UNIVERSITA' POLITECNICA DELLE MARCHE

DIPARTIMENTO DI SCIENZE AGRARIE, ALIMENTARI E AMBIENTALI

Cheesemaking with plant coagulants obtained from local wild flora of Marche region

PhD Thesis

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ABSTRACT
The aim of this doctoral thesis was to characterize the technological properties of the proteases found in raw aqueous extracts (CE) from wild flora of the Marche region, (*Carlina achantifolia* All.Subsp. *Achantifolia*). Microbiological dynamics, physico-chemical parameters, and volatile components (aroma) of cheese ("Caciofiore della sibilla") obtained by using vegetable coagulants, were also studied. CE prepared from separate anatomical parts of the thistle plant were compared with calf rennet and tested at different temperatures, concentrations of $CaCl_2$ and storage conditions. Analyses showed that the calf coagulant had the highest milk clotting activity (MCA) whereas vegetable coagulants had lower milk clotting times. The optimal MCA of CE was observed at a temperature of 55-60 °C. MCA of the flower extracts was greater than that of the stems. Addition of $CaCl_2$, in the range of concentration experimented, had a positive effect on the clotting activity of extracts. The amount of enzymes (CE) used was very discriminating for MCA. Storage at -20 °C had no effect on MCA.

The volatile components of the aqueous extracts obtained from *Carlina achantifolia* All. and of the cheese sampled different times of ripening were also quantified and partially identified by SPMEGC- MS. The number of volatile compounds identified in the flower aroma were greater than the stems. Many compounds identified in the early stages of the ripening were not detected in the final product. Other compounds were identified in all cheese samples but exhibited higher levels in the ripe cheese .

The impact of vegetable rennet on bacterial dynamics and on the overall diversity showed a great difference in the bacterial composition in the cheese while the contribution of the vegetable coagulant to the cheese fungal diversity had a marginal or no contribution.

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1. Introduction

Plant proteases have been used as milk coagulants in cheesemaking for centuries: the earliest reference is attributed to Lucius Junius Columella in his treatise De Re Rustica (50 BC).

A few papers have dealt with the use of plant extracts as vegetable coagulants for cheesemaking: plant proteases are mainly constituents of leaves or flowers, sometimes of latex and/or sap, fruits, roots, seeds. The genus Cynara L. is the most used vegetable coagulant in cheesemaking, and also the most investigated. The excessive proteolytic nature of most plant coagulants has limited their use in cheese manufacturing due to lower yields of cheese, bitter flavours and texture defects.

The search for new potential milk-clotting enzymes from plants is still continuing in order to meet the increasing global demand for diversified and good quality cheese production.

In the Mediterranean area, the exploitation of thistle rennet, especially obtained from *Cynara cardunculus L*., *Cynara scolymus L*. and *Cynara humilis L*., is not confined to Italy, where renowned peculiar cheeses, such as Caciofiore Aquilano and Caciofiore di Columella, are traditionally manufactured in Abruzzo and Lazio, respectively. The use of this type of vegetable coagulant is also particularly widespread in western Africa and the Iberian Peninsula, the latter boasting a large number of Protected Designation of Origin (PDO) cheeses. Even in Latin-American countries, such as Argentina and Chile, where 'cardo de Castilla' (C. *cardunculus L*.) grows vigorously, thistle rennet is used to produced a variety of ewes' milk cheeses. Crude extracts from flowers or even leaves of thistles are thermostable and characterized by a high proteolytic activity for the occurrence of aspartic proteases, known as cardonsins (or cynarases and cyprosins), with a high specificity for caseins.

Caciofiore cheeses produced in the foothills of Marche region are characterized by the use of local wild flora (*Carlina achantifolia* subsp. *achantifolia*, *Carlina acaulis* subsp. *caulescens*, *Onopordum tauricum*) for the preparation of aqueous extracts employed as milk coagulant.

"Caciofiore della Sibilla" is a speciality ewes' milk cheese traditionally manufactured in a foothill area of Marche (Central Italy) with a crude extract of fresh young leaves of *Carlina acanthifolia* All. subsp. *acanthifolia* as a coagulating agent, usually never weighing more than 1 kg,

Cheeses made with vegetable coagulants are normally produced on an artisanal scale, in a farmhouse or small dairy. However, they have an important socio-economical contribution to the dairy sector at local and regional areas of each country and thus play an important part in the local agricultural economy. We hope that the fully characterization of the Caciofiore could contribute to a promotion of this product and its production area.

2. Cheeses

According to the Italian legislation (r.d.l. No 2033 of 15/10/1925), "cheese is the product obtained from whole or partially skimmed milk, or from cream by acid or rennet coagulation, also by using ferments and salt. Cheeses can be classified according to a set of parameters that combine variously among them.

These parameters refer to

type of milk used
fat content
consistency, in relation to water content
technology used for production and processing
temperature of curd
seasoning period
denomination
coagulant employed.

Depending on the type of milk used, cheeses are distinguished in:

- cow milk
- sheep milk
- buffalo milk
- goat milk

If cheese is produced from milk differtent from the cow one, the indication of the species has to be indicated.

As to the fat contents, expressed in dry substance, has to be written (Leg n. 142/1992):

- fat cheeses, the fat content of which exceeds 35% of the dry matter are (Robiola, Gorgonzola, Fontina, Montasio, Grana Padano, Parmigiano Reggiano, Pecorino, etc.);
- light cheeses, where the fat content varies between 20% and 35% of the dry matter
- lean cheeses, prepared with skimmed milk, with a fat content of less than 20% of dry substance. I would like to point out that for many DOP cheeses obtained from partially skimmed milk (Grana Padano, Parmigiano Reggiano, Asiago, Raschera, etc.) the production discipline provides only a minimum fat content of the dry substance. So many of them, originally defined “Semigrassi”, nowadays belong to the category of fat cheeses.

Depending on the consistency, due to the percentage of water contained, can be distinguished:

- soft dough cheeses, when the water content is more than 45% (eg Robiola, Quartirolo, Stracchino, Crescenza, Mozzarella, Burrata, Gorgonzola, Caprini, Casatella, Squacquerone). They can be crusted (i.e. Taleggio) or not (i.e. Pannerone)
- semi-hard cheese, where the water content is between 35% and 45% (eg. Ragusa, Asiago, Bitto, Fontina, Bra, Castelmagno, Italic)
- hard-topped cheese, where the amount of water is less than 35% (eg. Grana Padano, Parmigiano Reggiano, Pecorino Romano, Montasio, Pecorino Sardo, Sardo Flower)

The water content of common cheeses is not defined by law and only for some cheeses DOP the Production Discipline provides a minimum moisture content. The texture of the cheese is also influenced by other parameters such as the advancement of seasoning.

Depending on the technology used and the process temperature of the curd, cheses can be classified as following:

- raw pasta cheeses, where, during processing, the curd is not subjected to any heating above the coagulation temperature (eg Robiola, Mozzarella, Crescenza, Gorgonzola)
- half-cooked cheese, when the curd warming does not exceed 48 ° C (eg.Asiago, Fontina, Italico, ...) cooked pasta cheeses, if obtained by heating the curd over 48 ° C (eg. Grana Padano, Parmigiano Reggiano, Montasio, Bitto,)
- woven pasta cheeses, characterized by spinning the curd in hot water at 70-90 ° C (eg Mozzarella, Fiordilatte, Caciocavallo, Provolone, Ragusano)
- grassy cheeses (“formaggi erborinati”), where the milk is deliberately inoculated with molds which will develop them selves within the cheese paste contributing to the maturation of it with specific enzymatic activities (eg. Gorgonzola, Castelmagno)

Depending on the seasoning period, can be recognized:

- fresh cheese, obtained by acid or pre - coagulation, and not subjected to

seasoning, without crust or superficial microflora and must be consumed within a few days from production (eg. Mozzarella, Fiordilatte, Crescenza, Casatella)

- short ripened, matured cheeses with a maturation period not exceeding 30 days (eg. Taleggio, Murazzano, Bra, Lombard Quartirolo, Asiago, Monte Veronese, Casciotta of Urbino)
- averege ripened cheeses,with a maturing period shorter than 6 months (eg. Fontina, Castelmagno, Raschera, Toma Piedmont, Valtellina Casera, Provolone Valpadana, Caciocavallo Silano, Pugliese Canestrato, Sicilian Pecorino, Sardinian Pecorino, Bitto)
- ripened cheeses with slow maturation, from 6 months onwards (eg. Grana Padano, Parmigiano Reggiano, Sardinian Flower)

According to the denomination, cheeses are distinguished in:

Protected Designation of Origin (PDO) cheese: they are "cheese produced in zones geographically delimited, observing loyal and consistent local uses and features commodities derive predominantly from the conditions of the production environment " (Chapter I, Article 2 L. 10.4.54, 125). This denomination is now sanctioned and protected at the level of EU by Council Regulation (EC) No 510/2006.

Among these cheeses are Asiago Dop, Bitto Dop, Bra Dop, Caciocavallo Silano Dop, Canestrato Pugliese Dop, Casciotta d'Urbino Dop, Castelmagno Dop, Flower Sardo Dop, Fontina Dop, Form de Mut of the Upper Val Brembana Dop, Gorgonzola Dop, Grana Padano Dop, Montasio Dop, Monte Veronese Dop, Bufala Mozzarella Camp Dop, Murazzano Dop, Parmigiano-Reggiano Dop, Pecorino Romano Dop, Pecorino Sardo Dop, Sicilian Pecorino Dop, Pecorino Toscano Dop, Provolone Valpadana Dop, Quartirolo Lombardo Dop, Ragusa Dop, Raschera Dop, Robiola of Roccaverano Dop, Sprale of the Giudicarie Dop, Taleggio Dop, Toma Piemontese Dop, Aosta Valley Fromadzo Dop, Valtellina Casera Dop.

Protected Geographical Indications (IGP): are "cheese produced on the national territory, observing loyal and consistent uses, whose product characteristics derive from particular features of the raw materials or of the production technique "(Chapter I, Article 2 L. 10.4.54 n. 125). This denomination is also nowadays sanctioned and protected at EU level by Reg. (EC) No 510/2006. Italy has not so far requested registration cheeses with this brand.

"**Traditional Guaranteed Specialty" (STG)** are "cheeses whose specificity

consists in respecting a detailed method of traditional production, while lacking a bond with a geographic area: they can therefore be produced throughout the national territory. They are protected by Council Regulation (EC) No 509/2006. In Italy the only example is Mozzarella STG.

T**raditional cheeses:** there are over 450 so-called 'regional' cheeses, eg Piave, Squacquerone, Fossa Cheese, Moena's Puzzone, Murge's Murge, Cacio Marcetto, Bagòss, Piacentinu of Enna, Casieddu di Moliterno, Casolet Val Camonica, Dobbiaco, Paglierina Rifreddo, Tosca del Primiero, Former Embriago, Morlacco del Grappa.

