clotting rate is

significantly reduced, and at refrigeration temperature virtually no curd is obtained (Singh & Waungana, 2001).

The temperature 30-35 ºC is necessary also to optimize the growth of mesophilic starter bacteria, which have an optimum growth temperature of about 27-28 ºC and will neither grow, nor even survive, above 40 ºC. In addition, the structure of the clots improved at lower temperature (Swaisgood et al., 2003).

2.5.4 Calcium concentration

Milk with higher calcium content has a shorter rennet coagulation time. Calcium ions are essential for aggregation and gelation of casein micells. An increase of Ca2+ activity can lead to faster aggregation of the casein micelles . Para-*k*-CN binds to calcium ions more strongly than *k*-CN. Addition of $CaCl_2$reduces the rate of rennet clot formation time and also increases rennet curd firmness. Milks that have tendency to form weak curd may be fortified with $CaCl_2$ prior to the addition of rennet. The addition of $CaCl_2$to milk has a positive effect on texture and cheese curd yield (Everett et al., 2017; Ben Amira et al., 2017).

2.5.5 Enzyme concentration

The effect of enzyme concentration on the coagulation is directly related to time of reaction and gel firmness. An increase in enzyme concentration (larger amounts of rennet added to milk) reduces the total time required for rennet clotting, due to a higher level of kappa-casein proteolysis. Hence, micelles need more kappa-casein cleavage to aggregate faster. As a result, the secondary phase of rennet action will also proceed much earlier, with the greater result in in gel firmness. Lowering the amount of rennet reduces the rate of curd firmness (Lucey, 2002).

Enzyme kinetics have been used to study these effects. Have been stated that the clotting time was inversely related to the concentration of rennet used to clot the milk, as follow:

Eq. (1) $$CT = {}^{K}/_{[E]} + A$$

Where ***CT*** is the clotting time, ***K, A*** are the constants, **[E]** is the enzyme concentration.

Clotting time is affected by both enzymatic and aggregation phases of coagulation, so A in this equation is refers to the time needed to the second phase, which is not enzyme dependent (Lucey, 2002).

2.5.6. Milk protein concentration

The quantities of casein influence the coagulation of milk and its performance. The coagulation time of milk decreases markedly with protein (and thus casein content).
The casein content of the milk also has an effect on the rate of firming; the gel increases with the concentration of casein.
The size of casein micelles present an impact also on milk coagulation: small micelles will lead to firmer curds.
Milk with smaller micelles aggregates faster and have firmer clot. Smaller micelles have a higher level of *κ*-casein, which can improve the bridging of proteins and calcium, resulting in a shorter coagulating time and firmer gel (Everett, 2017). Several small casein micelles have an increased surface area compared with fewer large micelles. The increased area leads to greater availability of hydrolytic cleavage, hence, faster aggregation. Smaller micelles also provided better cheese yield.

2.6 Milk coagulating enzymes

2.6.1 Animal origin rennet

Animal rennet is an extract isolated from fourth stomach (abomasums) of young unweaned ruminants (calves, lambs, kids) with the capability of clotting milk by enzymatic action. The major component of rennet is chymosin, but it can contain also pepsin in varying concentration. The proportion of chymosin and pepsin in rennet depend on the age and feeding regime of the animals from which enzymes are obtained. As calves become older and begin eating other feeds, the proportion of bovine pepsin in relation to chymosin increases. Traditional animal rennet contains 5% pepsin and almost 95% chymosin.
Today, animal rennets are produced mainly from frozen abomasums (earlier, dried abomasa were used), which are cut up in special grinders and their milk-clotting prochymosines extracted in a 3–10g per 100g NaCl brine solution. The prochymosines are activated to chymosin and pepsin by lowering the pH to about 2 for 1h and then adjusted to about pH 5.5

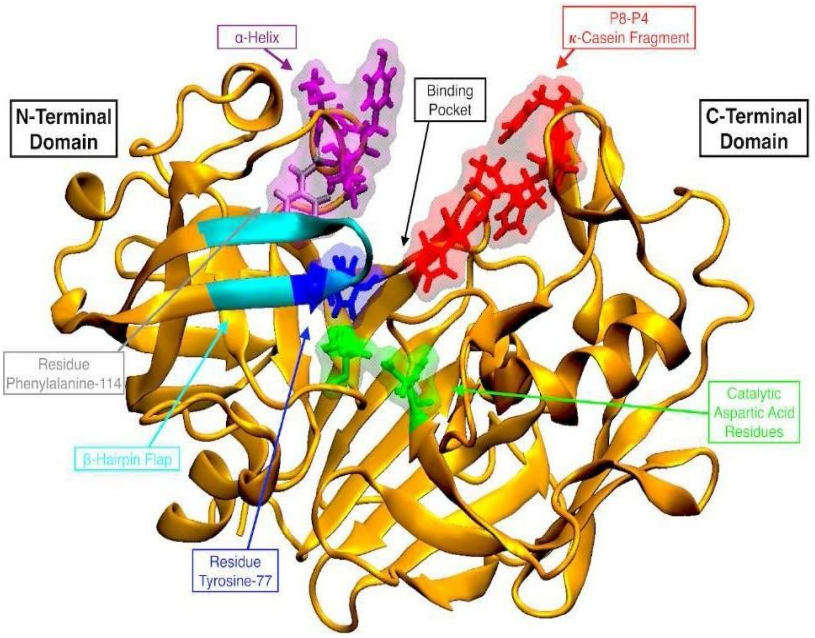
before filtering and concentrating the extract. The extract is further filtered to remove bacteria and the concentration of NaCl is then increased to about 20g per 100g. Finally, the rennet is diluted to a certain strength (total milk-clotting activity). Sodium benzoate, propylene glycol and salt are added as preservatives for the final rennet. Rennets are usually distributed as liquids, but they may also be in powder form.

Chymosin is the biochemical name of the enzyme that was formelly known as rennin. It belongs to the group of aspartic acid proteinases, that have a high content of dicarboxylic and hydroxyamino acids and a low content of basic amino acids. Its molecular mass is 36 kDa.

Like most proteases, chymosin is secreted in the inactivated form of zymogen prochymosin, and then is activated by autocatalytic proteolysis at a low pH value into the protein of chymosin with a molecular weight of 36 kDa.

Chymosin has the lowest total proteolytic activity with respect to milk-clotting activity (12%).The general proteolytic pH optimum of chymosin is 3.8, but it has high specific milk-clotting activity at the pH of milk, that is, 6.7.

Chymosin hydrolyses the Phe105-Met106 bond of the milk κ-casein molecule. This bond is much more susceptible to acid proteases than other peptide bonds in the protein system of milk. As a result, the micelles lose steric stabilization and become susceptible to aggregation, particularly in the presence of Ca2+ thereby leading to gel formation and phase separation of the milk into curds and whey (Andren A., 2011)



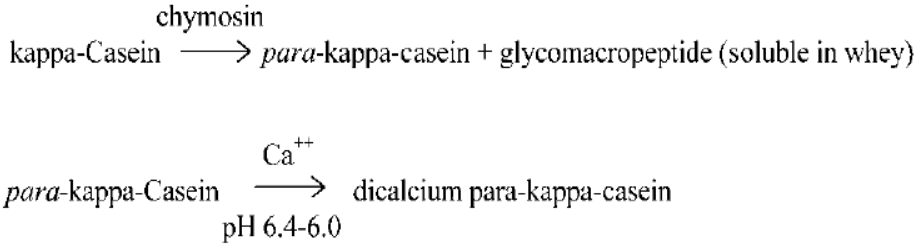


Figure 2-3:Destabilization of casein micelle by introduction of chymosin (Aktayeva et al., 2015).

2.6.2 Genetically modified rennet

During the1980s, recombinant DNA technology was used to develop microorganisms capable of producing chymosin, using the DNA sequence of chymosin from a calf abomasums cell.

Genetic technology has been used for the commercial production of a 100% pure chymosin product.

The microbes used for this type of rennet include non pathogenic microorganisms *Escherichia coli* K-12, *Kluyveromyces marxianus* var. *lactis* and *Aspergillus niger* var. *awamori*.

Prochymosin genes are extracted from an animal's stomach cells then are transferred through DNA plasmid intervention and implanted into yeast cultures that act as a host. Fermentation follows to produce prochymosin, cell destruction, activation of the prochymosin to chymosin,

and harvesting/producing large yields of pure, 100 % chymosin. The new chymosin enzymes are separated out and purified (Figure 2-4).

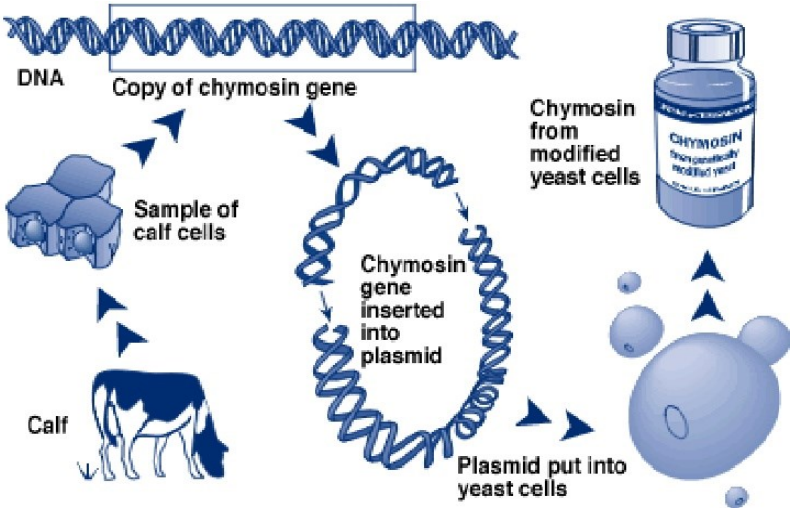


Figure 2-4: Production of recombinant chymosin (Kumar et al., 2010)

Since genetically modified chymosin is identical to animal chymosin (the same amino acid sequence), its properties are the same as those of calf chymosin. The only difference is that the producing organism could add some residues to the chymosin molecule. However, such modifications have not been observed to change the properties of genetically modified chymosin compared to animal chymosin.

These rennets have been widely studied to evaluate their impact on the quality and yield of cheese. An initial concern was due of the absence of pepsin from these types of rennet, proper cheese flavour might not develop. Most studies have generally concluded that there are no significant differences in flavour, texture, composition, and yield compared with animal rennet controls.

Recombinant chymosin have several advantages such as predictable coagulation behavior, low proteolytic activity and higher yield since it its 100% pure enzyme.

Due to the extensive knowledge about the genome, *E. coli* have been used as host organism of recombinant chymosin (Kumar et al., 2010). The perks of its ability to grow rapidly and on inexpensive media makes it a suitable protein factory. As well as it large number of cloning vectors and mutant host strains.

Yeast has several advantages as a factory of proteins. Low economic cost during cultivation, extensive knowledge about fermentation and the physiology as well as the genetics of the organism (Kumar et al 2010). *Pichia pastoris* is a commonly used host for protein expression

due to the previous mentioned reasons as well as; ease of manipulation, high expression level and its ability to perform eukaryotic post-translation modification. It has been used for the expression of both goat and bovine chymosin (Feijoo-Siota et al., 2018).

2.6.3Microbial-derived coagulants

Microbial rennets are proteolytic ezymes produced by microorganisms. There are able to induce the coagulation of milk in a way similar to the animal rennets. Advantages of microbial enzymes are easy to produce, low cost of production, compliance with the criteria of natural origin and vegetarian requirements.

The enzymes show, however, higher proteolytic activity during cheesemaking, which may lead to a loss of protein degradation products into the whey and thus negatively affect cheese yield (Jacob et al., 2010).

Microbial milk-clotting enzymes are usually bacterial or fungal origin. The aspartic proteinases from fungi are generally divided into two groups:

- Pepsin enzymes derived from *Aspergillus, Penicillium, Rhizopous and Neurospora*;
- Chymosin ezymes derived from *Cryphonectria* and *Rhizomucor spp.*

More than 100 fungal sources were reported by Garg and Johri (1994), which reflects the high scientific interest in alternative coagulants for cheese production. Fungi producing milk clotting proteases are ubiquitary and may easily be isolated from various environments (Tubesha and Al-Delaimy, 2003).