3. Cheesemaking process

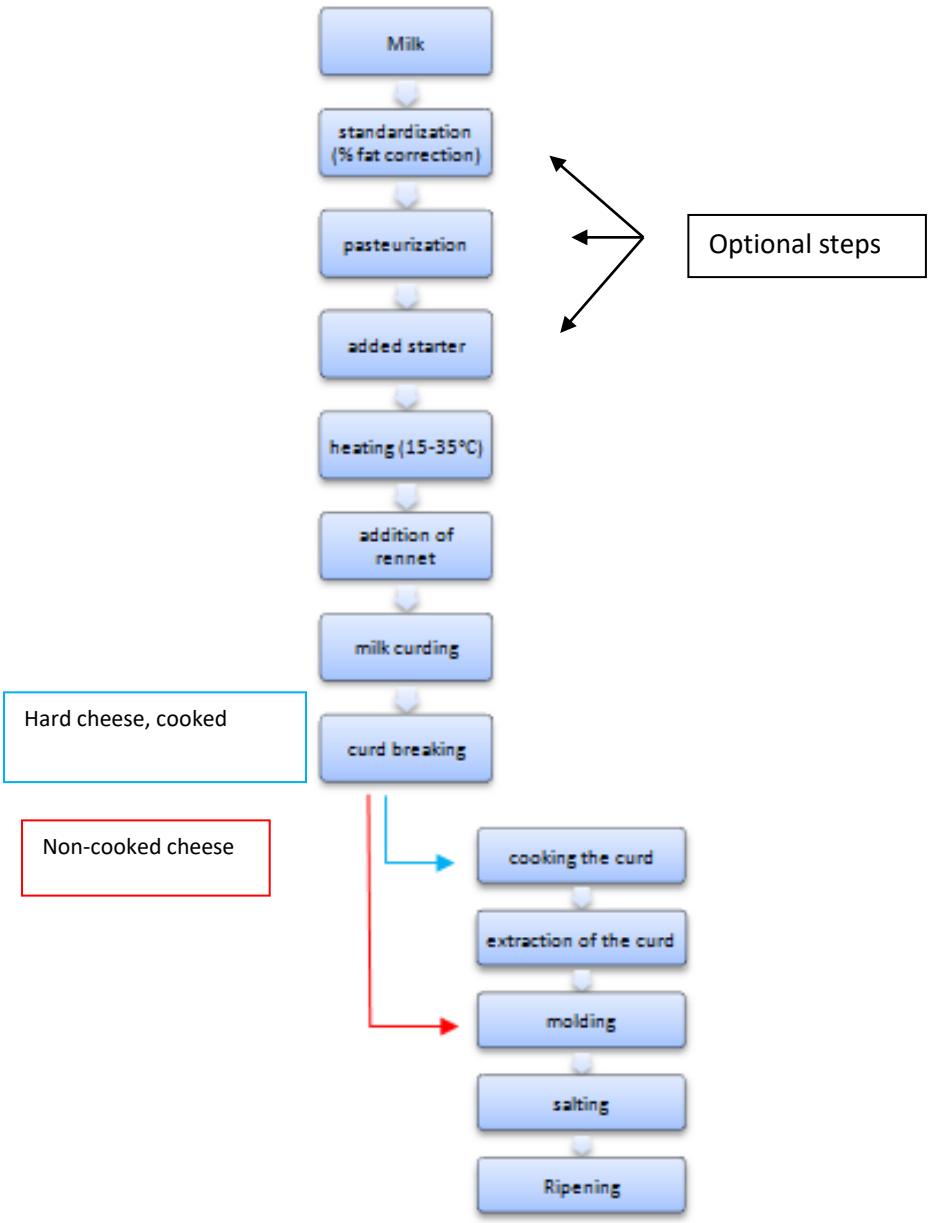

Fig 1: Cheese-making process

In the production of cheese is mainly used cow milk, this doesn't exclude the use of sheep milk, goat and buffalo one moreover, milk may have different characteristics such as: fat percentage, physical condition, enrichment, etc.

The milk, refrigerated in the stable, upon arrival in the dairy, is subjected to microbiological, chemical and physical analysis before being used in the production process.

3.1. Milk standardization (optional phase)

The standardization of milk fat content is indispensable because of the significant variations in the percentage of fat, especially of cow milk, even if it comes from the same breeding stock. This stage is also carried on depending on the type of cheese to be produced.

Generally, to perform this phase, a centrifuge or an overlapping skim is used, in which the milk components are separated according to their density.

3.2. Pasteurization (optional phase)

Pasteurisation is only applied to hard cheeses in order to reduce the natural microbial charge of milk to allow the development of inoculated microorganisms with insemination, to destroy the major responsible for early swelling, the coliforms that determine the production of carbon dioxide, shortly after formulation of the cheese and eliminating pathogenic microorganisms.

3.3 Adding starter (optional phase)

The addition of starter consists in enriching the milk, by the grafting of a particular microbial flora, suitable for the type of cheese to be produced. Starters are divided into: natural, if they are made of microorganisms naturally present in milk and selected, if appropriately chosen in case of pasteurized milk, the addition of starter is mandatory, but it is optional when the raw material has not undergone pasteurization treatment.

3.4 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, so as to create the ideal conditions for the enzyme and hence its coagulant activity and proteolytic activity**.**

3.5 Addition of rennet

Rennet is the raw enzymatic product obtained from the abomasum (fourth stomach) of infant ruminants (calves, lambs or little goats). Rennet is also called prune, chimase or rennin. What rennet makes in milk is due to two enzymes produced by the gastric mucosa of infant calves: chimosine (having coagulant activity) and pepsin (with produces proteolytic activity).

The addition of rennet is a decisive step for the final characteristics of the finished product. As a technological coagulant it results in the formation of a gel, called curd that in time tends to contract and expel serum.

3.6. Coagulation

The coagulation that determines the transformation of milk into curd, which then tends to contract (syneresis) by expelling the serum, is due to the gelatinization of the caseinic micelles. During this phase phosphocaseinase Ca (soluble) in the presence of rennet is converted into paracaseinate of Ca (insoluble) and caseinoglycopeptide (soluble). Coagulation can be of two types: acidic or enzymatic.

Enzymatic coagulation can be explained in three phases that really don't occur separately.

During the first phase, chimosine attacks the casein and solubilizes a small part of it (glycomacropeptide) and cleaves the peptide bond between methionine (106) and phenylalanine (105) of casein k. Following this break, casein k loses its protective colloid properties, resulting in the destabilization of the micelles, which are no longer protected by the polar groups of the glycocropeptide. The soluble fraction (hydrophilic and with a high negative charge) released from casein k is a caseinoglycopeptide having a molecular weight of 8000 Da, consisting of the sequence of amino acids 106-169 and containing all casein k kernels corresponding to about 30% of the total casein glucide. The insoluble fraction of casein k, paracasein k or parafosfocaseinate (residues 1-105) does not contain either carbohydrates or phosphate groups, but all sulphurous and aromatic amino acids of casein k. The primary phase is the only fully known and can occur in very large pH conditions (5.5 to 7) and temperature (4 to 45 ° C), no calcium ions.

In the secondary phase, the casein state passage, in the form of colloid, is present in the semi-solid clotting state, which initially occupies the whole volume kept by the milk. This phase takes place at temperatures above 15 ° C, to allow the formation of hydrogen bridges between the micelles that have lost the hydration state at a pH between 5.5 and 6.5 and in the presence of ionic calcium (Ca2 +).

During the third phase, the clot is contracted, increasing the number of intermittent bonds, expelling the serum (curd purging) from the internal spaces between the micelles and assuming an ever increasing consistency. At this phase there is also a slow, non-specific proteolytic activity, which consists in the release of some αs1 and ß casein peptides, which determines the maturation of cheese. Syneresis or purging of the gel can be spontaneous, when it is very slow or induced when it is faster, the latter generally occurs during the production of cheeses.

Coagulation and sineresis are the phases that have a major impact on the organoleptic characteristics of the cheese.

3.7 Curd breaking

Curd formation can take place into two ways depending on the type of coagulation (acid or enzyme). In the first case, the formation of the clot and subsequent serum release is slow and occurs at temperatures between 20-22 ° C. In the second case the clot is broken by mechanical action (the final size of clotting breaks depends on the type of cheese one wants to produce).

3.8 The processing of curd

The curd processing changes according to the type of cheese produced.

For raw and soft paste the curd is broken repeatedly and left under serum for 20-30 minutes to facilitate purge at the coagulation temperature in which it is located (35-38 ° C). Afterwards the curd is extracted and put into form without pressing.

As to hard and cooked paste one must break the curd into pieces of rice grain size, meanwhile the mass, always under agitation, passes from the coagulation temperature (32 ° C) to the one that

favours the losing of the serum (50- 56 ° C). The curd is then transferred into the molds and pressed in order to get a further purge of the serum and stabilize the clots of the curd;

As a to hard and semi-hard pastes the process is the same, but instead of reaching a cooking temperature of 50-56 ° C, a heat treatment at 42-44 ° C is carried out for a few minutes.

3.9. Salting

Salting can be done in two to ways: direct salting or brine. Direct salting consists in treating the piece surface with salt while the process brine consists in putting the cheese into a cold bath (10-12 ° C) consisting of a sodium chloride solution (NaCl) at a given concentration (15-20%). The brine should be periodically renewed to prevent infections due to the presence of unwanted microorganisms and to restore the initial saline concentration which decreases due to salt absorption and serum release from the cheese. Therefore, brine is regenerated daily, reintegrating saline concentration, and periodically boiling or filtering it, which eliminates all microorganisms and seroproteins that cause intorbidity.

3.10 Ripening

The ripening of cheese is a very important step because at this stage there are a profound physical and chemical-physical modifications of the product, which transform the components of the curd into substances that characterize, its taste, aroma, colour, appearance and the texture of the pasta.

The main changes that take place in the form of cheese during ripening are:

- loss of water;

- lactose conversion into lactic acid due to lactic ferments that result in a pH reduction of 6.5 to 5.5;

- triglyceride hydrolysis by lipases, fatty acids and glycerin, which in turn are degraded by oxidative processes in increasingly simple and qualifying compounds, that characterized the type of cheese;

-proteolysis of protein substances, especially of casein, resulting in the formation of peptides, free amino acids, carbon dioxide, carboxylic acids, etc. Which all together determine the characteristics: smell, flavour and texture of the cheese;

- crust formation;

3.11. Loss of water

The percentage of lost water depends on the type of cheese produced and the level of maturity in the ripening chamber; i order to limit this loss of water and consequently to increase the yield of the cheese, a surface treatment with vegetable oils or paraffin is sometimes useful.

3.12. Lactose conversion

The lactose transformation into lactic acid is linked with the presence of the flora of the cheese and the uncoated pasta, even in the presence of rhaggenii and original milk enzymes, especially if these have not been subjected to a strong thermal treatment. Immediately after forming cheese, lactose

undergoes fermentation (omolactic fermentation) forming: mainly lactic acid (CH3-CHOH-COOH, concentration ≥ 85%) at a decreasing speed from the starting to the end, methanol (CH3-CH2-OH) and acetaldehyde (CH3-CHO), acetic acid (CH3-COOH), acetone (CH3-CHOH-). In addition to this type of fermentation, lactose is attacked by etherolactic ferments that form, besides lactic acid, acetic acid and / or ethanol, carbon dioxide, in ratio 1:1 among them, acetone and diacetyl. The fermentation products are aromatic and therefore contribute to cheese flavour formation. Their aromatic power grows with the decrease in molecular weight, consequently it is lower for lactic acid and increases for acetic acid and ethyl alcohol, acetone and acetic aldehyde, diacetyl up to maximum for acetoin.

Lactic acid, in turn, is partially salified with calcium and in part forms the substrate for other biochemical processes by mold and / or propionic fermentation. The fermentation products of the latter are: propionic acid (CH3-CH2-COOH), acetic acid, water (H2O) and carbon dioxide, which determines the formation of the eyelids in Emmenthal and Gruyère. This kind of fermentation is badly seen in long-lasting cheeses.

The set of neoformation products,which is characteristic of each cheese, helps to regulate pH and ionic balance and to prevent the development and proliferation of harmful ferments.