Several milk-clotting enzymes of microbial origin have been commercialized and used in cheese making, such as aspartic proteases (APs) obtained from *Aspergillus oryzae*, *Rhizomucor miehei*, *Rhizomucor pusillus*, *Endothia parasitica*, and *Irpex lactis* (Neelakantan et al., 1999).

2.6.4 Plant coagulants

Ethical, religious and economic factors have led to the search for an alternative to animal derived rennet enzymes. The coagulants from plants are natural proteases, involved in plant development which have been found to have milk clotting activity. The milk clotting activity differs between plants as well as their parts from which the enzymes are extracted (Ben Amira, et al., 2017).

Increased interest to plant proteases due because there are natural products easily purified, can be eaten by vegetarians and may be certified as Kosher and Halal. Although several plant coagulants are able to coagulate milk, but most of them has been found to be inappropriate for cheese production due its excessively proteolytic character which causes bitterness and reduction of cheese yield. Excessive proteolysis can lead to a decrease in cheese yield (due to excessive non-specific proteolysis in the cheese and loss of peptides in the whey) and defects in the flavour (bitterness) and texture (softness) of ripened cheese (Jakob et al., 2011).

Despite this, the aqueous extract obtained from the flowers of *Cynara cardunculus* has been used for years in artisanal cheese making, especially in Mediterranean countries(Spain and Portugal). Fig latex is used in Italy and Turkey

The most studied plant species as source of milk coagulant are the species of the genera Cynara (*C. Cardunculus, C. scolymus, C. humilis*) (Zikiou A. et al.,2020; Liburdi K. et al., 2019; Gomes S. et al., 2018; Baraka Abo El-Yazeed Abd El-Salam et al., 2017; Esposito M. et al., 2016; Ordiales E., 2016) followed by the species of the genera Solanum (*S.tuberosum, S.betaceum, and S.elaeagnifolium*) (Rocío Tito et al., 2020; Gutiérrez-Méndez N. et al., 2018; Dely R. Chávez-Garay et al., 2016) and Ficus (*F.carica, F. johannis*) (Afsharnezhad M. et al., 2019; El-Hocin Siar et al., 2020; Liburdi K., 2019).

The vegetable proteases used to coagulate milk are divided into groups based on the catalytic mechanism used during the hydrolytic process. The main types are aspartic, cysteine and serine proteases (Shah et al., 2004).

2.6.4.1 Aspartic proteases

Aspartic proteases have two aspartic residues at their catalytic site. They are most active at acidic pH and show preferential specificity for cleavage at peptide bonds between hydrophobic amino acid residues responsible for the catalytic activity (Simoes I. et al., 2004).

Aspartic proteases with milk-clotting activity have been found, as you can see in the table 2-1, in artichoke (*Cynara scolymus* L.); milk thistle (*Silybum marianum*); *Onopordum turcicum*; rice kernels; *Centaurea calcitrapa* (Shah et al., 2014).

The most studied are aspartic proteases of the artichoke *Cynara cardunculus.* Cardoon (*Cynara cardunculus*) flowers are traditionally used in the Mediterranean region for cheesemaking. It produces cardosins and cyprosins, aspartic proteases that have been found to accumulate in mature flowers (petals and pistils) but not in leaves or seeds.

Cardosin A is an abundant aspartic protease from pistils of *C. cardunculus*. These flowers contain aspartic proteases, which have been shown to share specificity and kinetic parameters with chymosin and pepsin (Silva et al., 2004).

2.6.4.2 Cysteine proteases

Cysteine proteases, also known as thiol proteases, and the catalytic mechanism of these enzymes involve a cysteine group in the active site. Cysteine proteases have great potential in the food, biotechnology, and pharmaceutical industries owing to their property of being active over a wide range of temperature and pH (Shah et al., 2014).
Cysteine proteases include enzymes isolated from *Carica papaya* (papain), *Ananas comosus* (bromelain), *Ficus glabrata* and *Ficus racemosa* (ficin) (Aktayeva S. et al., 2015).

2.6.4.3 Serine proteases

Serine proteases have in their active sites serine residues. Their main role in plants is almost the same as the aspartic proteases, with some additional features. They are found in almost all taxonomic groups of plants and are present in almost all parts of plants (latex, flowers, leaves, roots, stems and seeds), but in the greatest quantity in fruits. Plant serine proteases are active over a wide range of pH 7-11, and temperature 20-50°C (Aktayeva S. et al., 2015).

Chapter 3
BOTANICAL CHARACTERISTICS OF *ONOPORDUM TAURICUM*

Onopordum tauricum Willd, (Taurian thistle) is a species of thistle belonging to the *Asteraceae* family (Fig. 3-1, 3-2).The representatives of genus Onopordum are native to Europe.

Figure 3-1: Onopordum tauricum – flower

Figure 3-2: Onopordum tauricum- plant

This is a biennial herbaceous plant producing a sticky, glandular, very spiny stem up to 2 meters tall. The spiny, bright light green leaves are up to 25 centimeters long and are divided

into triangular lobes. The inflorescence is made up of several large flower heads each up to 7 centimeters wide. They are lined with long, spiny phyllaries and bear pink-purple tubular flowers up to 3 centimeters long.

Anthesis occurs predominantly in the month of July-August.

The habitat of the plant are uncultivated, ruins, riparian areas, from 0 to 1500 m above sea level (Fig. 3-3). Sometimes it also appears under the aspect of a nitrophilous plant, preferring places with soils rich in deposits of animal excrement, such as in stables, sheep settlements or various farms. (https://en.wikipedia.org/wiki/Onopordum_tauricum).

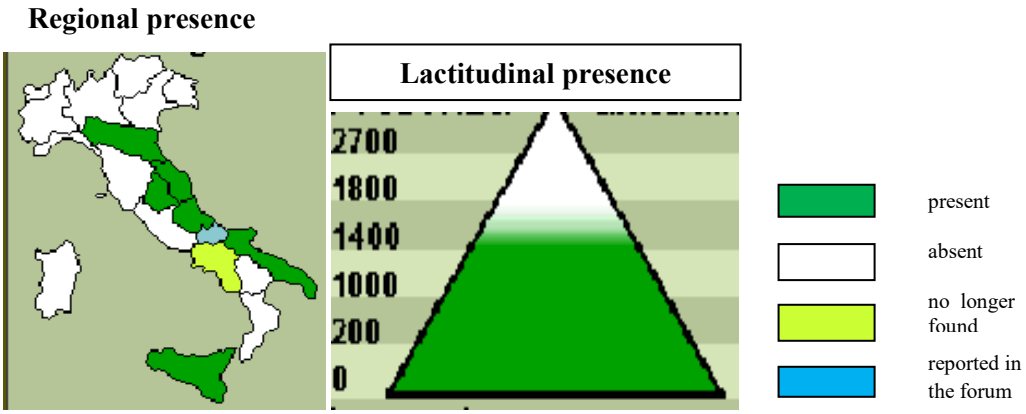


Figure 3-3: Regional and lactitudinal presence of Onopordum tauricum in Italy

Chapter 4
MATERIALS AND METHODS

4.1 Plant material and crude extract preparation

Spontaneously grown *O. tauricum* plants were collected in July 2019 along the outer fringes of the Monti Sibillini National Park, which extends in the hearth of Italy, between the Marche and Umbria regions. Tubular flowers (Figure 4-1) were manually separated from receptacle immediately after harvesting, and macerated in demineralized water (1:10 w/v) for 24 h at 4 °C. The liquid phase was recovered by filtration through a muslin cloth and subsequent centrifugation (5000× *g*, 10 min). Finally, the aqueous crude extract (CE) was freeze-dried (VirTis Advantage benchtop freeze dryer, Steroglass S.r.l., Perugia, Italy) and stored at −20 °C until it was used. At the time of use, the dried extract was reconstituted in demineralized water 1:10 w/v (reconstituted extract, RE).

Figure 4-1:Inflorescence (flower head) of Onopordum tauricum (a) whole; (b) section.

The milk clotting performance of RE was compared with a commercial liquid preparation of calf rennet (CR) (51 International Milk Clotting Units, IMCU) provided by Caglificio Clerici (Como, Italy).

4.2 Substrates preparation and chemical characterization

Cow's, goat's and ewe's milk were used as coagulation substrates. Partially skimmed UHT milks from cow and goat were purchased in a local grocery store. Crude ewe's milk was collected in a local farm (Azienda Agricola Zerbino Francesco, Senigallia, Italy), immediately refrigerated at 4 °C, and transferred to the laboratories of Università Politecnica delle Marche, where it was skimmed by centrifugation at 5000× *g* and 30 °C for 10 min. All skimmed milks were freeze-dried, and milk powders were stored under vacuum at −20 °C. Dry matter percentages were calculated on the basis of the amount of the freeze-dried products. Milk powders were analyzed for fat, protein, and ash contents, according to the procedures described in Roncolini et al.,2020, while lactose content was calculated by subtracting the quantified milk nutrients from the total solid content.

Milk powders were reconstituted in different buffer solutions (Fig. 3-2), based on their dry matter contents. To prepare the buffers, a solution of sodium acetate of 100 mM was adjusted to the desired pH values (4.5, 5.0, 5.5, 6.0, 6.5) by adding concentrated acetic acid.

The powder-buffer/distilled water mixtures were placed in ultrasonic bath and underwent sonication to ensure a complete dissolution of the milk powder.

4.3 Physicochemical parameters determination

The total protein amount of RE and CR was determined according to the Coomassie blue dye binding method (Bradford, 1976) using the Bio-Rad (Bio-Rad Laboratories S.r.l, Milan, Italy) ready-to-use reagent. A set of bovine serum albumin (Merck KGaA, Darmstadt, Germany) solutions (0.2–0.9 mg/mL) was used for calibration. Absorbance readings at 595 nm were carried out by using a UV-1800 Shimadzu (Kyoto, Japan) spectrophotometer.

The pH values of reconstituted milks were checked by a benchtop pH meter equipped with a glass electrode (Hanna Instruments, Padova, Italy) and the actual pH values were used for modeling the caseinolytic activity of *O. tauricum* proteases.

4.4 Experimental design

The experiments were designed according to the DOE D-optimal design with JMP statistical software to determine the response sequence and then to establish a model. Three independent variables were selected:

- temperature as continuous factor in the range 35-55 °C ;
- volume of raw extract as continuous factor in the range 300–500 μL;
- pH as 5-level discrete factor (4.5, 5.0, 5.5, 6.0, 6.5).

The selection of ranges of each factor was based on preliminary experimental results. The D-optimal criterion was used for designing the experiment, to obtain the maximum amount of useful information in a reasonable number of experiments to run, including two replicates of each run.

The software generated 20 experiments that were carried out in double. A second-order response surface, according to the following equation, was used to fit the experimental data matrix:

Eq (2) $$Y = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{3} b_{ii} X_i^2 + \sum_{i \neq j=1}^{3} b_{ij} X_i X_j$$

where Y is the response variable (MCA); Xi, Xj are the coded values of the input variables (temperature, volume of coagulant, pH); and b0, bi, bii, and bij are the regression coefficients for the intercept, linear, quadratic, and interaction terms, respectively.

The experiments were randomized to minimize the effects of unexplained variability. The experimental plan was also carried out using a commercial liquid preparation of CR. The volumes of CR used in each run were adjusted to have the same amounts of total proteins that were in the used volumes of RE.

To confirm the enzymatic milk-clotting activity of the *O. tauricum* extract, a control test tube was prepared for each run without adding the RE to the milk.

All the factors combinations and the real values of MCT (s), for each milk type are reported in Table 4-2.

The procedure of the clotting test was performed as reported in Figure 4-2.

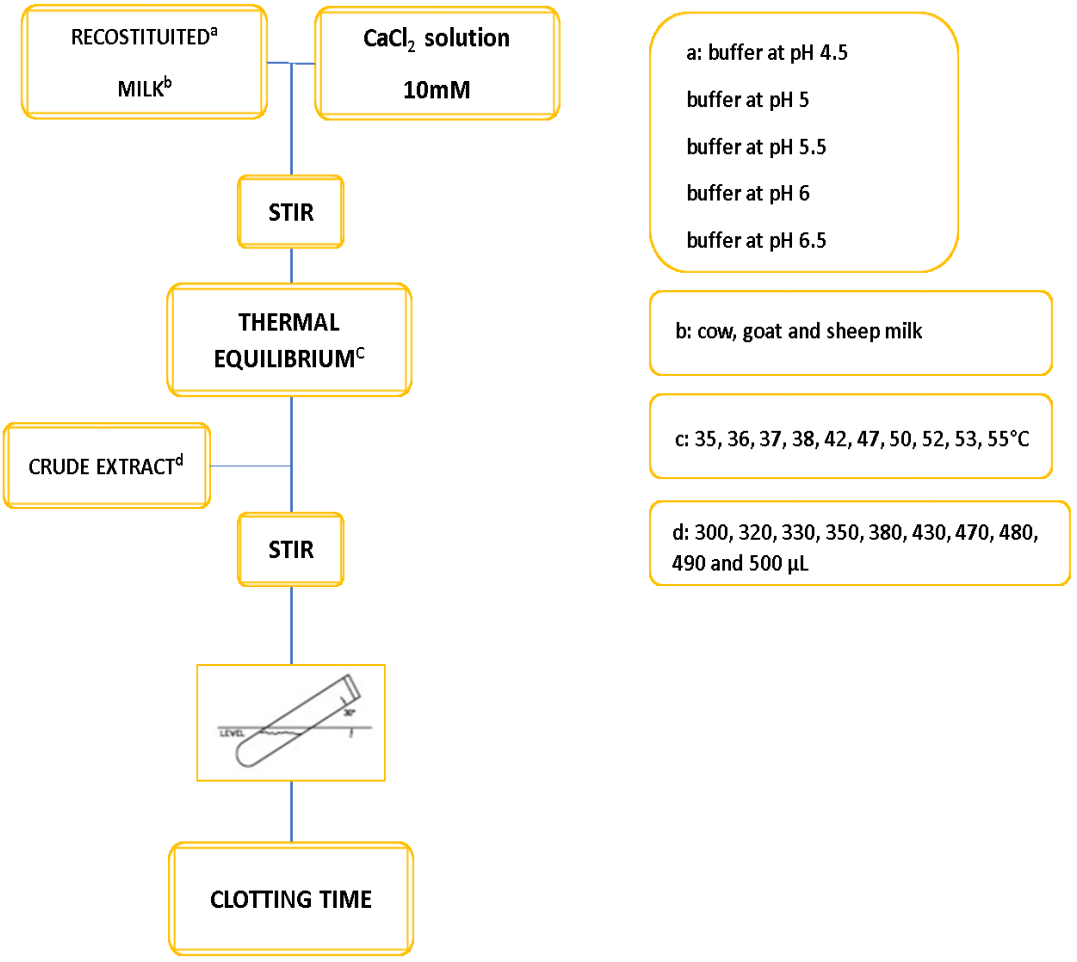


Figure 4-2: Schematic of testing procedure for determining the MCT

For clotting time determination 5 mL of reconstituted milk was pipetted into assay tube. A calcium chloride (Sigma-Aldrich, Milan, Italy) solution (500 g/L) was added to the substrate to achieve the final concentration of 10 mM. The assay tube was allowed to equilibrate for 5 min at the desired temperature in an M20 thermostatic water bath (Lauda-Königshofen, Germany) before the addition of the RE.

A predetermined amount of RE was injected directly into the assay milk just as a stop watch was started. The tube was shaken gently for 3 to 4 s to disperse the enzyme and attached to the rotating spindle. The clotting time was reordered for each run when the first flocculation was observed in the substrate film on the wall of the assay tube.

4.5 Milk clotting activity determination (MCA)

A Soxhlet's unit-related definition was used for quantifying the clotting activity. One unit of milk-clotting activity was arbitrarily defined as the volume of milk that can be clotted by one volume unit of RE in 40 min in the assay conditions of pH and temperature (Tabayehnejad et al., 2012):

Eq. (3) $$MCA(U) = \frac{2400}{T}\frac{S}{E}$$

where T is the clotting time (s); E is volume of RE (mL); and S = volume of milk (mL).

4.6 Effects of pH, temperature and volume of RE on Milk Clotting Time

Milk Clotting time (s) (MCT) (based on the IDF standard as the indicator of milk flocculation) indicated as time from raw extract/rennet addition to the formation of the first visible floccules were measured visually.

The effect of pH for the activity of the RE from *Onopordum tauricum* was determined by assaying MCT with buffers in the pH range 4.50–6.5 and by measuring the pH of reconstituted milk substrate.

To evaluate the effect of temperature for the activity of RE ,the substrate was pre-incubated with 10 mM $CaCl_2$ at different temperatures (35–55 °C) prior to addition of RE. The time taken for the milk to clot was taken as measure of enzyme activity.

To evaluate the effect of enzyme concentration, different concentrations of RE (300-500 µL) were added to milk at different temperatures (35 - 55°C), to study its effect on MCT. The time taken for the milk to clot was taken as measure of enzyme activity.

4.7 Statistical analysis

A response surface methodology (RSM) (Yolmeh et al., 2017) was used to explore the effects of three independent variables (temperature, pH, volume of RE) on the measured parameter (MCA) and the relationships among the explanatory variables. The software JMP Version 11.0.0 (SAS Institute Inc., Cary, NC,USA) was used to both design the experimental plan and analyze the data matrix.

CHAPTER 5
RESULTS AND DISCUSSION

5.1 Physicochemical characteristics of the milks of different animal species

The physicochemical characteristics of bovines, goat's and ewe's milks are reported in Table 5-1.

Table 5-1: Physicochemical characteristics of the milks of different animal species used for the assessment of the clotting activity of aqueous extracts from Onopordum tauricum flowers.

Milk	pH	Dry matter % w/w	Protein % w/w	Fat % w/w	Lactose % w/w	Ash % w/w
Bovine[1]	6.67	12.2	3.5	1.6	5.8	1.3
Goat[1]	6.72	10.8	3.6	1.6	4.8	0.8
Ewe[2]	6.76	13.9	5.9	1.8	5.3	0.9

[1]UHT, [2]raw

Bovine milk presented a slightly lower pH value (6.67) than goat's and ewe's milk (pH 6.72–6.76). The protein and fat composition of bovine's and goat's milk used in the experiment is very similar to each other. Ewe's milk samples in the dry matter, protein, fat values were higher than other milks.

As the skimming had no or very little effect on the rennet coagulation time in cow's, ewe's, and goat's milk (Calvo M., 2002), we used partially skimmed milks to reduce foaming and better catch the beginning of flocculation. In most of the experimental studies, the clotting properties of vegetable extracts were studied on reconstituted milk prepared from commercial bovine skim milk powder (Brutt i et al., 2012; Tavaria et al., 2001), while only a few authors used different substrates, namely whole and low-fat pasteurized milk (Gutiérrez-Méndez et al., 2019; Mazorra-Manzano et al., 2013; Silva et al., 2020), and thermized (55 °C for 15 s) milk (Liburdi et al., 2019). To the authors' knowledge, only Liburdi et al. compared the clotting performance of vegetable extracts in milk of different origin (bovine, buffalo, goat, and ewe).

5.2 Characterization of crude extract from *Onopordum tauricum*

The flower heads of *O. tauricum* yielded 8.23 g of dry extract/100 g of fresh flowers. The total protein content measured on the RE was 3.61 µg/µL, while the total protein content of the liquid bovine rennet used as reference was 2.64 µg/µL.

The preliminary experiments with crude extracts of different parts of the inflorescence (tubular flowers, receptacle), and of the stem and leaves showed that only RE from the flowers was able to coagulate the milk. The REs from the receptacle and leaves extracted with water and acetate buffer pH 5.0 did not show milk-clotting activity after 120 min. Furthermore, no clotting activity was detected in the flower extracts upon heat treatment (100 °C for 5 min), clearly indicating the enzymatic nature of the milk coagulation.

5.3 Effect of $CaCl_2$ on the raw extract MCT

Calcium chloride supplementation in milk, a current practice in cheese making, has been reported to play an essential role in the aggregation of casein micelles and in curd firmness. It has been suggested that the main effect of calcium ions occurs during the second phase of the clotting process, where calcium ions makes bridges between micelles of para-casein indicating that, the more there are calcium ions (0 – 20 mM) in milk, the more there will be linkage and the faster will be milk clotting. For this reason, the addition of CaCl2 to milk, especially to thermally processed ones, is the simplest way to reduce the clotting time and increase the curd firmness during cheesemaking.

According to Kethireddipalli and Hill (2015), a fortification with 0.1-0.2 g/L of CaCl2 (1–2 mM) is sufficient to obtain an optimal clotting of pasteurized milk, but up to 0.3-0.6 g/L (3–6 mM) are needed for milks that were subjected to more intense heat treatments.

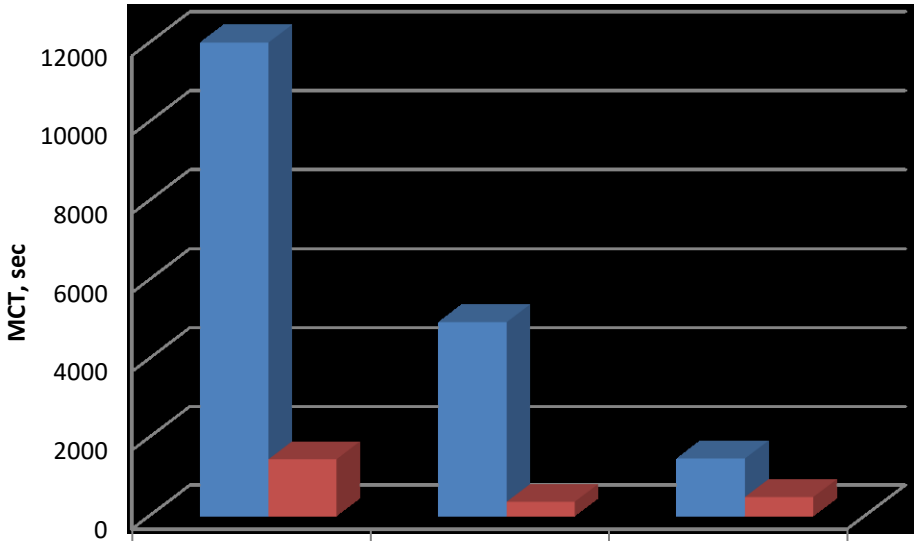
In order to evaluate the effect of $CaCl_2$ on clotting properties, the RE of *Onopordum tauricum* (300 µL) was tested in 5 mL of the different milks (goat's, cow's and ewe's) at their natural pH and the temperature 35 ºC, without and with a 10 mM $CaCl_2$ addition, this concentration was used based on previous studies (Silva et al, 2004; Tejada et al., 2008; Brutti et al., 2012; Llorente et al., 2004; Lo Piero et al, 2011; Lufrano et al., 2012; Pontual et al., 2012; Maorra-Manzano et al., 2013; Ordiales et al., 2012).

According to the results presented in Figure 5-1 it can be seen that control samples without added $CaCl_2$ had lengthened clotting time, while experimental samples with added 10 mM of $CaCl_2$ coagulated faster. The addition of 10 mM CaCl2 reduced the clotting time of ewe's,

goat's, and cow's milk by 3-fold (24 vs. 8 min), 8.3-fold (200 vs. 24 min), and 13.6-fold (82 vs. 6 min), respectively.

As the clotting times of non-fortified goat's and cow's milk largely exceeded the useful value for cheesemaking, all experiments were carried out with calcium fortification. The consistent improvement in the clotting performance of thistle extract in goat's and cow's milk could be ascribed to the severity of the thermal process that they underwent.

The impaired rennet clotting properties (longer clotting time, weaker curd) of heat-treated milks have been mainly attributed to the interactions between denatured whey proteins and casein micelles, which interfere with the micelle aggregation.



	Goat's milk	Cow's milk	Ewe's milk
no CaCl2	12000	4920	1458
10 mM CaCl2	1440	360	480

Figure 5-1: Effect of calcium chloride on MCT in crude aqueous extract of Onopordum tauricum

As it can be seen from Figure 5-2 MCT decreases faster in cow's milk than goat's milk. It can be explained by the available information in literature (O'connel & Fox, 2000;Libourga et al., 2008). According to Libourga (2008), when using kid or calve rennet, milk clotting time decreases faster when using cow milk than goat milk, which is consequently influenced by its micelles size; it decreases while micelles size decreases (O'Connell and Fox P.F., 2000). Goat milk micelles are bigger than cow milk casein micelles. For the same volume substrate volume, there are more bridges between para-casein micelle in cow milk than in goat milk. Para casein micelle network is formed more rapidly with cow milk than with goat milk.