3.13. Triglyceride hydrolysis

Hydrolysis of fat, during the ripening, plays an important role in the formation of the aroma of the product, but does not involve profound modifications of cheese weaving. The fat, during maturation, undergoes qualitative and quantitative variations. More interesting under the aspect of cheese flavour, are the changes due to the activity of the lipases that share triglycerides into glycerine and fatty acids. Lipase actives in cheese comes mainly from microorganisms, molds and micrococci. Natural milk lipase is not active, especially in cooked pasta because it is easily destroyed by the pasteurization of milk, unlike microbial lipases that are heat-resistant enough (up to 76° C). Lipolysis is generally measured according to the amount of free fatty acids, which have varies considerin the type of cheese. In soft pastry cheese, butyric acid (CH3-CH2-CH2-COOH) prevails, while in Emmenthal, hard cheese, it is pro-prionic acid which prevails. The index or degree of lipolysis in cheese is indicated by the capric acid content. Normally free fatty acids account for 2-3% of the fat fraction, but in some cheeses they can reach 7%.

3.14. Crust formation

The crust has two important functions: it is forms support and a separation structure, such as a barrier, between the outer environment and the cheese internal paste, limiting the loss of moisture and the gaseous and / or volatile products originated during maturation.

4. Rennet type

4.1. Animal rennet

Rennet (animal) is obtained from the fourth stomach (abomasum) of non weaned ruminants (calves, lambs, little goats) contains all the enzymes necessary for the digestion of milk and in particular chimosine (or renin) and pepsin, which act directly on the protein chains. The adult animal stomach can't be used precisely because they are lactose-free and they have other enzymes necessary for the digestion of the food. Traditional rennet is obtained by maceration of dried abomaso fragments or by salting with 10% NaCl (facilitating the extraction of enzymes), adding antiseptics for 10-12 hours at 20 ° C and pH 4. The juice, then filtered, is clarified and dried. Commercial rennet are all standardized and carried on a fixed basis: the rennet is the amount of coagulated milk of 1 cc of rennet in 40 minutes at 37 ° C; it is therefore an extremely important parameter for dairies. The enzymes present in animal rennet are of two categories: coagulant enzymes, very abundant in gastric juice of uninhabited animals as chimosine which has an optimum action when it is at about neutral pH; peptonizing enzymes whose concentration increases in weaned individuals, such as pepsin having an acid pH-optimum, that is about 3. The proportion of these enzymes on coagulation of milk varies according to the age of the calf, the separation method of rennet and pH value at the time of measurement but it is usually 88/94% for chimosine and 6/12% for pepsin. These two enzymes are the main enzymes contained in the rennet, and lipase, gastric and lysozyme have recently been introduced into milk coagulation.

Chimosine structure

Chymosin, or rennin, is a protein with catalytic activity, called enzyme, which is present in the stomach of infant ruminants in the inactive form of prochimosin which is activated at pH 4.7 or 2.1. It is part of the category of hydrolysis or enzymes that catalyze hydrolysis reactions, bonding

reactions by the use of a water molecule. Chymosines are involved in the peptide bonding that binds the amino acids in the proteins, therefore they occupy proteolysis or a process of degradation of proteins, so they are called proteases. The optimal working temperature is about 40 ° C but it develops in milk too at 4 ° C but it is very slow. Its main task is the hydrolysis of the casein, it is responsible for the formation of the clot from selective hydrolysis of the polypeptide chain of the K casein between the phenylalanine residue Phe 105 and meth methionine residue Met 106, resulting in to two fragments, paracasein K and a glycopeptide. In this way, paracasein K at the presence of Calcium ions, precipitates and the clot is formed. Proteases are highly specific and their activity is closely related to certain parameters such as pH, temperature and other factors such as irradiation. Pepsin, which is also a protease, is secreted as a zymogen, an inactive form that acquires functional capacity only after a precise structural modification. It is especially the hydrochloric acid secreted by the parietal cells of the stomach that transforms the pepsinogen, its precursor, into pepsin, by a proteolytic cut that leads to the removal of about forty amino acids. Active pepsin, in turn, promotes the formation of new pepsin by directly acting on pepsinogen. Under normal conditions, i.e., a temperature of about 37 ° C and a pH of 1.5 / 2, pepsin can digest up to 1,000 times its protein weight in an hour. At pH above 3.5, pepsin loses of its proteolytic activity much until irreparably denatured to more than 5. Because of the action of pepsin, food proteins are reduced to peptones, smaller fragments but in a size still excessive to be absorbed. Pepsin is about 45 times more proteolytic than chemosine, it also acts on K casein by clotting milk, but much less specific than chimosine. Lipase is always an idylase but catalyzes the hydrolysis and synthesis of acylglycerols (triglycerides). Lipases greatly affect the quality of products, they are used to release short chain fatty acids, thus giving new flavours and aromas to cheeses. They are also widely used in the food industry to produce oils and fats containing polyunsaturated fatty acids (PUFAs) of high nutritional value. When lipolysis is carried out too long, it can have unpleasant consequences in taste, according to the type of cheese, with the formation of typical spicy flavours and aromas.

4.2. Genetically modified rennet

In 1990 the recombinant version of calf chimosine was the first aid to those foods produced with recombinant DNA. This technology was registered by the US Food and Drug Administration. In fact, genetically engineered chymosin is used predominantly in the United States, but other countries are also showing increasing attention to this type of genetically modified enzyme. The recombinant chemosine “Bos Taurus” technique is by far the most important recombinant enzyme in engineering genetic technique. After cloning preprochimosin or pro-chimosine it is inserted into the cDNA of bacteria, yeasts, filamentous fungi, in order to reach the enzymatic expression of the gene. Then, after insertion of DNA carrier, this can be transferred into microorganisms such as *Escherichia coli, Bacillus subtilis, Lactococcus lactis, Saccharomyces cerevisiae, Kluyveromyces lactis, Aspergillus niger, Aspergillus orza or Trichoderma reesii.* Pro-chimosine is usually activated at pH 2 similar to the one derived from a calf. Recent studies aimed to better activation conditions have reported a significant increase in pro-chimosine gene expression by *E. coli* DNA manipulation. The improved production of chimosine from filamentous fungi such as A. niger was achieved through the glycosylation of the molecule resulting in a 100% yield increase over the native enzyme. Active chimosine extracts can be industrially purified by filtration, precipitation, flocculation, centrifugation, chromatographic or gel filtration with resins of different functionality.

Research has also been carried out on recombinant chymosin derived from other animals such as deer, buffalo, antelopes, giraffes, sheep, goats, pigs and more recently camel (Camelus dromedarius) expressed in A. niger var. awamori. (Mandy *et al* 2011)

4.3. Microbial rennet

Many extracellular proteases of microbial origin act similarly to chimosine and are partially suitable for cheese production. These coagulants can easily be produced by fermentation and therefore it is possible to have almost unlimited availability. Since enzymes are not derived from ruminant tissues, there are no constraints on spongiform encephalopathy, and the cheeses produced with microbial enzymes are accepted by the category of lacto-vegetarian consumers. However, these enzymes have superior proteolytic activity in chewing that can lead to a loss of products resulting from the degradation of whey proteins and thus adversely affect cheese yield. Since 1960, the fungus coagulants used in the dairy industry heve been more than 100 to demonstrate the growing interest in these enzymes. The mushrooms producing milk curd are ubiquitous proteases that can easily be isolated from various environments. In particular, there are three large-scale species: *Rhizomucor miehei, Rhizomucor pusillus and C. parasitica.* The aspartic protease produced by *R. miehei* consists of a single polypeptide chain with a high resemblance in the three-dimensional structure to the chimosine one. This protease (40.5KDa, optimal phagocytic activity pH 5.6, optimal proteolytic activity pH 4.1, 50% loss of proteolytic activity after 30 min 45° C) is the most common used microbial coagulant to produce cheese and it is available at different levels of stability and purity in commerce. The protein has no activity on the sulfhydrin groups and the contributions of Phe, Thr and Lys are equivalent to the calf chimosine one. Recently it has been reported that proteases derived from *R.pusillis* show only one single band at the electropherogram after extensive purification, corresponding to a molecular mass of 49KDa. The coagulation activity of milk is better at 50° C, and the sensitivity to pH and $CaCl_2$ has to be similar to the calf rennet one. Cryphonectria parasitica is less characterized but is generally but its proteolytic activity is higher. Solid state fermentation has often been successfully used. The best results were obtained for *R.miehei* (9.0: 1) and *R. pusillus* (12.5: 1) through fermentation carried out for 5 days in a temperate chamber at the presence of solid bran, yeast extract or potato, dextrose; the enzyme yield increase was also monitored. After purification, chemical modifications are usually applied to reduce thermal stability. Thermal stability has in fact been the major disadvantage of the first generation of fungal proteases, because even after the cheese was cooked, the enzyme remained intact and caused an increase in the breakdown of casein during maturation. Information on appropriate procedures is poor even if Havera and Humphreys have reported an oxidation methionine treatment with H_2O_2 in combination with the addition of maleic anhydride which has led to an increase in thermo-labile and coagulation activity for *R. pusillis* proteases. Another potential pathway is that of mutagenesis for fungal production. Two mutants at the presence of a single exchanged amino acid (Ala 101 instead of Thr101 and Gly186 instead of Asp186) in the proteases of *R. pusillus* showed a marked decrease in thermal stability. In addition to improving the commercial properties of microbicidal coagulants, research focuses on the search for new proteases from different sources.(Mandy *et al* 2011)

4.4. Vegetable rennet

There are not only coagulant enzymes of animal origin, but other coagulant enzymes of other nature can also be used nowadays, for reasons related to dietary and ethical choices such as vegan, and to retrieve old traditions increasingly producers tend to use coagulant plants. They are not yet very used nowadays in the production of cheeses as in the past, but they are the subject of many studies. It is obtained from flowers of spontaneous mountain plants and in particular from the *Cynara Cardunculus* (Wild Cardo). At first, its use, which appeared to be quite common among the mountain margins (nomadic shepherds), was justified by purely economic reasons, but experience showed that it was possible to obtain particularly delicate and creamy cheeses and therefore even more enjoyable. What shares the action of plant clotting is the proteolytic activity of the contained protease, excessively above the coagulant activity, which, on the contrary, is very low. These characteristics result in a fall in cheese yield (Kg of cheese that can be obtained from a given amount of milk), texture defects (consistency), and pronounced the bitter taste during ripening of cheeses due to the development of some hydrophobic peptides by hydrolysis of the protein fraction of the ß casein. The problem lies on the composition of plant extracts, which contain a complex set of enzymes whose activity is very difficult to control. But why should we then encourage the production of dairy products based on vegetable coagulants at the expense of the animal ones? The reasons are many, the worldwide increase in cheese production and at the same time the difficulties of finding calf poultry (due to reduced slaughter of calves for the production of rennet, which are instead bred to produce meat for feeding), has led to an increase in the purchase price of the rennet and consequently to look for coagulant substitute. One of the alternatives of growing interest is precisely plant proteases. The introduction of these coagulants into the caseinates begins to be considered for various reasons:

• Restrictions of religious origin

• Diet type (the spread of vegetarianism among consumers has led to the consequent demand for products of plant origin and ethically correct production)

• Legislative prohibitions (in some countries such as Germany, the Netherlands and France it is not allowed to use veal rennet)

The use of these coagulants has brought many advantages, especially at artisan level, the use of coagulants obtained from plants allows the production of niche cheeses with flavours and aromas that characterize the product. The cheese thus obtained will be fresh, with a creamy consistency, acidic flavour, sometimes spicy, caracterized by a bitter note.

5. Proteases

5.1. Vegetable proteases

Vegetable rennet is protease, characteristic enzymes of plants, involved throughout their all life cycle, from the mobilization of reserve proteins during seed germination at the beginning of cell death and senescence (Schaller, 2004).

Plant proteases are divided into groups according to the catalytic mechanism used during the hydrolysis process. The main typologies are aspartic, serine, cysteine and metalloproteases (Bah *et al*., 2006), but plant proteases used to coagulate milk belong only to the first three types. Serine and cysteine proteases are different at catalytic level from aspartics and metalloproteases, as the are nucleophile of the catalytic site and part of an amino acid.

5.2. Aspartic proteases

Most of the plant's aspartic proteases have an extra insertion between the N-terminal and the C-terminal group, which consists in about one hundred amino acids and called PSI, or specific plant insertion (Yegin and Dekker, 2013). Aspartic proteases are active at acid pH and have a specificity in the cleavage of the peptide bonds between the hydrophobic amino acid residues responsible for the catalytic activity (Domingos *et al*., 2000). Aspartic proteases with milk coagulant activity have been found, as shown in Table 1, in artichoke (Cynara scolymus L.) (Llorente *et al*., 1997); (Tamer, 1993), in rice (Asakura *et al.,* 1997) and Centaurea calcitrapa (Domingos *et al*., 2005), in the marian thistle (Silybum marianum L.) (Vairo;Cavalli *et al.*, 2000). In thistle (*Cynara cardunculus*) we find cardosina and ciprosina, aspartic proteases that accumulate in ripe flowers (petals and pistils), but we do not find them in leaves or seeds (Cordeiro *et al*., 1998). Cardin A is composed of two subunits with a molecular weight of 31 and 15 kDa, while cardosin B consists of two subunits with a molecular weight of 34 and 14 kDa. Cardin A has been highly studied for its high resemblance to action specificity with k casein and it is seen that it breaks the same peptide bond Phe105 --- Met106 on which chimosine acts. Cardin B is similar to pepsin in terms of specificity and action (Egito *et al*., 2007). A unique feature of cardin A and which lacks in cardosin B, is the presence of the functional sequence Arg --- Gly --- Asp (RGD), which is known as the binding sequence in the integrin. It has been seen that the RGD sequence has the function of binding receptor on the cell surface. From the dried flowers of *C. cardunculus*, three cypresses with coagulant activity have been found, they have been isolated, purified and characterized (Yegin and Dekker, 2013).

5.3. Cysteine proteases

Cysteine proteases have a catalytic mechanism that involves a cysteine group in the active site. Cysteine proteases are widely used in food, biotechnology, and pharmacy sectors for their wide range activity of temperatures and pH. Belonging to this group is ficin, isolated from the latex of several species of Ficus. In particular, the ficus isolated from the latex of *Ficus racemosa* has been shown because it can digest casein, indicating a milk coagulation capacity (Devaraj *et al*., 2008). Also enzymes extracted from *Albizia lebbeck* and *Helianthus annuus* belong to this group and have coagulant activity (Egito *et al.*, 2007) as well as the actinidine isolated from mature fruit of kiwi

(*Actinidia chinensis*) (Katsaros *et al*., 2010) and proteases extracted from the ginger root (Zingiber officinale) (Hashim *et al*., 2011).

5.4. Serine proteases

Serine proteases possess a serine residue in their active site and are spread among various taxonomic groups are present in almost all parts of the plant, but especially in fruits. Serine plant proteases have been found and extracted from latex, seeds, flowers, leaves and roots. Blackpholine, a serotype-like protease similar to chimitropsin, was purified from the latex of Euphorbia neriifolia (Yadav *et al.,* 2011). Another enzyme, S. blackpholine, a serine dimeric protease of 94kDa molecular mass, has a coagulant activity in milk and was purified by E. neriifolia latex (Yadav *et al.,* 2012). Religious, Religious B and Religious C were isolated from the latex of the religious Ficus (Kumari *et al*., 2012). *Streblus asper* has been purified by a thermostable enzyme with a molecular mass of 63 kDa, streblin (Tripathi *et al*., 2011). Still cucumber from Cucumis apple tree (Uchikoba and Kaneda, 1996) and the dairy from *Lactuca sativa* (Lo Piero *et al*., 2002) were used as coagulants in milk.

Vegetable species cited in literature

Actinidia chinensi	*Lactuca sativa*
Albizia julibrissin	*lemon juice*
Aloe variegata	*melon*
Angiospermae	*Moringa oleifera*
Arabidopsis	*Nepenthes distillatoria*
Arabidopsis thaliana	*Nocardiopsis sp.*
Balanites aegyptiaca	*Onopordum acanthium*
Benincasa hispida	*Onopordum turcicum*
Brassica napus	*Opuntia ficus-indica*
Bromelia hieronymi	*orange Citrus aurantium*
Calotropis gigantea	*Oryza sativa L.*
Calotropis procera	*Papilionoideae species*
Centaurea calcitrapa	*pineapple peel*
Chinese Ginger	*rice wine*
Cirsium vulgare	*Sideroxylon obtusifolium*
Citrus sinensis L.	*Silybum marianum*
Cucumis trigonus Roxburghi	*Solanum aethiopicum Shum*
Cynara cardunculu	*Solanum dobium*
Cynara scolymus	*Solanum elaeagnifolium*
Euphorbia amygdaloides	*Solanum esculentum,*
Ficus carica	*Solanum tuberosum L*
Ficus racemosa (L.)	*Streblus asper*
Ficus religiosa	*Synadenium grantii*
Ginger	*Withania*
Helianthus annus	*Wrightia tinctoria*
Jacaratia corumbensis	
kiwi juice	

6. Research and bibliographical analysis

This work has allowed a reasoned bibliographic research aimed to analyzing knowledge and get information about the chemical-physical, functional, microbiological and nutritional properties of plant-derived coagulants, their use and specific properties on end products (cheese).

Initially, in order to carry out bibliographic research, keywords have been identified, which have been inserted into two databases (ISI Web of Knowledge and Scopus).

Bibliographic analysis was carried out considering two aspects: the form (descriptive catalogue) and the contents (semantic catalogue).

Descriptive cataloguing presents the document from a formal point of view, expressing it through concretely identifiable formal elements (bibliographic data about the author or the title on the title page) and physical examination (size, number of pages, etc.), in order to allow it to be identified. These formal elements are collected and organized according to a predetermined rule code, in order to represent the description in the most formalized way (using, for example, the ISBD standard).

Semantic cataloguing describes the document from its conceptual entity with regard to the subject that the text communicates, that is, its content of information. It is used for indexing languages, among which the most common are the subject and the bibliographic classification. Rigorous use of precise and specific terminology allows to avoid ambiguity of meanings.

A bibliographic database is an archive that collects quotations, that is bibliographic references to parts of publications (articles on magazines, essays or chapters within monographs, theses, congresses, etc.). Through bibliographic references it is then possible to go back to the original document in order to read it in its entirety.

The record is the descriptive unit of documents within the database. The information that is compiled is divided into fields (eg: Author [Article of the author] / Title [Title of article] / Source [Publication, volume, file and pages] / Publication Type [Type in the article: review, meta-analysis, etc.] In many cases, it is accompanied by an abstract, or a summary of the content of the publication that helps to understand whether the document in question is really interesting for the purpose of the research and goes therefore found in full.

Search in a database

bibliographic

• Be distinguished:

-the structure of the database

- IR software (= information retrieval, information retrieval)

Search for free words

It only detects the presence of that specific string of letters in any record field (the one specified in the sw mask).

• It can deliver numerically higher but qualitatively less relevant results (more "noise").

Search for tesauro words (subject descriptor)

PREFERENTIAL METHOD:

• Retrieves all documents that contain that descriptor among those assigned in the "Subject Headings" field, regardless of the term used by the authors to express the concept. Avoid therefore having to think about possible synonyms.

• Allows you to use the explosion to expand / narrow the search field.

• Allows you to find targeted documents even with generic titles and no abstract.

The thesaurus is a controlled and structured vocabulary whose terms, defined descriptors, uniquely indicate every concept regardless of its possible synonyms and are organized in such a way as to make explicit the semantic relations (gender / species, part / all) existing between the various terms (tree structure).

In order to indicate the topics covered within individual documents, each database uses a controlled vocabulary, or tesauro, whose words are reported within the record in the field of subject terms. Tesauro's controlled terms are used by catalogers of various databases to ensure uniformity in assigning subjects to articles that handle the same subject. Each document is indexed, as far as the content is concerned, with as many descriptors as are the topics discussed. The most important concepts are reported with an asterisk (* = FOCUS).

ubsequently, archiving took place on JabRef (a free multiplatform program that allows you to manage your own bibliographies).

import db in jabref

duplication elimination

elimination "non-attendant"

The following steps were to organize the resources into thematic groups, to select the resources useful to the set goals

Finally, the numerical and graphical processing of the resources made in the database was made, referring to:

a) Abstract and citation database of peer-reviewed literature

b) the topics (topic) of the dabase that were most relevant to the present apprenticeship;

c) the time interval investigated;

d) the type of documents

e) the year of publication of the articles

f) the country of documents(corresponding author)

g) author of documents

h) the affiliation of documents

i) the Journals of documents

l) Vegetable species cited in literature

6.1. Abstract and citation database of peer-reviewed literature: results of queries

To start the research project a bibliographic research was carried out using the databases (such as scopus and web of science). various combinations of keywords were inserted and the search results were processed according to different parameters which will be described below (Fig.2).

Document type	Article		Review		Book/ Inbook		Conference paper/ review		Other		Patent		Total docs	
Keywords	**SC**	**WoS**	**SC**	**WoS**	**SC**	**WoS**	**SC**	**WoS**	**SC**	**WoS**	**SC**	**WoS**	**SC**	**WoS**
vegetable* AND rennet	56	80	5	7	2	1	2	1	1		21	4	87	93
vegetable* AND coagulant*	87	90	6	7	2			3	2	1	80	50	177	151
"plant coagulant*"	39	30	2	3			13	3	5		3	3	62	39
plant AND protease* AND cheesemaking	8	10	1	2	1								10	12
cheesemaking AND vegetable*	9	6	2	2	1	1	2						14	9

Fig 2: Preliminary analysis of the results obtained from the bibliographic research for key words in the various databases. SC = Scopus (Elsevier); WoS = Web of Science (Thomson Reuters); Other = note, short survey, undefined

6.2. Documents by type

The bibliography analyzed (247 documents) was divided by document type. The categories that are part of this group are: articles, Book / In BOOK, Conferences, Patents (Fig 3).