Kumar et al. (2020), in the experiment with cow's skimmed milk, using crude extract from *Cucurbita moschata* seeds, observed that clotting activity of the seed extract was increased with the increasing concentration of the $CaCl_2$ from 0.01 to 0.1M. Maximum curdling activity was observed at 0.02M $CaCl_2$ concentration and the gelation time was also found to be directly proportional to the concentration of $CaCl_2$.

5.4 Milk clotting activity

Most of the experimental studies on the effect of the independent variables(pH, temperature, RE volume, calcium ion concentration) on clotting activity used a univariate approach, disregarding how response (MCA) could be affected by the interactions among variables.

The generated reports of the RSM are summarized in Tables 5-2, 5-3, 5-4, 5-5.

Table 5-2: Experimental design matrix (D-optimal criterion) and observed responses (clotting time) in cow's, goat's, and ewe's milk.

		Cow's milk[2]			Goat's milk			Ewe's milk		
Run	**T**	**EV**	**pH**	**CT[1]**	**EV**	**pH**	**CT[1]**	**EV**	**pH**	**CT[1]**
***Onopordum tauricum* extract**										
1	53	417	6.45	100	320	6.5	320	368	6.9	195
2	35				500	4.9	130	575	5.05	90
3	35	430	6.5	360	330	6.7	1440	379	6.9	480
4	38				350	4.9	100	402	5.05	80
5	55	630	6.5	35	480	6.7	500	554	6.9	180
6	50				470	4.9	53	540	5.05	40
7	47	417	5.7	147	320	5.7	390	368	5.95	115
8	52	650	6.5	90	500	6.7	570	575	6.9	200
9	55	459	6.06	35	350	6.1	190	402	6.87	100
10	42	643	5.7	80	490	5.7	455	563	5.95	120
11	47	499	6.45	110	380	6.5	670	437	6.9	238
12	35	630	6.45	320	480	6.5	920	554	6.9	297
13	42	565	6.5	200	430	6.7	870	494	6.9	255
14	55				300	4.9	47	345	5.05	35
15	55	390	6.5	110	300	6.7	377	345	6.9	250
16	38	390	6.45	320	300	6.5	900	345	6.9	320
17	36	565	5.7	140	430	5.7	390	494	5.95	213
18	37	650	6.5	250	500	6.7	930	575	6.9	360
19	53	499	5.7	30	380	5.7	180	437	5.95	85
20	35				300	4.9	160	345	5.05	130
Commercial calf rennet (liquid form)										
1	53	438	6.45	8	438	6.5	25	438	6.9	11
2	35	680			680	4.9	35	680	5.05	5
3	35	451	6.5	19	451	6.7	80	451	6.9	37
4	38	480			480	4.9	25	480	5.05	10
5	55	655	6.5	7	655	6.7	17	655	6.9	12
6	50	640			640	4.9	15	640	5.05	3
7	47	438	5.7	8	438	5.7	600	438	5.95	5
8	52	680	6.5	8	680	6.7	30	680	6.9	14
9	55	478	6.06	6	478	6.1	18	478	6.87	8
10	42	670	5.7	10	670	5.7	900	670	5.95	26
11	47	520	6.45	7	520	6.5	45	520	6.9	10
12	35	656	6.45	18	656	6.5	65	656	6.9	28
13	42	588	6.5	13	588	6.7	50	588	6.9	20
14	55	410			410	4.9	5	410	5.05	2
15	55	410	6.5	8	410	6.7	20	410	6.9	13
16	38	410	6.45	11	410	6.5	85	410	6.9	18
17	36	588	5.7	14	588	5.7	1800	588	5.95	150
18	37	680	6.5	16	680	6.7	55	680	6.9	31
19	53	520	5.7	7	520	5.7	1920	520	5.95	5
20	35				410	4.9	40	410	5.05	7

[1]Average values of two replicates of each run.[2]Runs n. 2, 4, 6, 14, and 20 were not investigated in cow milk due to casein precipitation in acetate buffer at pH 4.5.T = temperature (°C), EV = volume of coagulant (μL), CT = clotting time (s).

Table 5-3:Estimated coefficients of the predicted second-order polynomial model for the milk clotting activity of O.tauricum extract

Term	Ewe's milk			Goat's milk			Cow's milk		
	Estimates	**F-ratio**	**p-value**	**Estimates**	**F-ratio**	**p-value**	**Estimates**	**F-ratio**	**p-value**
Intercept	2105.10			5553.6 *			-32,483.55		
Linear									
T	83.30	4.66	0.0563	57.42	3.10	0.1087	39.73	0.23	0.6493
EV	0.34	0.01	0.9107	-0.34	0.01	0.9129	14.48	5.14	0.0726
pH	-1145.98	4.75	0.0543	-2153.02	21.90	0.0009 *	9198.39	1.77	0.2408
Quadratic									
T × T	0.29	0.50	0.4962	0.43	1.58	0.2367	2.01	12.12	0.017 *
EV × EV	-0.00	0.16	0.6989	-0.00	0.05	0.8205	-0.01	4.68	0.0827
pH × pH	114.41	7.37	0.021 *	202.00	27.55	0.000 *		1.06	0.3504
Interactions									
T × EV	-0.03	3.07	0.1104	-0.03	2.82	0.1238	0.04	2.32	0.1885
T × pH	-13.05	39.00	<0.0001*	-12.27	40.19	<0.0001 *	-34.89	12.32	0.017 *
EV × pH	0.30	2.75	0.1281	0.35	3.38	0.0957	-1.25	2.75	0.1581

* Level of significance $p< 0.05$. EV, volume of coagulant

Table 5-4: Estimated coefficients of the predicted second-order polynomial model for the milk clotting activity of commercial calf rennet

Term	Ewe's milk			Goat's milk			Cow's milk		
	Estimates	**F-ratio**	**p-value**	**Estimates**	**F-ratio**	**p-value**	**Estimates**	**F-ratio**	**p value**
Intercept	31,246.16			47,947.18 *			-211,167.38		
Linear									
T	1508.17	3.59	0.0873	193.76	0 20	0.6669	362.66	1.25	0.3145
EV	-59.52	1.31	0.2791	-22.67	0.56	0.4723	-19.89	0.65	0.4558
pH	-13,742.13	1.62	0.2316	-15,695.82	6.58	0.0282 *	69,645.77	6.49	0.0514
Quadratic									
T × T	1.71	0.04	0.8435	5.44	1.43	0.2594	-3.86	2.85	0.1524
EV × EV	0.04	0.97	0.3476	0.01	0.37	0.5579	0.01	0.25	0.6390
pH × pH	1395.31	2.60	0.1378	1411.55	7.59	0.0203 *	-5846.04	6.61	0.0500 *
Interactions									
T × EV	-0.55	2.78	0.1262	-0.35	3.53	0.0898	-0.01	0.01	0.9145
T × pH	-187.60	18.96	0.0014 *	-69.72	7.24	0.0227 *	12.30	0.10	0.7672
EV × pH	4.83	2.32	0.1588	3.26	3.01	0.1134	1.39	0.23	0.6505

* Level of significance $p< 0.05$. EV, volume of coagulant

The quality of the fit to the polynomial models was checked by the regression coefficient R^2,which measures the amount of total variability explained by the model, and the adjusted R^2, which shows the percentage of variation explained by only the independent variables (T, pH, volume of coagulant) that significantly affect the dependent variable (MCA) (Table 5-5). The results showed that the second-order model (Equation (2)) was significant for all milks, clotted by both the plant extract and animal rennet. Fisher's F-test and *p*-value showed the significance of each coefficient (Tables 5-3and 5-4). It was observed that the volume of coagulant and, in a more general way, the milk/coagulant ratio, did not influence in a significant way the MCA of both the vegetable and animal rennet in all milks.

Table 5-5: Models for the milk clotting activity (MCA).

MCA[1]	R^2	Adjusted R^2	F Ratio	p-Value
	Ewe's milk			
RE = 114.41(pH)^2- 13.05 (T)(pH)	0.9648	0.9330	30.43	<0.0001 *
CR = - 187.60(T) (pH)	0.9315	0.8698	15.11	0.0001 *
	Goat's milk			
RE = 5553.69 - 2153.02 (pH) - 12.27 (T) + 202.00 (pH)^2	0.9698	0.9427	35.72	<0.0001 *
CR = -47,947.18 - 15,695.82 (pH) - - 69.72(T)(pH) + 1411.55 (pH)^2	0.8622	0.7383	6.95	0.0028 *
	Cow's milk			
RE = 2.01(T)^2 - 34.89 (T)(pH)	0.9636	0.8980	14.70	0.0043 *
CR = -5846.04(pH)^2	0.9701	0.9163	18.03	0.0027 *

[1]Each model equation is presented using the significant experimental values ($p< 0.05$). RE reconstituted extract of *O. tauricum* flowers. CR, commercial calf rennet. * Level of significance $p<0.05$. EV, volume of coagulant.

The clotting properties of *O. tauricum* extracts in ewe's milk were strongly affected by pH and the interaction T × pH, while the latter factor alone characterized the behavior of calf rennet in ewe's milk. In goat's milk, negative coefficients for pH and T × pH demonstrated linear and interactive effects to increase MCA, as well as the positive coefficients for pH × pH revealed a quadratic effect to increase the MCA of both the thistle extract and calf rennet. Two effects (T × T and T × pH) had *p*-values less than 0.05 in cow's milk added by plant extract, indicating they had a significant influence on the MCA, while only the quadratic pH affected the MCA of animal rennet in cow's milk.

5.5 Effect of technological variables on the clotting activity of *O.tauricum* extract and commercial rennin in bovine's milk

The three-dimensional plots of the effect of temperature, pH, and volume of coagulant on the MCA are given in Figures 5-4, 5-6 and 5-8.

The findings highlighted that, within the explored range of the independent variables, a negative interaction between pH and temperature affected the general behavior of the MCA: the two variables had to move in opposite directions to cause a strong increase in the MCA.

The performance of *O.tauricum* extract was compared with that of commercial calf rennet in the same experimental conditions. The quadratic pH effect on the clotting of cow's milk by calf rennet was reflected by the dome-shaped surface with a maximum in the pH range 5.8–6.2.

It was observed that MCT of cow's milk decrease gradually with the increase of temperature from 35 to 55 ^{o}C and RE volume from 300 to 500 µL. The rates of protein aggregation as well as firming of the gel are highly changed in response to elevated temperature (Fig. 5-2). It is stated that high temperatures can increase some hydrophobic interactions leading to the shrinkage of matrix (Ahmed et al., 2012).

An obvious decrease of MCT has been observed by the decrease of the pH of substrate from 6.5 to 4.5.

Similar behavior was observed also for goat's and ewe's milk. By raising coagulation temperature, RE volume and decreasing pH of substrate lower values of MCT and fast flocculation were observed. Similar results were obtained for *Fœniculum vulgare* (Bey et al., 2018).

For the cow's milk the observed MCT ranged among 30- 360 sec. The response surfaces (Fig. 5-3) showed lowest values of MCT 30 and 35 sec at 53 and 55 °C, 499 and 630 µL of RE volume and pH of milk 5.7 and 6.06 respectively. While highest values of MCT (360 sec) were recorded for the milk coagulated at temperature 35 °C, 430 µL of RE volume and pH 6.5.

In comparison, the observed MCT with calf rennet ranged among 7- 19 sec. The 7 sec was reported at 47, 53 and 55 °C, RE volume 438, 520 and 655 µL and pH 6.45, 5.7 and 6.5 while 19 sec at 35 °C, RE volume 451 µL pH 6.5 respectively.

MCT of cow's milk with *O.tauricum* extract was approximately 19 times longer than cow milk with calf rennet at 35 ^{o}C (run 3); 4.3 times longer respectively at 53 ^{o}C (run 19) (Table 5-2).