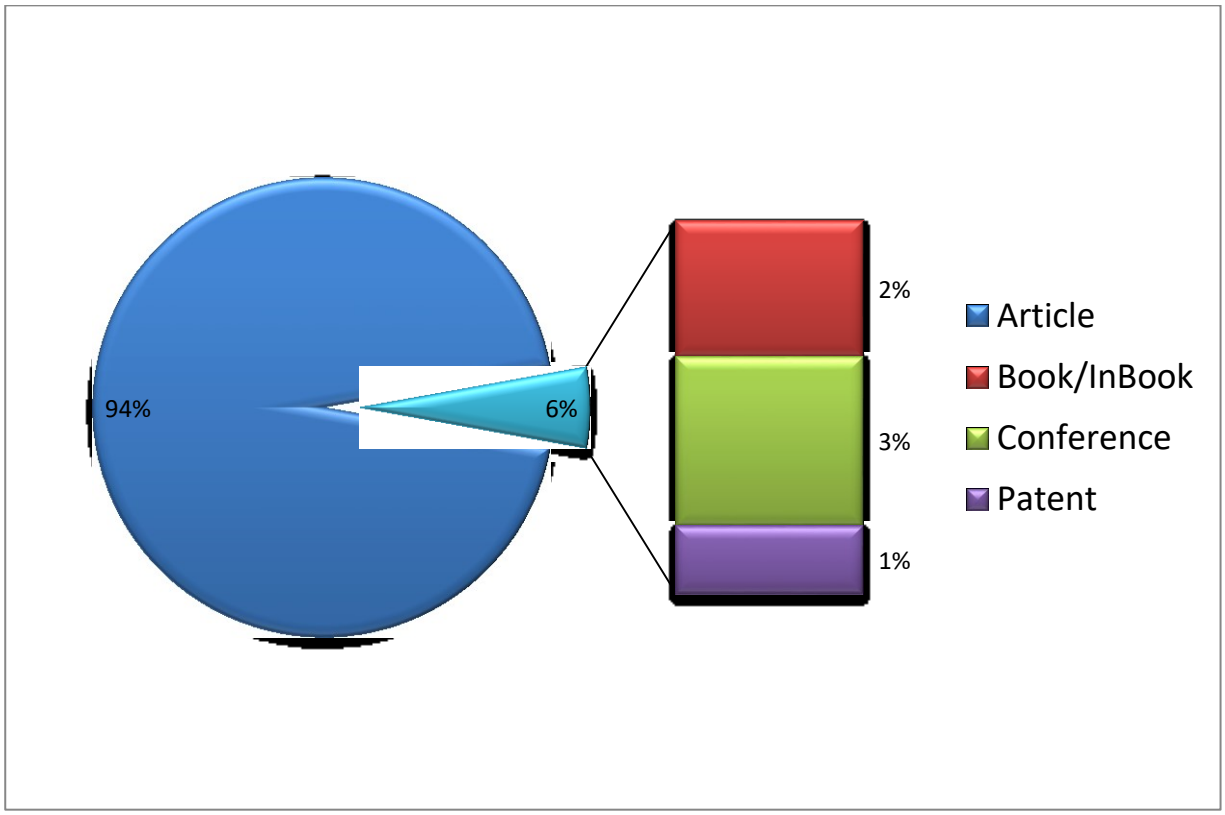

Fig 3: Documents by type-Date range: 1960 to present

6.3. Documents by year

The bibliographic sources have been cataloged according to the year of publication (Fig.4).

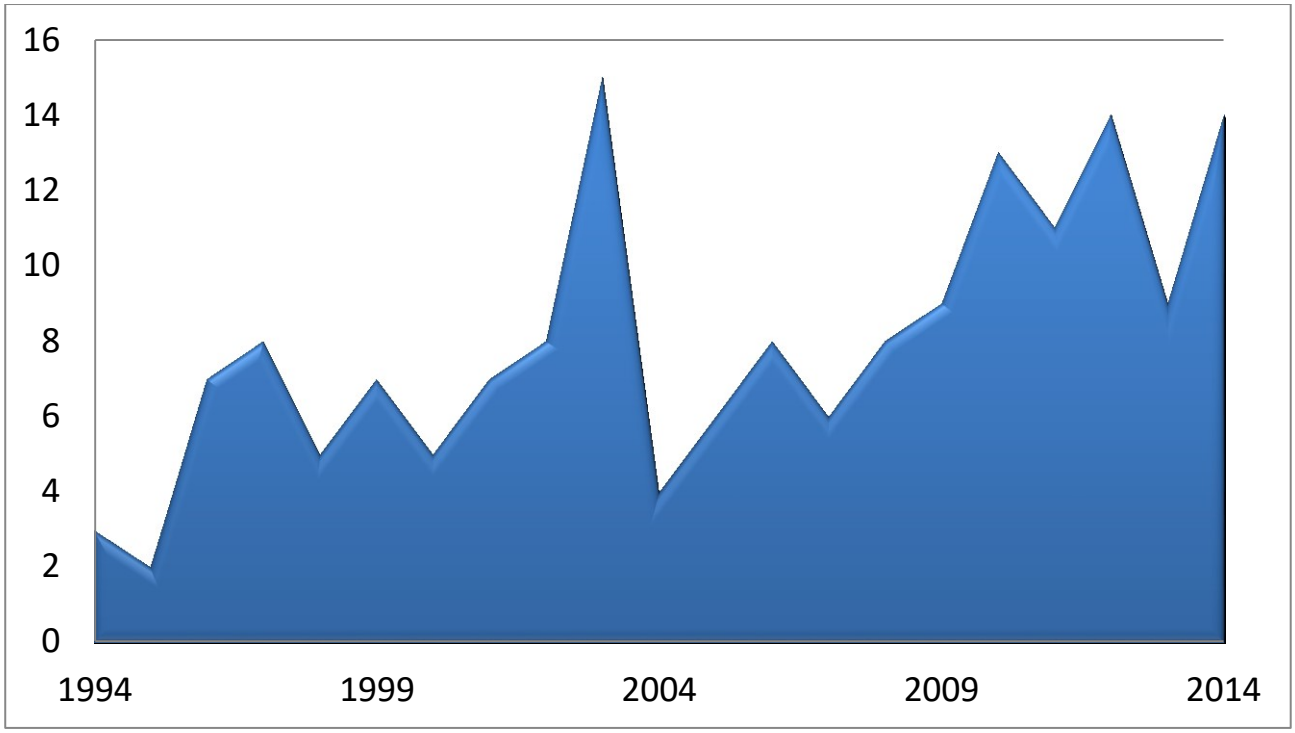

Fig4: Documents for year. Date range: 1960 2014

6.4. Documents by country (corresponding author)

All the bibliographic sources have been cataloged considering in the country where they were published and specifically considering the country of the first author (Fig.5).

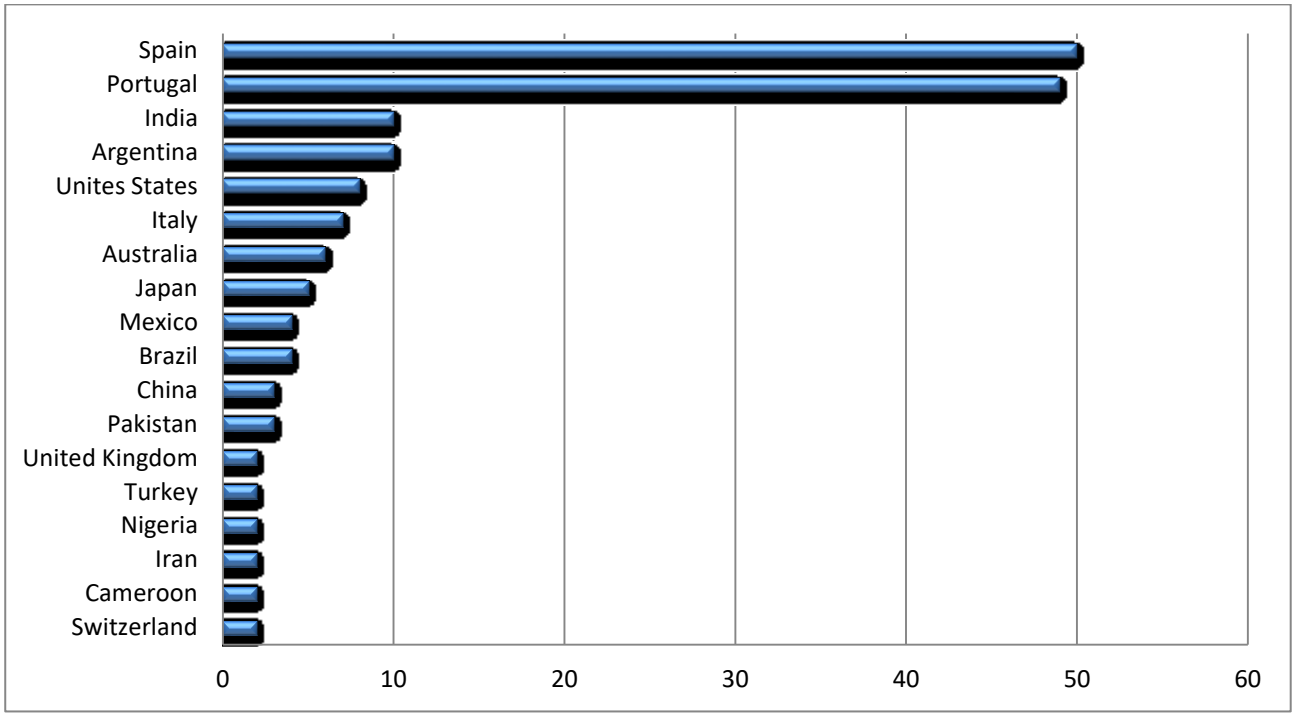

Fig5: Documents by country (other 11 citations from 1988 to 1993). Follow Other with 1 document: UK, Algeria, Cameroon, Denmark, Ireland, China, Germany, Switzerland, Tunisia.

6.5. Documents by author

All the bibliographic sources have been analyzed identifying and classifying according to the authors. they were ordered considering the number of documents produced on the topic of interest (Fig.6).

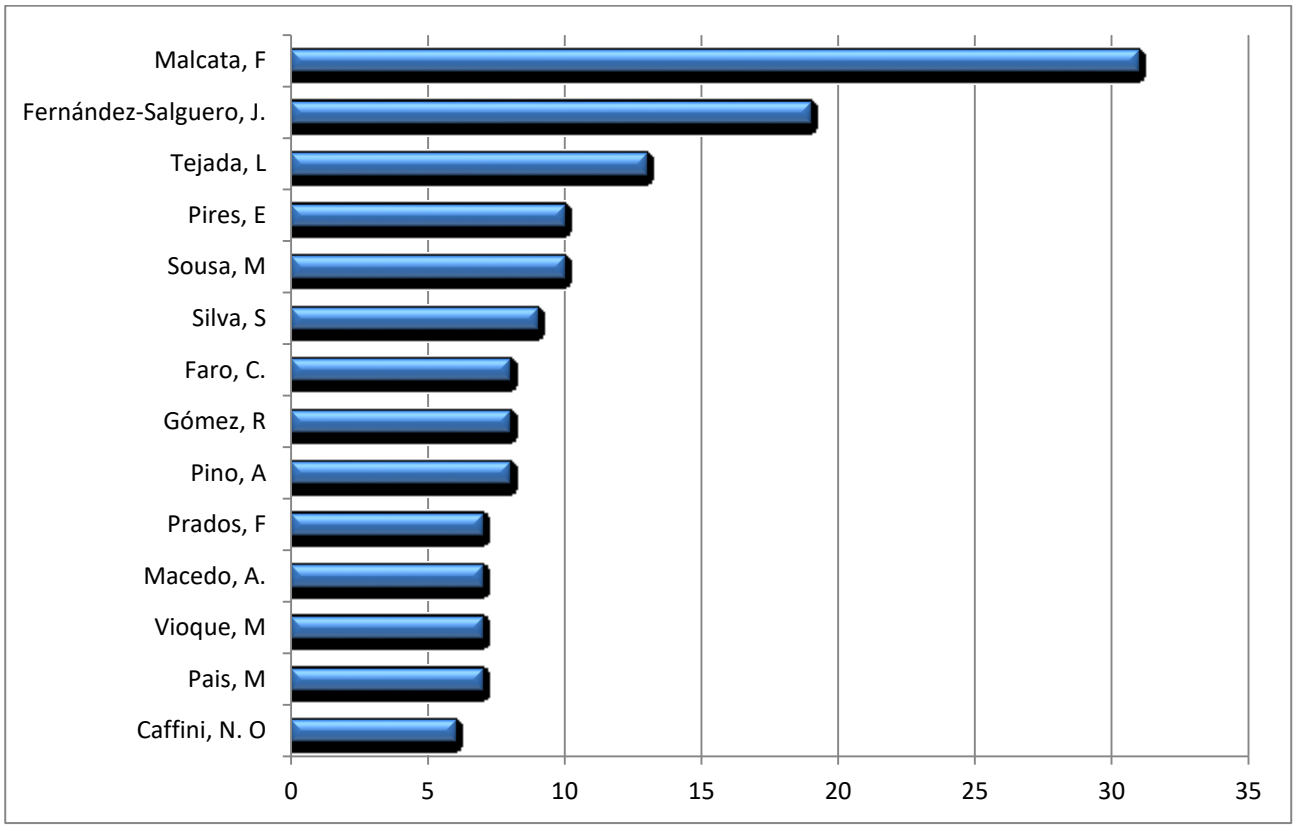

Fig 6: Authors who published the May issue of documents on the topic of interest (Malcata, F. Department of Chemical Engineering, University of Oporto, Portugal; Fernández-Salguero, J.Departamento de Bromatología y Tecnología de los Alimentos, Universidad de Córdoba, Spain; Tejada, E.Universidad Católica San Antonio de Murcia, Departamento de Tecnología de la Alimentación y Nutrición, Spain). Follow:7 authors with 4 publications 21 authors with 3 publications 22 authors with 2 publications 120 authors with 1 publication.

6.6. Documents by affiliation

The documents of the bibliography have been classified according to the affiliation in order to identify the institutions that have taken the most interest from the topic of interest (Fig.7)

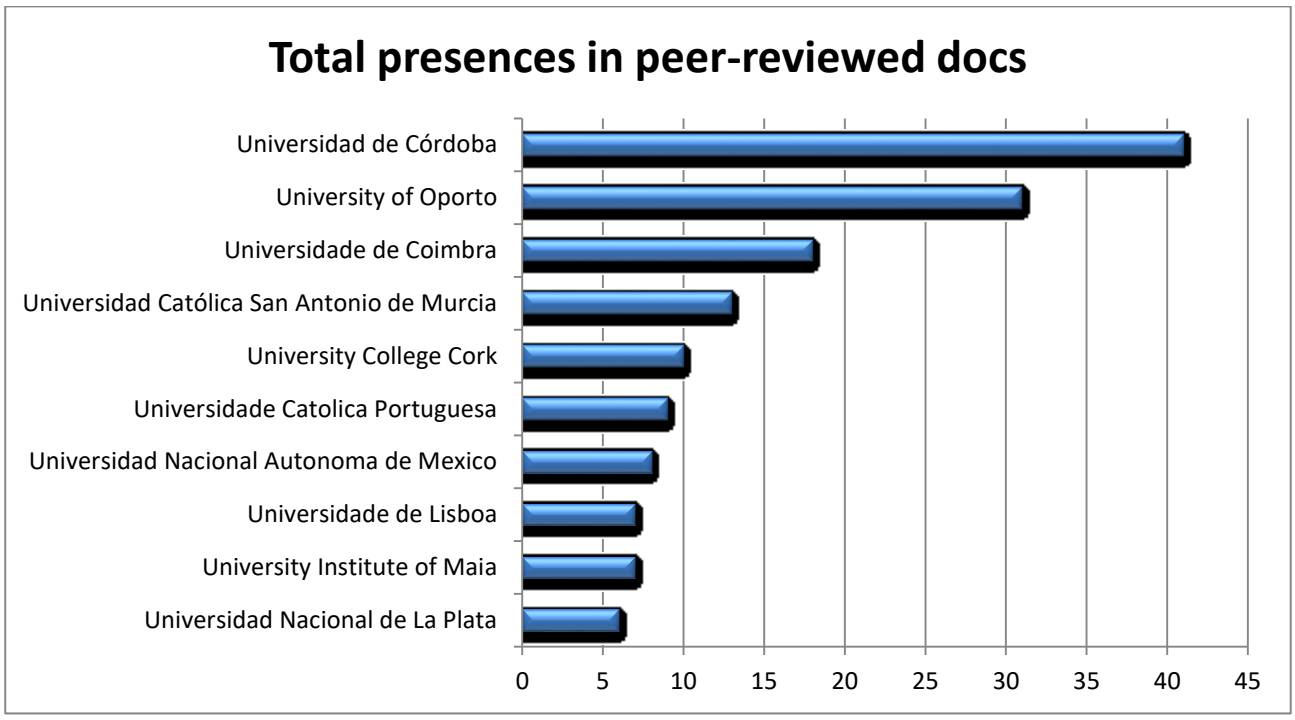

Fig 7 Documents according to the affiliation.

6.7. Journals

The documents were classified identifying the journals on which the articles were published, also indicating the value of the impact factor, the number of artiche and quartile,respectively, for each journal (Fig.8-9).

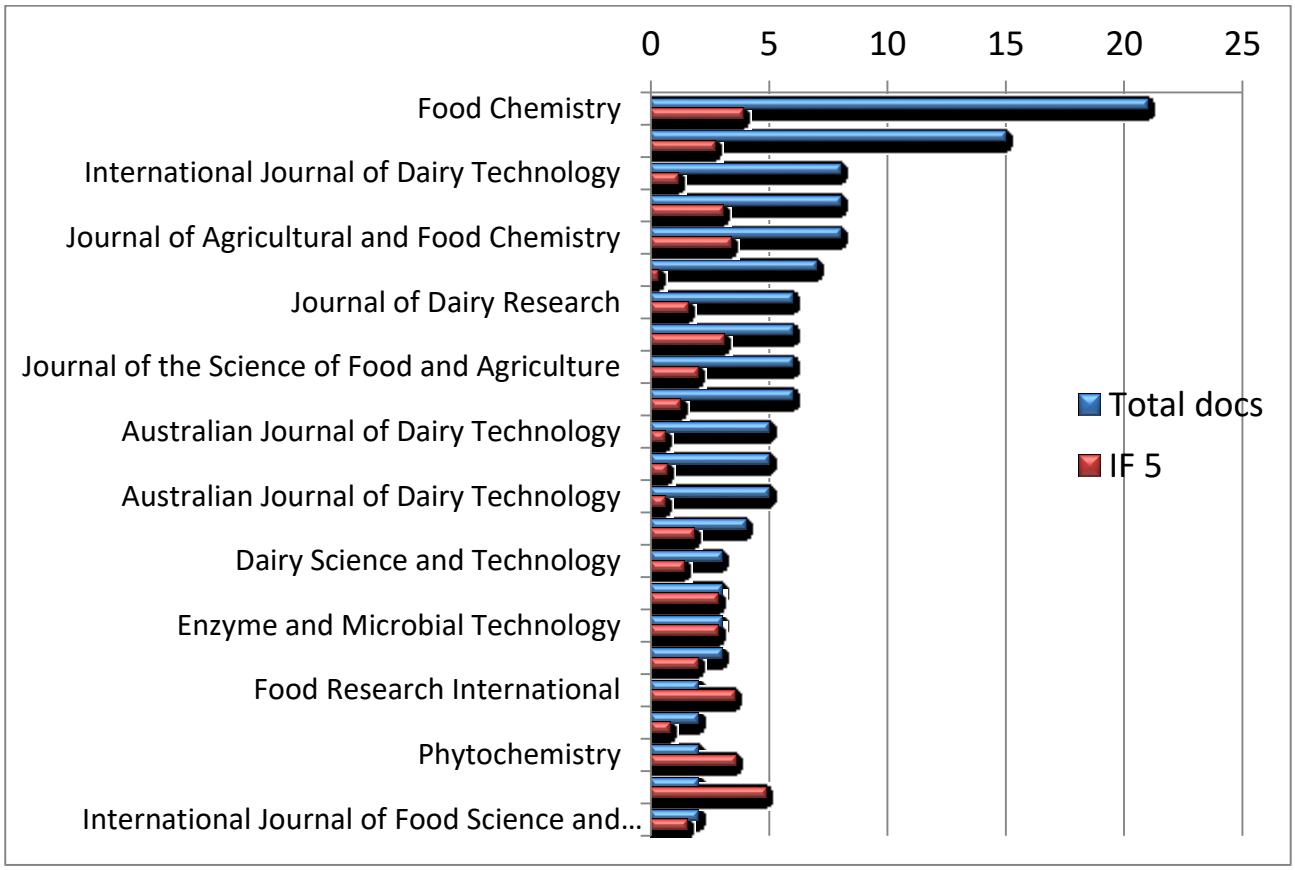

Fig 8 Publications journal and impact factor for 5 years

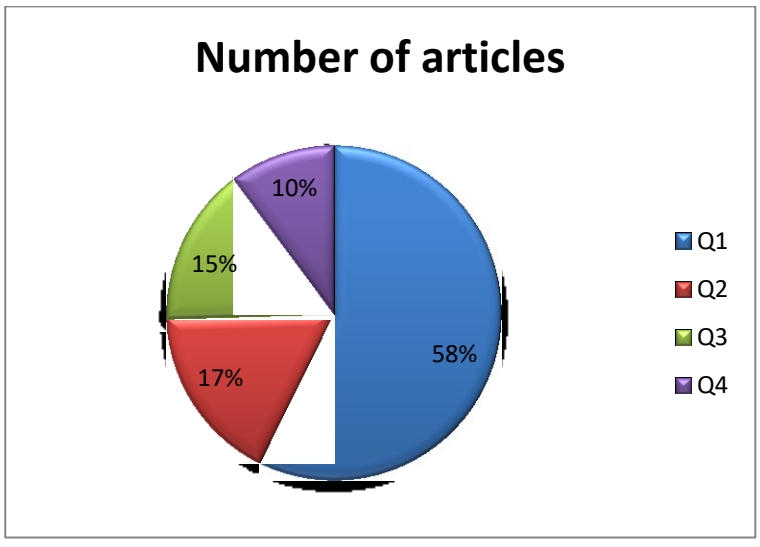

Fig 9 Quartile of the articles in journa.

6.8. Thematic groups

All the documents have been consulted and common interest groups have been identified. Each document was then assigned to each respective group (Fig.10).

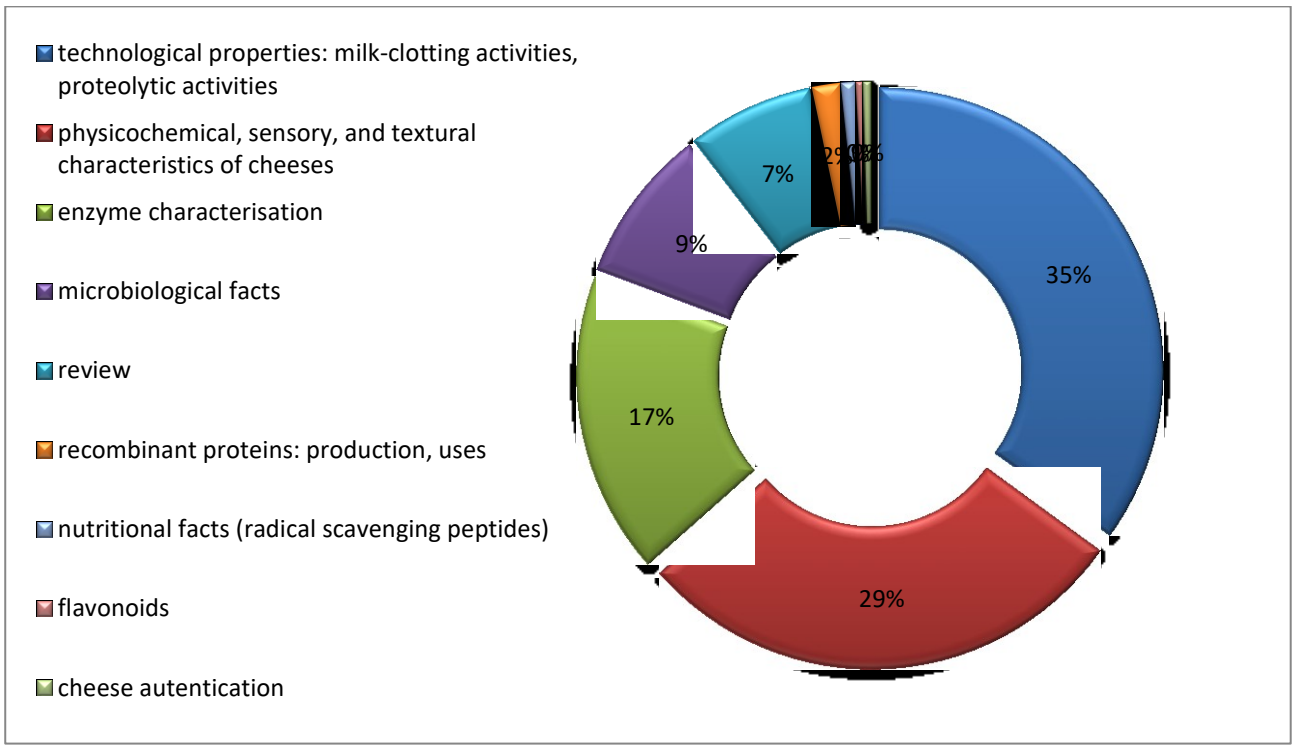

Fig 10 The topics of the dabase that were most relevant to the present apprenticeship.