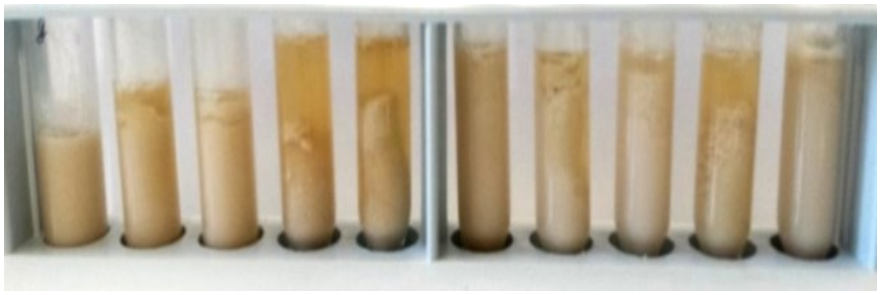
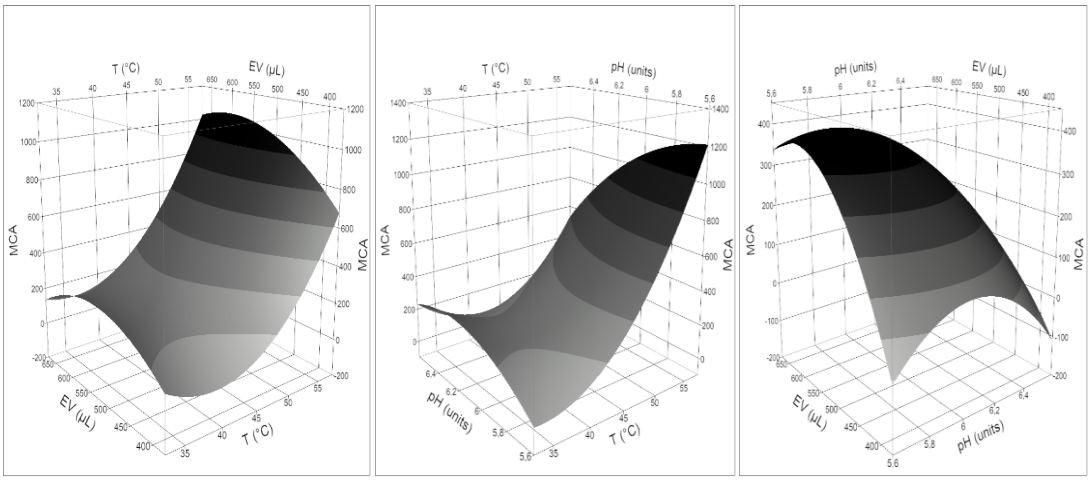
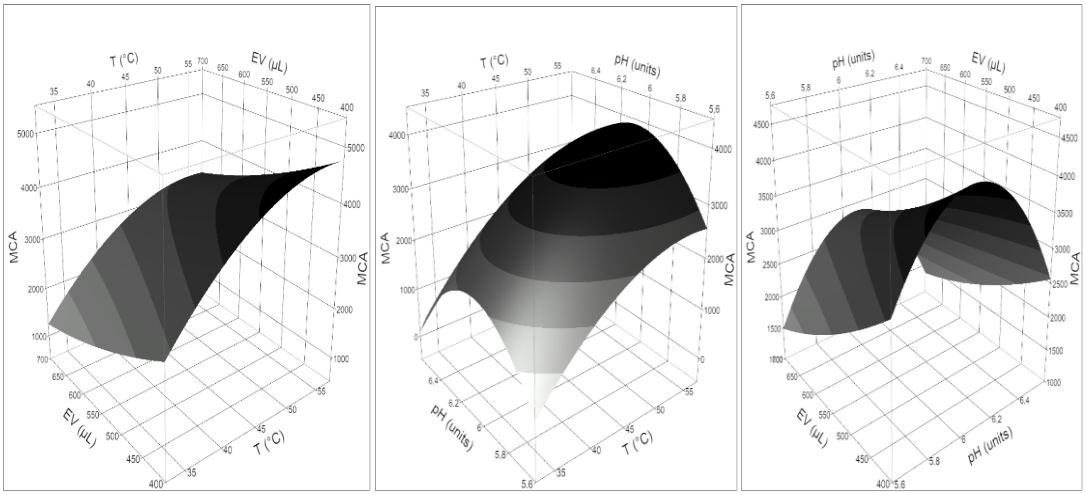


Figure 5-2: Coagulated bovine's milk samples



(a) **(b)** **(c)**



(d) **(e)** **(f)**

Figure 5-3:Response surface plots for the effects of (a)coagulant volume and temperature; (b) pH and T; (c) pH and coagulant volume, on the clotting activity of O. tauricum extract in cow's milk; (d)coagulant volume and temperature; (e) pH and T; (f) pH and coagulant volume, on the clotting activity of commercial rennin in cow's milk.

5.6 Effect of technological variables on the clotting activity of *O.tauricum* extract and commercial rennin in goat's milk

For the goat's milk the observed MCT ranged among 47- 1440 sec. The response surfaces (Fig. 5-5) showed lowest values of MCT 47 and 53 sec at 55 and 50 °C, 300 and 470 µL of RE volume and pH of milk 4.9 respectively. While highest values of MCT (1440 sec) were recorded for the milk coagulated at temperature 35 °C, 330 µL of RE volume and pH 6.7.

In comparison, the observed MCT with calf rennet ranged among 5- 1920 sec. The 5 sec was reported at 55 °C, RE volume 410, 520 µL and pH 4.9 while 1920 sec at 53 °C, RE volume 520 µL pH 5.7 respectively.

However, the plant RE added to the goat's milk at pH 5.7 reported lower values of MCT (180-455 sec). This means that the chymosin of calf rennet exhibits lower activity at pH 5.7 and consequently higher MCT (600-1920 sec) compared respectively to the high pH (6.5 and 6.7) and to pH 5.7 in goat's milk by using *Onopordum tauricum* extract.

The results showed that goat's milk has higher values of MCT compared to cow's and ewe's milk under the same experimental conditions. In fact, MCT of goat's milk was approximately 2.9-fold longer than cow's milk and 3.3-fold longer than ewe's milk at 35 ºC, pH 6.5 and RE volume480 µL; 6-fold and 2.1-fold longer respectively at 53 ºC, pH 5.7 and RE volume 380 µL.

Recently, it have been shown that the notable differences in chemical composition of cow's and goat's milk not observed.

It is reported in literature that pH change influences both the enzymatic and aggregation reaction. Although the effect of pH on the enzymatic phase is minor compared to its effects on aggregation. In fact, acidification decreases the stability of casein micelles through the neutralization of their negative charges. Decreasing milk pH leads to faster increase of micelles aggregation due to the reduction of their potential. Therefore, the variation of milk pH could strongly affected the coagulant properties of the milk.

In general, the casein fraction of the cow milk precipitates at pH of 4.6, however, the minimum solubility of the caseins in goat's milk is achieved at pH 4.1. This behavior indicates the different nature of proteins in the two types of milk, in fact, goat milk has bigger micelles compared to cow milk (Libouga et al., 2008). Based on protein difference, also various rheological characteristics could assume the final curd, softer for goat compared to cow milk.

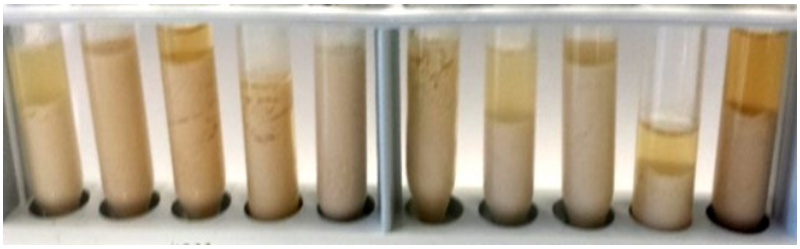


Figure 5-4: Coagulated goat's milk samples

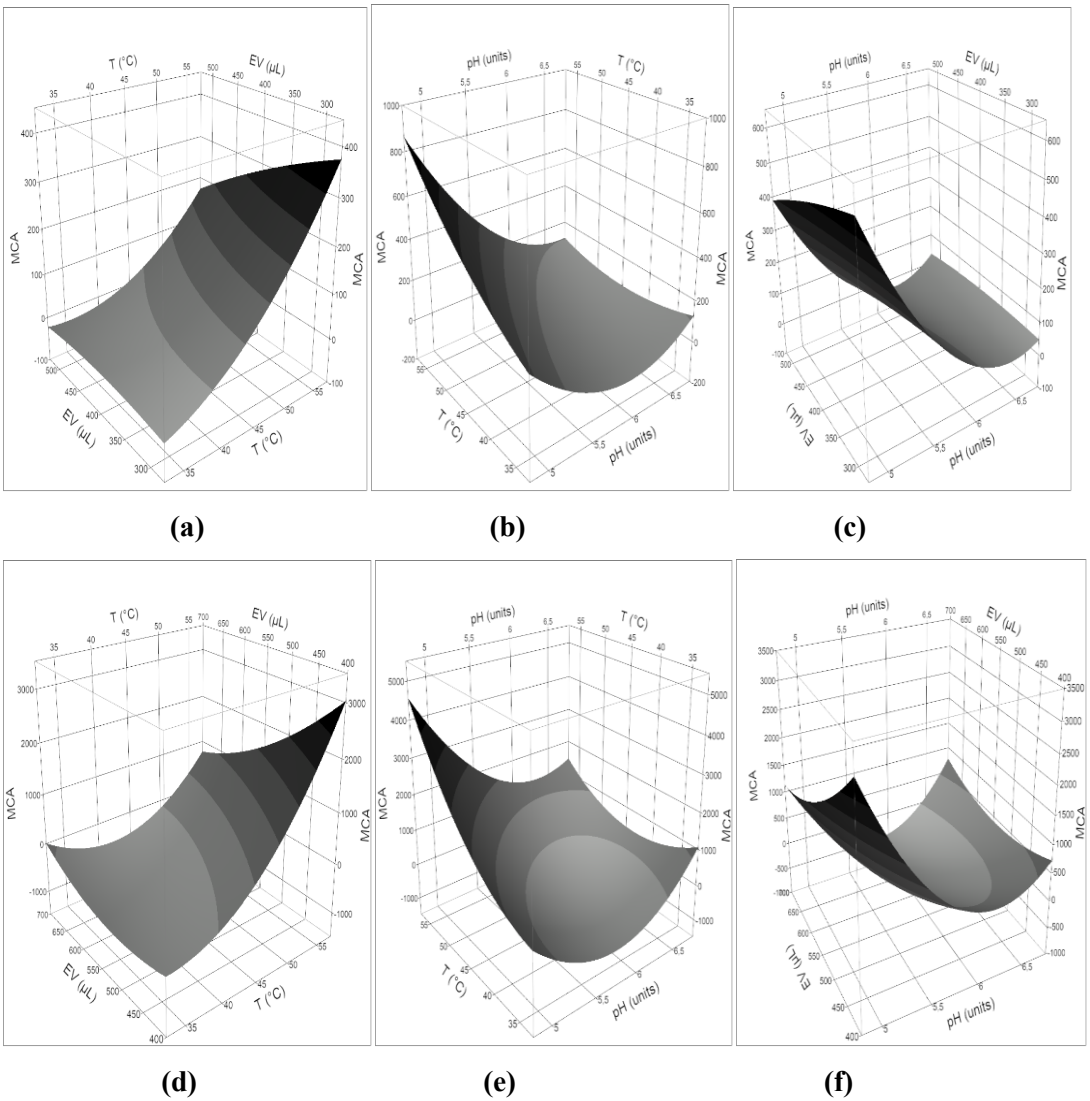


Figure 5-5:Response surface plots for the effects of (a) coagulant volume and temperature; (b) pH and T; (c) pH and coagulant volume, on the clotting activity of O. tauricum extract in goat's milk;(d)coagulant volume and temperature; (e) pH and T; (f) pH and coagulant volume, on the clotting activity of of commercial rennin in goat's milk.

5.7 Effect of technological variables on the clotting activity of *O.tauricum* extract and commercial rennin in ewe's milk

For the ewe's milk the observed MCT ranged among 35-480 sec. The 35 sec was reported at 55 °C and pH 5.05, while 480 sec at 35 °C and pH 6.9 (Figure 5-7). In this case, no influence of the RE volume was noticed (345 and 379 μL). Considerable influence of the RE volume was observed in the runs 3 and 18 were the MCT was respectively 480 sec at 35 °C and 360 sec at 37 °C at the same pH (6.9).Thus, the different RE volume and temperature (2 °C) provoked a difference of 2 min for the runs with low temperature and RE volume (Table 4-2).

In contrast, for ewe's milk with commercial rennet the observed MCT ranged among 2-150 sec.

The clotting properties of *O. tauricum* extracts in ewe's milk were strongly affected by pH and the interaction T × pH, while the latter factor alone characterized the behavior of calf rennet in ewe's milk.

From a qualitative viewpoint, the clotting properties of ewe's and goat's milks appeared more affected by the kind of milk than the type of clotting agent.

As previously mentioned, thermal and mechanical treatments have opposite effects on the time for milk casein to clot. Ewe's milk should be the better substrate for specific proteases, as it was crude and it had the highest protein content (Table 4-1) and the more favorable casein/whey protein ratio (Fox et al., 2015). By contrast, cow's and goat's milk underwent both a mechanical (homogenization) and a thermal (UHT) process. As a result, the range of the measured clotting times (30–360 s, 35–480 s, 47–1440 s, for cow's, ewe's, and goat's milk, respectively) of the calculated MCAs (60–802, 66–994, 25– 851 for the cow's, ewe's, and goat's milk, respectively) and of the previously reported estimated MCA values at 35 °C were comparable for all the milk types used in the experiment.

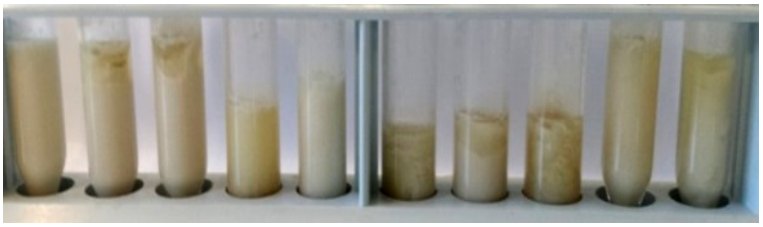


Figure 5-6: Coagulated ewe's milk samples

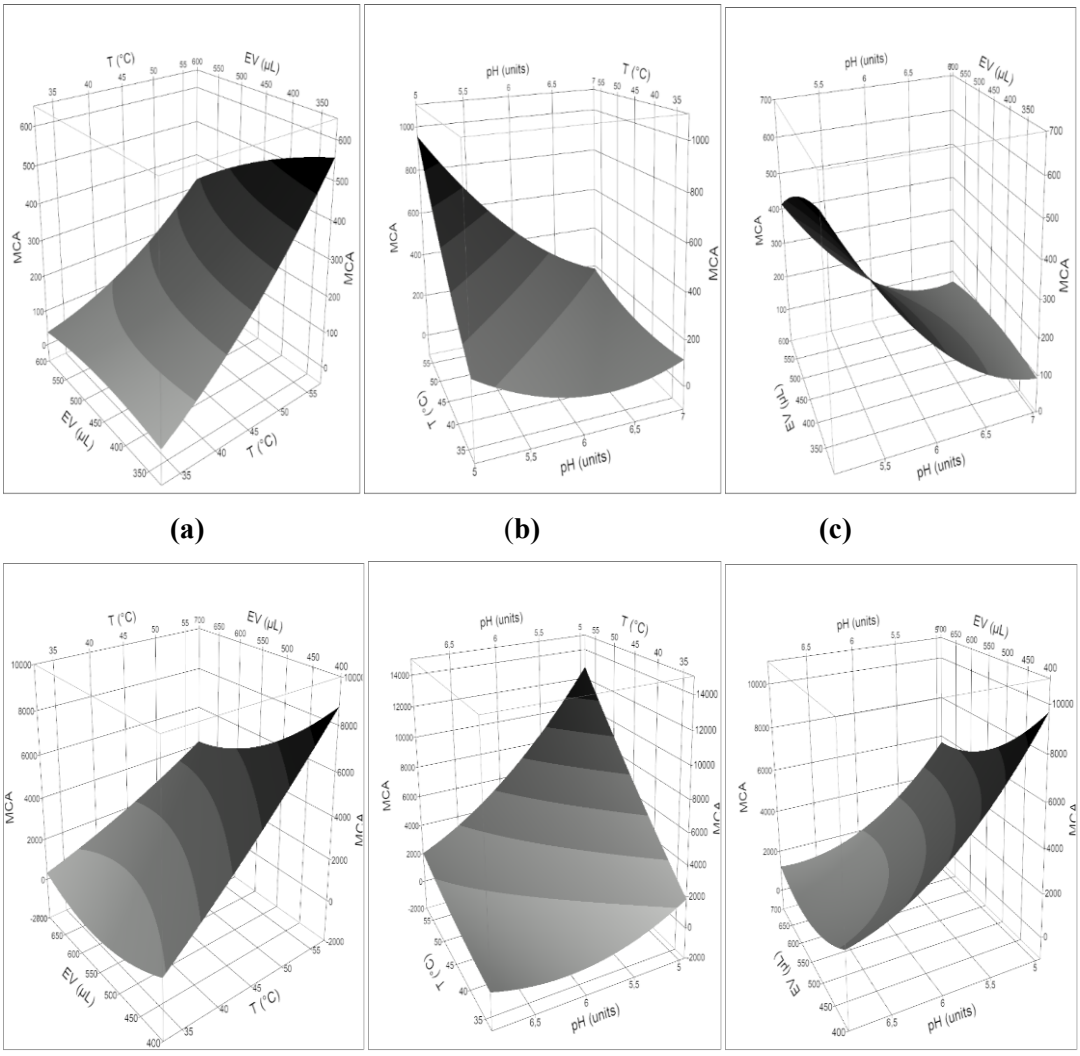


Figure 5-7:Response surface plots for the effects of (a)coagulant volume and temperature;(b) pH and T; (c) pH and coagulant volume, on the clotting activity of O. tauricum extract in ewe's milk;(d) coagulant volume and temperature; (e) pH and T; (f) pH and coagulant volume, on the clotting activity of commercial rennin in ewe's milk.

5.5 Optimal conditions for milk clotting

Account of optimal coagulation temperature, pH, RE volume is necessary, as it affects the activity of the enzyme which helps in successful industrial applications.

Each enzyme has an optimum temperature and pH for maximum enzyme activity.

MCA is the most important property of enzymes used in cheesemaking as it describes the ability of the enzyme/extract to specifically hydrolyze the Phe105–Met106 bond of κ-casein, thus causing the destabilization of casein micelles, which in turn results in their aggregation. Therefore, the production of coagulants with a high specific MCA and the optimization of the clotting conditions are always the first goals to achieve. The desirability function was used to maximize the MCA and to estimate the predicted responses (Table 5-6). The input variables were kept within the ranges studied.

The optimal conditions of MCT obtained by fitted response surface are reported in the Table 5-6.

Table 5-6: Optimal conditions for milk clotting

	T (°C)	EV(µL)	pH(Units)	Predicted MCA [1]	Desirability	Measured MCA
Ewe's milk +RE	55	300	5.0	1005	0.9852	989
Ewe's milk + CR	55	400	4.9	15,015	0.9829	14,634
Goat's milk +RE	55	300	4.9	798	0.8711	821
Goat's milk +CR	55	400	4.9	5155	0.8645	5254
Cow's milk + RE	55	500	5.7	940	0.9946	892
Cow's milk + CR	55	400	6.1	4775	0.9965	4651

[1] Maximize desirability EV,volume of coagulantRE, reconstituted extract of *O.tauricum* flowers. CR commercial liquid calf rennet.

In all the milk types, the optimal temperature for clotting was the highest (55°C) in the range explored (35–55°C) and the optimal pH value (4.9–6.1) was the lowest in the actual range studied (5.0–6.9 for ewe's milk, 5.7–6.5 for cow's milk, 4.9–6.7 for goat's milk). A general decrease in the MCA was observed by increasing the volume of coagulant, so that the optimal range for this parameter was 300–400 µL. The behavior of the thistle extract in cow's milk was an exception: the higher the volume of the extract, the higher the clotting activity, so that the optimal value for this parameter was the highest used (500µL). In the conditions that maximized the clotting activity, the performance of calf rennet was 5.0, 6.5, and 15.0 times better than thistle extract in cow's, goat's, and ewe's milk, respectively.

At the temperature of 35 °C and the natural pH of studied milks (Table 5-1),the estimated MCA of the *O. tauricum* extract was 72–87, 69–86, and 75–151, in goat's, ewe's, and cow's milk, respectively, and in the range of the coagulant volume used. In comparison, the MCA of calf rennet was 5.4–4.9, 3.3–14.7, and 4.9–16.7 times higher than plant extract in goat's, ewe's, and cow's milk, respectively.

Some authors have compared the milk-clotting activity of plant proteases with calf rennet in the same conditions. Nestor et al. (2012) compared the milk-clotting activity of enzyme extract from *Solanum elaeagnifolium* berries with calf rennet and found that the milk-clotting activity was 39.4 and 2,474 at 32 °C respectively. Ahmed et al. (2009) compared the milk-clotting activity of enzyme extract from *Solanum dubium* with calf rennet and found that the milk-clotting activity was 880 and 2,496 at 37 °C, respectively.

Brutti et al. (2012) reported a much lower clotting activity (1400 times lower than chymosin) for a partially purified extract of *O. acanthium* (30 °C, pH 6.5, 10 mM CaCl2). However, the same authors highlighted that "onopordosin" showed a more favorable clotting/proteolytic ratio than the aspartic protease of *C. cardunculus* and of proteases from other plants (*Asclepias fruticosa*, *Bromelia balansae*, *Bromeliahieronymi*, *Philibertia gilliesii*).

The clotting performance of *O. tauricum* aqueous extract was consistent with the findings of Chazarra et al. (2007) in crude extracts of dried flowers of *Cynara scolymus* L. (clotting times 100–500 s, 30–60 °C, pH 5.5–7.0), and of Guiama et al. (2014) in extract produced from *Solanum aethiopicum* fruits (MCA 30–140).

CONCLUSIONS

The study found that aqueous extract from *Onopordum tauricum* flowers has the property of curdling milk. The clotting properties of such extract were tested for the first time in milk of different species (cow, goat, ewe)and compared with the clotting performance of commercial calf rennet. No clotting activity was detected in both aqueous and acidic (pH5.5) crude extracts of other aerial anatomical parts (receptacle, leaves, stems).

According to a second-order response surface model, the combination of three independent variables (temperature, pH, volume of coagulant) that maximize the clotting activity of *O. tauricum* extract was obtained at a temperature of 55 °C, pH of 4.9–5.7, and volume of coagulant equal to 300–500 μL. Under these optimal conditions, the predicted MCA was 798–1005, with the highest value measured in crude ewe's milk. The clotting activity of Taurian thistle decreased as the temperature decreased (from 55 to 35 °C) and the pH increased (from 4.9 to 6.8). The estimated MCA of the *O. tauricum* extract at 35 °C at natural milk pH demonstrated similar performance to the crude extracts of dried flowers of artichoke (*Cynara scolymus* L.) (Chazarra et al., 2007) and of *Solanum aethiopicum* fruits (Guiama et al., 2014).

Thus, *O.tauricum* extract promises to be a new resource of protease with potential use as a milk coagulant agent for cheese production as alternative to commercial rennet.

To better understand the technological behavior of *O.tauricum* extracts, comparative studies of caseinolytic and non-specific proteolytic activities must be carried out and completed with studies on rheological properties of milk gels and on sensory properties (texture, flavor, color) of ripened cheeses.