6.9. Vegetable species cited in literature

The species mentioned in the literature were ordered by citation number. it is evident that the sort of election is the *Cynara Cardunculus* (Fig.11).

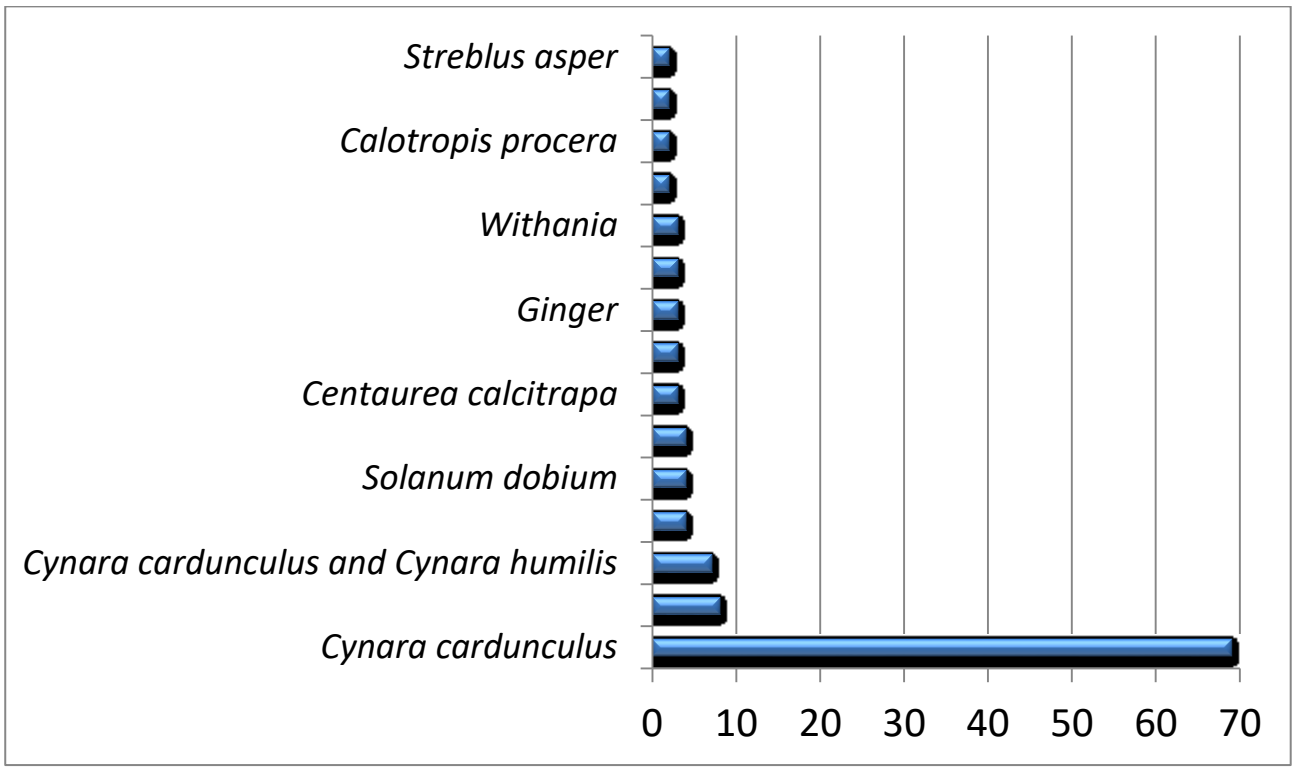

Fig 11: Species mentioned in the bibliographyanother 15 species with a citation.

7. Thematic groups of the experimental part from the bibliography

The following steps were to organize the information of bibliografy into thematic groups, to select the resources useful to the set goals referring to:

- Preparation of crude enzyme (CE)
- Purification and fractionation of crude enzyme (CE)
- Storage stability of crude extracts
- Evaluation of technological properties (crude extracts)
- Proteolytic (caseinolytic) activity (CA)
- Milk-Clotting activity (MCA)
- Milk-Clotting index
- Effect of temperature, pH, calcium ions, enzyme concentration on clotting activity
- Effect of temperature, pH, calcium ions on proteolytic activity - Optimum pH, T, calcium ions
- Enzyme stability
- Enzyme characterization
- Other studies
- Physicochemical, sensory and textural characteristics of curds/cheeses
- Manufacture of miniature cheese
- Manufacture of cheese
- Cheeses produced with vegetable rennet

7.1. Preparation of crude enzyme (CE)

Plant	Anatomic part	Homogenisation	Separation	Storing crude extracts	Ref
Calotropis gigantea	stem, leaves, flowers	5 g / 25 mL 0.05 M sodium acetate buffer (pH 5.5) - pestle and mortar	1) filtered muslin cloth 2) centrifuged 4,000 rpm for 20 min	deep freezer (-20 °C)	Anusha et al, Eur Food Res Technol 2014
	latex	1 mL / 5 mL 0.05 M sodium acetate buffer (pH 5.5) - mixing	1) filtered muslin cloth 2) centrifuged 4,000 rpm for 20 min	deep freezer (-20 °C)	
kiwi fruit (Actinidia deliciosa), ginger (Zingiber officinale)	pulp, rhizomes (slices)	1:1 (w/v) with 20 mM Na phosphate buffer pH 7.2 - kitchen blender	1) centrifuged 5,000 g for 30 min at 4 °C 2) filtered double cheesecloth	refrigeration 4 °C (used tha same day) lyophilization (powder kept - 15 °C)	Mazorra-Manzano et al, Food Chem 2013
melon (Cucumis melo)	pulp (slices)	tq kitchen blender	1) centrifuged 5,000 g for 30 min at 4 °C 2) filtered double cheesecloth	refrigeration 4 °C (used the same day) lyophilization (powder kept - 15 °C)	
sour orange (Citrus aurantium L.)	flowers	1:5 with cold buffer (20 mM Tris-HCl pH 7.2) - kitchen blender	1) filtered cheese cloth 2) centrifuged 7,500 g for 30 min at 10 °C	on ice or refrigeration 4 °C (used tha same day)	Mazorra-Manzano et al, LWT 2013
Cynara scolymus	flowers	1 g/3 mL 0,1M potassium phosphate buffer (pH 6) 1mM EDTA- pestle and mortar	1) filtered two layers of gauze 2)centrifuged 16000 g for 20 min at 4°C		Llorente et al,Food Chemistry 2014
Sideroxylon obtusifolium	latex	1g latex/100 mlL0.1M phosphate buffer (pH 6)	1) stirred 1h 24°C 2) filtered paper	Frozen -20°C	Da Silva et al Food Science And Technology 2013
Onopordum acanthium L.	Stigmas and stiles	1 g/3 mL 0.1M citric acid-sodium citrate buffer (pH 3) containing 1mM EDTA	1) stirred 30 min 2) centrifuged 5000g 20min 4°C		Brutti et al LWT 2012
Solanum elaeagnifolium var. Cavanilles	berries	macerated 0.1 M phosphate buffer (pH5) (24 h; 4 °C)	1) Centrifuged 2000 g 5min 4°C 2) filtered Whatman nr 1 paper 3) Centrifuged again under same condition	Frozen -20°C	Guetièrrez-Mèndez et al J Food Sci 2012
Cynara scolymus	roots, rhizome, leaves, midribs, receptacles, stems, flowers	1 g/3 mL potassium phosphate buffer 0.1 M (pH 6) cold - Electric mixer	1) filtered 2) centrifuged 16000 g per 20 min 4°C 3) filtered Whatman N. 1	Kept -20°C	Llorente et al, J Agric Food Chem 2004
Actinidia chinensis	fruit	1 : 1.25 buffer (200 mM mannitol; 70 mM sucrose; 20 mM Hepes-KOH pH 7.5; 10 mM cysteine) - homogenizer	1) filtered 2) centrifuged 24,400 g 20 min 4°C		Lo Piero et al, Eur Food Res Technol, 2011
Cynara scolymus	dry stigmas	100 g + 800 mL citrate buffer 50 mM pH 3.0 containing 1 M NaCl - food mixer	1) filtered muslin 2) centrifugation 24,000 rpm 20 min 3) filtered Whatman N. 4 4) UF cut off 10,000 5) dialyzed		Sidrach et al, Phytochemistry 2005

Cynara cardunculs	-flower opening; only some styles; stigmas -flowers fully open -flowers begin to dry	**Dried flowers** extract Macerating 0,25 g + 5 mL water	1)Room T° 4h 2)Homogenate was filtered Whatman N. 4	Kept cool dry	Ordiales et al Food Chemistry 2012
Moringa oleifera	Dried flower	50 g + 500 mL NaCl 0,15 M Ammonium sulphate 60% saturation	1)homogenized in magnetic stirrer 4 h 4 °C 2)filtration gauze 3)centrifugation 9000g 15 min 4°C 4)treated with ammonium sulphate 5)precipitate protein fraction collect by centrifugation 6)60% supernatant fraction dialysed (10 mL;3,5KDa cut-of membrane) 7) distilled water 4h and NaCl 2h (using a volume of 2 L for dialysis fluid)		Pontual et al Food Chemistry 2012
Cynara cardunculus	Dry flower	70 g E + 1L water mortar Extract divided into three parts	1)Room T° 24h 2)filtered Lyophilised (prior freezing at between -25°C and -35°C) liophilisation at 4-13 Pa for 24-36 h stored in sealed room T° away from the light second part of aqueous extract was placed in eppendorf kept 4°C the remaining placed in eppendorf kept -30°C	room T° kept 4°C kept -30°C	Tejada et al Journal of the science of Food and Agricolture 2008
Cynara scolymus	Dried flower	15 g E + 50 mL sodium citrate buffer 50mM pH 3	1) homogenizad 2) centrifugation 50000g 30 min **Purified three cynarese fraction (A,B,C)**		Chazarra et al International Dairy Journal 2007
Cynara cardunculus	Dried flower	Cardosins extracted from dried flower Lyophilized enz reconstituted in 10 mM citrate buffer (pH 6,2) or 10 mM citrate buffer (pH 5) conteining 20 g/L NaCl	Extraction Gel filtration Ion-exchange chromatography 10°C 20 h		Silva et al Food Chemistry 2005