BIBLIOGRAPHY

- Afsharnezhad M., Shahangian S.S., Sariri R., 2019. A novel milk-clotting cysteine protease from *Ficus johannis*: Purification and characterization. *International Journal of Biological Macromolecules,* 121, pp.173-182.
- Ahmed I.A.M, Morishima I, Babiker EE, Mori N., 2009. Characterisation of partially purified milk-clotting enzyme from *Solanum dubium* Fresen seeds. Food Chem 116:pp. 395–400.
- Aktayeva S., Akishev Zh., Khassenov B., 2015. Proteolytic enzymes in cheese making. *Biotechnology*.
- Almena-Aliste M., Mietton B., 2014. Cheese Classification, Characterization, and Categorization: A Global Perspective. *Microbiology spectrum.*
- Andre´n A., 2011. Rennets and Coagulants. Swedish University of Agricultural Sciences, Uppsala, Sweden.
- Araújo-Rodrigues H., Freni K.Tavaria˙,Dos Santos M.T., Alvarenga N., Pintado M.M., 2020. A review on microbiological and technological aspects of Serpa PDO cheese: An ovine raw milk cheese. *International Dairy Journal*, 100.
- Baraka Abo El-Yazeed Abd El-Salam, Osama Abd El-Hamid Ibrahim, Howida Abd El-Razek El-Sayed, 2017. Purification and Characterization of Milk Clotting Enzyme from Artichoke *(Cynara cardunculus L.)* Flowers as Coagulant on White Soft Cheese. *International Journal of Dairy Science*, 12 (4), pp. 254-265.
- Ben Amira A., Besbes S., Attia H., Blecker Ch., 2017. Milk-clotting properties of plant rennets and their enzymatic, rheological, and sensory role in cheese making: A review. *International Journal of Food Properties*, 20, pp.76–S93.
- Benett, R.J., and Johnston, K.A. (2004). General aspects of cheese technology. Cheese, Chemistry, Physics and Microbiology. Vol.2, *Elsevier Academic Press. Amsterdam*, pp. 23-50.
- Bey N., Debbebi H., Abidi F., Marzouki M.N., Salah A.B., 2018). The non-edible parts of fennel *(Fœniculum vulgare)* as a new milk-clotting protease source. *Industrial Crops & Products,* 112, pp.181-187.

- Bobe, G., Beitz, D. C., Freeman, A. E., & Lindberg, G. L.,1998. Separation and quantification of bovine milk proteins by reversed-phase high-performance liquid chromatography. *Journal of Agricultural and Food Chemistry*, 46(2), 458-463.
- Bradford, M.M. , 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.*, *72*, pp. 248–254.
- Brutti C.B,Pardo M.F., Caffini N.O., Natalucci C.L., 2012.*Onopordum acanthium L.* (Asteraceae) flowers as coagulating agent for cheesemaking. *Food Science and Technology*, 45 (2), pp. 172-179.
- Bylund, G., 2003. Dairy processing handbook. Publisher Tetra Pak Processing systemsAB, Lund, Sweden, p. 221.
- Calvo M.M., 2002. Influence of fat, heat treatments and species on milk rennet clotting properties and glycomacropeptide formation. *Eur. Food Res. Technol*., *214*, 182–185.
- Cardinali F., Osimani A., Taccari M., Milanovic V., Garofalo C., Clementi F., Polverigiani S., Zitti S., Raffaelli N., Mozzon M., Foligni R., Franciosi E., Tuohy K., Aquilanti L., 2017.Impact of thistle rennet from *Carlina acanthifolia All*. subsp. acanthifolia on bacterial diversity and dynamics of a specialty Italian raw ewes' milk cheese. *International Journal of Food Microbiology,* 255, pp. 7-16.
- Castillo M., Payne F.A., Hicks C.L., and lope M.B., 2000. Predicting cutting and clotting time of coagulating goat's milk using diffuse reflectance: Effects of pH, temperature and enzyme concentration. *International Dairy Journal*, 10, pp. 551-562.
- Chávez-Garay D.R., Gutiérrez-Méndez N., Miriam E. Valenzuela-Soto, García-Triana A., 2016. Partial characterization of a plant coagulant obtained from the berries of *Solanum Elaeagnifolium*. *Journal of Food*, 14, pp.200–205.
- Chazarra, S.; Sidrach, L.; López-Molina, D.; Rodríguez-López, J.N. , 2007. Characterization of the milk-clotting properties of extracts from artichoke *(Cynara scolymus*, L.) flowers. *Int. Dairy J*., 17, pp. 1393–1400.
- Consultancy and Market Research. Food and Dairy, www.clal.it.
- Colombo L.M., Agustina Fernández Cecilia, V.Cimino, Liggieri C., Bruno M., FaroC., C.Veríssimo,Vairo-Cavalli S., 2018. Miniature cheeses made with blends of chymosin and a vegetable rennet from flowers of *Silybum marianum*: Enzymatic

characterization of the flower-coagulant peptidase. *Food Chemistry,*26615, pp.223-231.

- Conti F., Abbate G., Alessandrini A., Blasi C., 2005. An Annotated Checklist of the Italian Vascular Flora, Palombi Editori, Roma.
- Galán E., F. Prados, A. Pino, L. Tejada, J. Fernández-Salguero**,** 2008. Influence of different amounts of vegetable coagulant from cardoon *Cynara cardunculus* and calf rennet on the proteolysis and sensory characteristics of cheeses made with sheep milk. *International Dairy Journal*, 18, pp. 93-98.
- Garg S.K. and Johri B.N., 1994. Rennet: current trends and future research. *Food Reviews International*,10, pp. 313–355.
- Gomes S., Belo A.T., Alvarenga N., Joao Dias, Pinheiro C., Pinto-Cruz C., Bras T., Duarte M.F., Martins A., Lage P., 2018. Characterization of *Cynara cardunculus* L. flower from Alentejo as a coagulant agent for cheesemaking. *International Dairy Journal.*
- Goulding D.A., Fox P.F., Mahony J.A., 2020.Milk proteins: An overview. *Food*, pp.21-98
- Grandison, A.S., and Ford, G.D., 1986. Effects of variations in somatic-cell count on the rennet coagulation properties of milk and on the yield, composition and quality of Cheddar cheese. *Journal of Dairy Research*, 53, pp. 645-655.
- Guiama, V.; Beka, R.; Ngah, E.; Libouga, D.; Vercaigne-Marko, D.; Mbofung, C., 2014. Milk-coagulating extract produced from *Solanum aethiopicum* Shum fruits: Multivariate techniques of preparation, thermal stability and effect on milk solids recovery in curd. *Int. J. Food Eng*., *10*, pp. 211–222.
- Gutiérrez-Méndez N., Balderrama-Carmona A., Socorro E. García-Sandoval,Ramírez-Vigil P., Martha Leal-Ramos, García-Triana A., 2019. Proteolysis and Rheological Properties of Cream Cheese Made with a Plant-Derived Coagulant from *Solanum elaeagnifolium*. *Foods*, 8, 44.
- Haenlein G.F.W., and Park Y.W., 2010. Milk production. In: *Goat Science and Production*. S. Solaiman, Ed. Wiley-Blackwell Publ., New York. pp. 275-292.
- Haung, A., Hostmark, A.T., Harstad O.M., 2007. Bovine milk in human nutrition – a review. Lipids in health and disease, 25.
- Domingos A.,P.C. Cardoso, Z. Xue, A. Clemente, P.E. Brodelius, M.S. Pais., 2000. Purification, cloning and autoproteolytic processing of an aspartic proteinase from *Centaurea calcitrapa*. *FEBS Journal*, 267, pp. 6824-6831.

- Esposito M., Prospero Di Pierro, Dejonghe W., Mariniello L., Porta R., 2016. Enzymatic milk clotting activity in artichoke *(Cynara scolymus)* leaves and alpine thistle (*Carduus defloratus)* flowers. Immobilization of alpine thistle aspartic protease. *Food chemistry* 204, pp.115-121.
- Everett, D. W., 2017. Cheese: Chemistry, Physics and Microbiology. 4:th ed. London,United Kingdom: Elsevier Academic Press.
- Farrell, H. M., Jimenez-Flores, R., Bleck, G. T., Brown, E. M., Butler, J. E., Creamer, L. K., Hicks, C.L., Hollar, C.M., Ng-Kwai-Hang, K.F. & Swaisgood, H. E., 2004. Nomenclature of the proteins of cows' milk—Sixth revision. *Journal of Dairy Science*, 87(6), pp. 1641-1674.
- Feijoo-Siota F., José Luis R. Rama, Sánchez-Pérez A., Tomás G. Villa, 2018. Expression, activation and processing of a novel plant milk-clotting aspartic protease in *Pichia pastoris*. *Journal of Biotechnology 268*, pp.28-39.
- Fox, P. F., J. Law, P.L.H. McSweeney, and J. Wallace,1993. Biochemistry of cheese ripening. Cheese: Chemistry, Physics and Microbiology, Vol. 1. P. F. Fox, ed. Chapman & Hall,London, pp.523-556.
- Fox, P.F., Guince, T.P., Cogan, T.M., and McSweeney, P.L.H., 2000. Fundamentals of Cheese Science. *Aspen Publishers Inc*., Gaithersburg MD.
- Fox P.F. & McSweeney P.L.H., 2004. Cheese: chemistry, physics and microbiology. Vol. 1. General aspects. London: Elsevier Academic Press.
- Fox, P.F. Brodorb, A., 2008. The casein micelle: historical aspects, current concepts and significance. International Dairy Journal, 18, pp.677-684.
- Fox, P.F., Lowe, U.Y., Paul, L.H., McSweeney, P.L.H.,2015. Dairy chemistry and biochemistry; 2nd edition, *Springer International Publishing*, Switzerland.
- Jacob M, Jaros D., Rohm H., 2011.Recent advances in milk clotting enzymes. *International Journal of Dairy Technology*, 64 (1), pp. 14-33.
- Kelly A.L., Huppertz T., Jeremiah J. Sheehan, 2008. Pre-treatment of cheese milk: principles and developments. *Dairy Science & Technology*, 88 (4-5), pp.549-572.
- Kethireddipalli, P.; Hill, A.R., 2015. Rennet coagulation and cheesemaking properties of thermally processed milk: Overview and recent developments. *J. Agric. Food Chem*., *63*, pp. 9389-9403.
- Kubarsepp, I., Henno, M., Kart, O., and Tupasela, T., 2005. A comparison of the methods for determination of the rennet coagulation properties of milk. *Acta Agriculturae Scand Section A*, 55, pp.145-148.

- Kumar, A., Grover, S., Sharma, J. and V.K Batish , 2010. Chymosin and other milk coagulants: sources and biotechnological interventions. *Critical reviews inbiotechnology,* vol 30:4, 243 – 258.
- Kumar A., Sasmal S., 2020. Rheological and physico-chemical properties of milk gel using isolate of pumpkin (*Cucurbita moschata*) seeds: A new source of milk clotting peptidase. *Food Hydrocolloids.*
- Law, B.A., and Tamime, A.Y., 2011 Technology of Cheesemaking, 2nd edition. *Wiley-Blackwell*, p.1949.
- Li Z., Scott K., Otter D., Zhou P., Hemar Y., 2017. Effect of temperature and pH on the properties of skim milk gels made from a tamarillo (*Cyphomandra betacea*) coagulant and rennet. *J. Dairy Sci.*
- Libouga D.G., Ngah, Y., Jiokap Nono and L. Bitjoka, 2008. Clotting of cow (*Bos taurus*) and goat milk (*Capra hircus*) using calve and kid rennets. *African Journal of Biotechnology* Vol. 7 (19), pp. 3490-3496.
- Liburdi K., , Boselli C., Giangolini G., Amatiste S., Esti M., 2019. An evaluation of the clotting properties of three plant rennets in the milks of different animal species. *Foods*, 8, p. 600.
- Liburdi K, Emiliani Spinelli S., Benucci I., Lombardelli C., Esti M., 2018. A preliminary study of continuous milk coagulation using *Cynara cardunculus* flower extract and calf rennet immobilized on magnetic particles. *Food chemistry*,239, pp.157-164.
- Llorente, B.E.; Brutti, C.B.; Caffini, N.O., 2004. Purification and characterization of a milk-clotting aspartic proteinase from globe artichoke (*Cynara scolymus* L.). *J. Agric. Food Chem*., *52*, pp. 8182–8189.
- Lo Piero, A.R.; Puglisi, I.; Petrone, G., 2011. Characterization of the purified actinidin as a plant coagulant of bovine milk. *Eur. Food Res. Technol*., *233*, pp. 517–524.
- Lucey, J.A., 2003. Rennet coagulation of milk. In: Encyclopedia of Dairy Sciences. *Academic press. Amsterdam*, pp.286-293.
- Lufrano, D.; Faro, R.; Castanheira, P.; Parisi, G.; Veríssimo, P.; Vairo-Cavalli, S.; Simões, I.; Faro, C., 2012. Molecular cloning and characterization of procirsin, an active aspartic protease precursor from *Cirsium vulgare* (Asteraceae). *Phytochemistry*, *81*, pp. 7–18.

- Mazorra-Manzano, M.A., Perea-Gutiérrez, T.C., Lugo-Sánchez, M.E., Ramirez-Suarez, J.C., Torres-Llanez,M.J., González-Córdova, A.F., Vallejo-Cordoba, 2013. B. Comparison of the milk-clotting properties of three plant extracts. *Food Chem*., *141*, pp.1902–1907.
- Mazorra-Manzano Miguel A., Moreno-Hernández Jesús M., Ramírez-Suarez Juan C., 2018. Milk clotting plant proteases for cheese making. Biotechnological Applications of Plant Proteolytic Enzymes, pp 21-41.
- McSweeney P.L.H., 2007. Cheese problems solved. Woodhead Publishing. pp. 50-71.
- Montilla, A.; Balcones, E.; Olano, A.; Calvo, M.M., 1995. Influence of heat treatments on whey protein denaturation and rennet clotting properties of cow's and goat's milk. *J. Agric. Food Chem.*, *43*, pp. 1908–1911.
- Moreno-Hernández J.M., Bañuelos Pérez M.G., Osuna-Ruiz I., Salazar-Leyva J.A., CRamirez-Suarez J., Mazorra-ManzanoM.A., 2017. Exploring the milk-clotting properties of extracts from *Bromelia pinguin* fruit. *Journal of Microbiology, Biotecnology and Food Science,* 7(1), pp.62-66.
- Moudra K., Pachlova V., Cerníkova M., Sopík T., Bunka F., 2017. The combined effects of fat content, calcium chloride, and coagulant concentration on the development of cheese curd structure. *International Dairy Journal*, 73, pp.92-97.
- Neelakantan S., Mohanty A.K., Kaushik J.K., 1999. Production and use of microbial enzymes for dairy processing. *Current Science*, vol. 77, no. 1, pp. 143-148.
- Néstor G.M., Rubí CGD, Héctor JC., 2012. Exploring the milk-clotting properties of a plant coagulant from the berries of *S. elaeagnifoliun* var. Cavanillies. *J. Food Sci.,* 71:pp. 89–94.
- Ng-Kwai-Hang, K. F.,2011. Milk Proteins | Heterogeneity, Fractionation, and
- Isolation, In *Encyclopedia of Dairy Sciences (Second Edition)*, edited by John W. Fuquay, Academic Press, San Diego, pp. 751-764.
- O'Connell J.E., Fox P.F.,2000. The two-stage coagulation of milk proteins in the minimum of the heat coagulation time-pH profile of milk: effect of casein micelle size. *J. Dairy Sci*. 83: pp. 378-386.
- Ordiales, E., Martín, A., Benito, M.. Hernández, A., Ruiz-Moyano, S., Córdoba, M., 2012. Technological characterisation by free zone capillary electrophoresis (FCZE) of the vegetable rennet (*Cynara cardunculus*) used in "torta del Casar" cheese-making. *Food Chem*.,*133*, pp. 227–235.

- Park, Y., and Haenlein, G., 2010. Milk production. Goat Science and production. S.Solaiman, Ed. *Wiley-Blacwell Publ*., New York.
- Pellegrini O., Remeuf F., Rivemale M., and barillet F., 1997. Renneting properties of milk from individual ewes: influence of genetic and non-genetic variables, and relationship with physicochemical characteristics. *Journal of Dairy Research*, 64, pp. 355-366.
- Pontual, E.V.; Carvalho, B.E.; Bezerra, R.S.; Coelho, L.C.; Napoleão, T.H.; Paiva, P.M., 2012. Caseinolytic and milk clotting activities from *Moringa oleifera* flowers. *Food Chem*., *135*, pp.1848–1854.
- Potocnik, K., Ganther, V., Kuterovac, K., and Cividini, A., 2011. Mares milk: composition and protein fraction in comparison with different milk species. Mljekarstvo, 61, pp.107-113.
- Ramesh C., Kilara A., Shah N., 2008. Dairy processing and quality assurance. *Wiley-Blacwell*, Oxford, UK.
- Rajagopalan A., Soundararajan M., Sukumaran B.O., 2019. Proteases from Calotropis gigantea stem, leaf and calli as milk coagulant source. *Turkish Journal of Biochemistry*, 44, pp. 240-24.
- Rajagopalan A., Bindhu Omana Sukumaran, 2018. Three phase partitioning to concentrate milk clotting proteases from *Wrightia tinctoria R*. Br and its characterization. *International Journal of Biological Macromolecules,* 118, pp. 279-288.
- Rayanal, K.; Remeuf, F., 1998. The effect of heating on physicochemical and renneting properties of milk: A comparison between caprine, ovine and bovine milk. *Int. Dairy J.*, *8*, pp.695–706.
- Rayanatou I.A., Mahamadou E.G., Garric G., Harel-Oger M., Leduc A., Jardin J., Briard-Bion V., Cauty Ch., Adakal H., Grongnet J.F., Gaucheron F., 2017. Physico-chemical characterization of dairy gel obtained by a proteolytic extract from *Calotropis procera* – A comparison with chymosin. *Food chemistry,* 232, pp. 405-412.
- Reis M., Malcata F., 2011.Current state of Portuguese dairy products from ovine and caprine milks. *Small Ruminant Research*,101, pp.122-133.
- Ricceri J., Barbagallo R.N., 2016. Role of protease and oxidase activities involved in some technological aspects of the globe artichoke processing and storage. *Food Science and Technology*, 71, pp. 196-201.

- Rocío Tito F., Pepe A., Tonon C.V., Daleo G.R., Guevara M.G., 2020. Determination and characterisation of milk-clotting activity of two Solanum tuberosum aspartic proteases (StAPs). *International Dairy Journal*, 104.
- Roncolini, A.; Milanovic, V.; Aquilanti, L.; Cardinali, F.; Garofalo, C.; Sabbatini, R.; Clementi, F.; Belleggia, L.; Pasquini, M.; Mozzon, M.; et al., 2020. Lesser mealworm (*Alphitobius diaperinus*) powder as a novel baking ingredient for manufacturing high-protein, mineral-dense snacks. *Food Res. Int*., *131*, 109031
- Roseiro, L. B., Barbosa, M., Ames, J. M., &Wilbey, R. A., 2003. Cheesemaking with vegetable coagulants-the use of *Cynara L.* for the production of ovine milk cheeses. *Int J Dairy Technol*, 56(2), pp. 76–85.
- RincóA.A., V.Pino, M.R.Fresno, A.I.JiménezAbizanda, S.Álvarez, J.H.Ayala, A.M.Afonso., 2017. Influence of vegetable coagulant and ripening time on the lipolytic and sensory profile of cheeses made with raw goat milk from Canary breeds. *Food Science and Technology International*,23(3), pp.254-264.
- Rukke E.O., Sorhaug T., Stepaniak L., 2011. Heat treatment of milk: Thermization of Milk. *Encyclopedia of Dairy Sciences*, pp. 693-698.
- Rusconi M.E., N.S. Priolo, L.M.I. López, S.E. VairoCavalli, 2011.Action of *Silybum marianum* (L.) Gaertn peptidases on bovine caseins**.** *Alimentaria*, 421, pp. 90-95.
- Sarmento A., Lopes, H., Cláudia S. Oliveira, Vitorino R., Bart S., Sergeant K, Debyser G.,Jozef Van Beeumen, Domingues P., Amado F., Pires E., M. Rosário M. Domingues & Marlene T. Barros, 2009. Multiplicity of aspartic proteinases from *Cynara cardunculus L. Planta*,230, pp.429–439.
- Salvadori del Prato, 2001. Trattato del tecnologia casearia. *Edagricole.*
- Sato K., Goto K., Suzuki A., Miura T., Endo M., Mura K., Tanimoto M., 2018. Characterization of a Milk-clotting Enzyme from *Hericium erinaceum* and Its Proteolytic Action on Bovine Caseins. *Food Science and Technology Research*, 24 (4), pp.669-676.
- Say D. and Guzeler N., 2016.Production of Traditional Cheese Coagulant in Farmhouse and Coagulating Effect.*1st International Multidisciplinary Conference on Nutraceuticals and Functional Foods*, pp.133-137.
- Shah M.A., Shabir Ahmad Mir, Mohd Amir Paray, 2014. Plant proteases as milk-clotting enzymes in cheesemaking: a review. *Dairy Sci. & Technol*., 94, pp. 5–16.
- Silva M.Z.R., Oliveira P.B., Ramos M., Davi F. Farias, Chayenne A. de Sá, Juliana A.C. Ribeir, Ayrles F.B. Silva, Jeanlex S. de Sous, Rafael A. Zambelli, Ana C. da

Silva, Gilvan P. Furtad, Thalles B. Grangeiro, Mirele S. Vasconcelos, Sandro R. Silveira, Cleverson D.T. Freitas, 2020. Biotechnological potential of a cysteine protease (CpCP3) from *Calotropis procera* latex for cheesemaking. *Food Chemistry*,307.

- Silva, S.V.; Malcata, F.X., 2004. Influence of the coagulant level on early proteolysis in ovine cheese-like systems made with sterilized milk and *Cynara cardunculus. J. Food Sci.*, *69*, pp.579–584.
- Silva S.V., F.X. Malcata., 2000. Action of cardosin A from *Cynara humilis* on ovine and caprine caseinates**.** *Journal of Dairy Research*, 67, pp. 449-454.
- Simoes I., Faro C., 2004. Structure and function of plant aspartic proteinases. *Eur J Biochem*, vol. 271 no. 11, pp. 2067-2075.
- Singh, H., and Waungana, A., 2001. Influence of heat treatment of milk on cheese making properties. *Cheese Ripening Technology* 11, pp. 543-551.
- Swaisgood, H., 2003. Chemistry of the caseins. In Advanced Dairy Chemistry, Vol.1, *Kluwer academic/Plenum Plubishers*, pp.139-202.
- Yolmeh, M.; Jafari, S.M., 2017. Applications of response surface methodology in the food industry processes. *FoodBioprocess Technol.*, *10*, 413–433.
- Tabayehnejad, N.; Castillo, M.; Payne, F.A. , 2012. Comparison of total milk-clotting activity measurement precision using the Berridge clotting time method and a proposed optical method. *J. Food Eng*. ,*108*, pp. 549–556.
- Tamer, I., 1993. Identification and partial purification of a novel milk clotting enzyme from *Onopordum turcicum*. *Biotechnol.*,*15*, 427–432.
- Tamer M., Mutlu M., 1994. A new approach to modelling enzyme kinetics by a novel enzyme from *Onopordum turcicum* and powdered calf rennet. *The Chemical Engineering Journal and the Biochemical Engineering Journal*, 56, pp. 87-90.
- Tavaria, F.K., Sousa, M.M.F., 2001. Storage and lyophilization effects of extracts of *Cynara cardunculus* on the degradation of ovine and caprine caseins. *Food Chem*., *72*, 79–88.
- Tejada, L.; Vioque, M.; Gómez, R.; Fernández-Salguero, J., 2008 Effect of lyophilisation, refrigerated storage and frozen storage on the coagulant activity and microbiological quality of *Cynara cardunculus* L. extracts*. J. Sci. Food Agric.*, *88*, pp. 1301–1306,

- TrochT., LefébureE., Baeten V., Colinet F., Gengler N., Sindic M., 2017. Cow milk coagulation: process description, variation factors and evaluation methodologies. A review. *Biotechnol. Agron. Soc. Environ.*, 21.
- Tubesha Z.A. and Al-Delaimy K.S., 2003. Rennin-like milk coagulant enzyme produced by a local isolate of Mucor. *International Journal of Dairy Technology*, 56, pp. 237–241.
- Walstra P., 1979. The voluminosity of bovine casein micelles and some of its implications. Journal of Dairy Research 46(2), pp.317-323.
- Walstra P., Wouters, J., Geurts, T., 2006. Dairy science and technology, 2nd edn.CRC Taylor and Francis Group, Boca Raton.
- Zikiou A.,Esteves A.C., Esteves E.,Gomes S., Louro Martins A.P., Rosa N., Zidoune M.N.,Barros M., 2020. Algerian cardoon flowers express a large spectrum of coagulant enzymes with potential applications in cheesemaking. *International Dairy Journal*, 105.