Lactuca sativa	Fresh lettuce	Standard buffer 220 mM mannitol , 70 mM sucrose, 1 mM EGTA, 10 mM cysteine, 5 mM HEPES-KOH pH 7,5 solid (NH4)2SO4 55%saturation	Washed with bidistlled water Homogenized with standard buffer ratio 1:1,25 w/v Filtered Centrifuged 27200 g 30 min Again centrifuged 150000g 60 min Precipitated with (NH4)2SO4 The pellet resuspended in standard buffer Applied to a Sephacryl-S-300		Lo Piero et al Agricultural and Food Chemistry 2002
Cynara cardunculus	Dry flowers	1g/10mL buffer 0,1 M aqueous citric acid pH 3.0 Ammonium sulfate 30 % (w/v) saturation	1 g 10 mL buffer Macerated Added ammonium sulfate After 30 min Centrifuged 10000 rpm 10 min 4 °C Redissolved water (twice volume pellet) Added ammonium sulfate 70 % (w/v) saturation After 30 min Centrifuged 10000 rpm 4°C Redissolved water (twice volume pellet) Dialyzed o/n 4°C Lyophilized		Silva et al Journal of Food Science 2002
Cynara cardunculus	Dried flower	5 g E + 50 mL buffer 0,1M citrate buffer pH 5,4	Centrifuged 6000 g 5 min One part of these extract was used promptly Another part kept 4°C Remaining part lyophilized and reconstituted in water or citrate buffer	Keep cool Keep 4°C	Tavaria et al Food Chemistry 2001
Cynara cardunculus	Fresh flower	Styles 0,2 g 0,1 mL 0,1 M citric acid pH 3 Mortar and pestle Homogenized	Centrifuged 12000 g 10 min		Verissimo et al Biotechnology Letters 1995
Streblus asper	latex	Collected in 0,01 M tris-HCl buffer pH 8	Centrifuged 20000g 40 min	Frozen -20 °C 24h	Tripathi et al Food Chemistry 2011

7.2. Purification and fractionation of crude enzyme (CE)

Plant	Anatomic part		Ref
Actinidia chinensis	fruit	- pH CE adjusted to 4.4 - centrifuged 150,000 g per 60 min 4 °C - ion exchange chromatography (DEAE-Sepharose CL 6B) - active proteolytic fraction were combined and precipitated with ammonium sulphate (70% saturation) - centrifugation 24,400 g per 30 min 4 °C - pellet redissolved buffer (25 mM sodium acetate ph 4.4; 5 mM DTT; 1 mM EDTA) - dialyzed against 4 L buffer	Lo Piero et al, Eur Food Res Technol, 2011
Cynara scolymus	Flowers	-extract loaded into HiTrap Desalting column (Sephadex G25) - depigmented extract recovered and then loaded onto HiTrap Q column (anion exchange)	Llorente et al,Food Chemistry 2014
Onopordum acanthium L.	Stigmas and stiles	- SEC (Sephadex G25)	Brutti et al LWT 2012
Solanum elaeagnifolium var. Cavanilles	berries	- salting-out ammonium sulfate 20 % w/v saturation - supernatant salting-out ammonium sulfate 40 % w/v saturation - new supernatant salting-out ammonium sulfate 60 % w/v saturation	Guetièrrez-Mèndez et al J Food Sci 2012
Cynara scolymus	dry stigmas	- ion exchange chromatography (Q-Sepharose fast Flow) - fractions showing proteinase activity were pooled, dialyzed and lyophilized	Sidrach et al, Phytochemistry 2005
Cynara scolymus	flowers	- decoloration with 2.5, 5 and 10% w/v activated charcoal on ice for 30 min - anion exchange chromatography (DEAE-Sepharose Fast Flow) - active proteolytic fraction were pooled and loaded onto a column pepstatin-agarose (affinity chromatography) - washing and eluition of AP	Lorente et al, J Agric Food Chem 2004
Cynara cardunculs	**Dried flower** -flower opening; only some styles; stigmas -flowers fully open -flowers begin to dry	Pre-extraction: 1g cardoon + methanol (3:10 W/V) for 5 min room T° Suspension vortex periodically Centrifuged 5800g 5 min From an aliquot of 0,5 mL with chloroform(1:2 v/v) Centrifuged 24000g 5 min Pellet cleansed twice chloroform and pigment free pellets were collect and suspended in 100 µL of 30 % (v/v) acetonitrile -stigmas (100g) homogenized in food mixer in 800 mL aqueus citrate buffer 50 mM (pH 3) containing 1M NaCl Filter through muslin Centrifugation 24000rpm 20 min Filtered Whatman N°4 **(crude extract)** Ultrafiltration against 25mM tris-HCl buffer pH 7,6 Loaded in Q-Sepharose Fast Flow column + FPLC system 214 nm The enzyme were displaced with step gradients of 0,30-0,35-0,5 M NaCl in the previous buffer	Ordiales et al Food Chemistry 2012
Cynara cardunculus	Fresh flower	Supernat 0,2 mL applied to Hi-Load Superdex 200 column Active fraction was applied to Mono Q HR 5/5 column	Verissimo et al Biotechnology Letters 1995
Streblus asper	latex	Supernat was subjected to anion-exchange chromatography on a DEAE-Sepharose fast flow column Fractions of 2,5-3 mL were collected	Tripathi et al Food Chemistry 2011

7.3. Storage stability of crude extracts

Plant	Anatomic part	Storage method	Parameter assayed	Frequency	Ref
Calotropis gigantea	stem, leaves, flowers, latex	deep freezer (-20 °C)	milk-clotting activity	every third day	Anusha et al, Eur Food Res Technol 2014

7.4. Evaluation of technological properties (crude extracts)

7.4.1. Proteolytic (caseinolytic) activity (CA)

Plant	Anatomic part	Substrate	Incubation	Measure	1 Unit of protease activity =	Ref
Calotropis gigantea	stem, leaves, flowers, latex	1% w/v casein in 0.05 M citrate phosphate buffer pH 7.5	- 1 mL CE appropriately diluted / 1 mL substrate for 1 h at 37 °C - reaction stopping: 3 mL cold 10 % TCA; stand for 1 h at 2 °C	Abs 280 nm of clear supernatant	One unit of the protease activity was defined as the amount of the enzyme that liberated 1 µg of tyrosine under standard assay conditions	Anusha et al, Eur Food Res Technol 2014
kiwi fruit (Actinidia deliciosa), ginger (Zingiber officinale), melon (Cucumis melo)	pulp, rhizomes (slices)	- 1 % BSA in in 0.1 M phosphate buffer pH 7.0 - 1 % casein in in 0.1 M phosphate buffer pH 7.0	- 450 µL substrate + 50 µL CE for 60 min at 40 °C (kiwi), 50 °C (melon), 60 °C (ginger) - reaction stopping: 500 µL of 5% w/v TCA; stand on ice for 30 min	- 100 µL clear supernatant + 200 µL 0.2 N NaOH + 100 µL Folin-Ciocalteu/water 1:2 - color developing at 35 °C for 15 min - measuring Abs 660 nm	One unit of enzyme activity (U) was defined as the amount of protein that gave rise to an increase in the absorbance by one unit at 660 nm under the described conditions.	Mazorra-Manzano et al, Food Chem 2013
sour orange (Citrus aurantium L.)	flowers	- 1 % BSA in in 0.1 M phosphate buffer pH 7.0 - 1 % casein in in 0.1 M phosphate buffer pH 7.0	- 450 µL substrate + 50 µL CE for 60 min at 50 °C - reaction stopping: 500 µL of 5% w/v TCA; stand on ice for 30 min	Abs 280 nm of clear supernatant	amount of E that give rise to an increase in Abs by 1 unit under assay conditions One unit of enzyme activity (U) was defined as the amount of protein that increased the absorbance by one unit at 280 nm under the described conditions	Mazorra-Manzano et al, LWT 2013

Sideroxylon obtusifolium	latex	-azocasein (1% tris-HCl buffer)	100 µL substrate+ 60 µL CE 1h Room T° -reaction stopping:480 µL TCA 10% w/v -centrifugate 8000g 5min 4°C - 320 µL supernatant added to 560 µL NaOH 1 M	Abs 440 nm	Change optical density of 0.01 Abs unit/min	Da Silva et al Food Science And Technology 2014
Cynara scolymus	Different organ of globe artichoke	- 1%casein (w/v) in 0,1M potassium phosphate buffer (pH 6) -	- 0.1 mL CE + 1.1 mL substrate, 37 °C for 30 min - stopping: 1.8mL TCA 5% w/v - Centrifuged 4000 g 20 min	Abs 280 nm	amount of E that give rise to an increase in Abs by 1 unit per minute	Llorente et al, J Agric Food Chem 2004
		- haemoglobin Hemoglobinolytic activity		Abs 280 nm		
		- azocasein in 0.1 M Tris-HCl buffer pH 6.0		Abs 440 nm		
Actinidia chinensis	fruit	- total casein, α-casein, β-casein, κ-casein 2% w/v in 67 mM NaH_2PO_4 pH 7.2 and 2.5 mM DTT - pasteurized whole and semi-skimmed milk	- 40 µg E + milk (vol corresp. 20 mg proteins) or 1 mL caseins incubated 55 °C for 20 min - reaction stopping: 1.5 mL 5% TCA - centrifugation 9,000 g per 10 min	Abs 280 nm of clear supernatant	amount of enzyme that yields a 0.001 Abs change per min	Lo Piero et al, Eur Food Res Technol, 2011
Onopordum acanthium L.	Stigmas and stiles	- denatured haemoglobin 20 g/L pH 4.0	- 0.1 mL E + 0.5 mL substrate, 37 °C - stopping: 1 mL TCA 50 g/L	Abs 280 nm of clear supernatant	amount of enzyme that produces an increase of 1.0 Abs unit per minute in the assay conditions	Brutti et al LWT 2012
		- casein 1% in 0.1 M phosphate buffer pH 6.5	- 1.1 mL substrate + 0.1 mL E, 37 °C - stopping: 1.8 mL TCA 50 g/L	Abs 280 nm of clear supernatant	amount of enzyme that produces an increase of 1.0 Abs unit per minute in the assay conditions	
Cynara scolymus	dry stigmas	1% azocasein in 5 mM phosphate buffer pH 7.2	- 1 mL substrate + 0.1 mL E at 35.5 °C per 15 min - stopping: 2 mL 5% TCA	Abs 345 nm of clear supernatant		Sidrach et al, Phytochemistry 2005
		syntethic peptide [H-Pro-Thr-Glu-Phe-p-(NO_2)-Phe-Arg-Leu-OH] 0.2 mM in 50 mM sodium acetate buffer pH 5.0	- substrate + E at RT	Abs 310 nm	amount of E that hydrolysed 1 mol substrate/min at RT	
Cirsium vulgare	flower buds	(MCA)Lys-Lys-Pro-Ala-Glu-Phe-Phe-Ala-Leu-Lys(DNP)	recombinant E: substrate 1:10 in 50 mM Na acetate buffer pH 4.0 + NaCl 0.1 M, overnight at 37 °C	RP-HPLC C18, monitoring Abs 220 nm		Lufrano et al, Phytochemistry 2012

		(MCA)Lys-Lys-Pro-Ala-Glu-Phe-Phe-Ala-Leu-Lys(DNP) 2 µM in 50 mM sodium acetate buffer + 0.1 M NaCl (MCA)Lys-Leu-His-Pro-Glu-Val-Leu-Glu-Lys(DNP) 2 µM (MCA)Lys-Lys-Leu-Ala-Asp-Val-Val-Asn-Ala-Leu-Glu-Lys-Lys(DNP) 2 µM (MCA)Lys-Ser-Glu-Val-Asn-Leu-Asp-Ala-Glu-Phe-Lys(DNP) 2 µM in 50 mM sodium acetate buffer + 0.1 M NaCl + 8% v/v Me_2SO Agr-Glu(EDANS)-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-Lys(DABCYL)-Arg 2 µM Agr-Glu(EDANS)-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Thr-Lys(DABCYL)-Arg 2 µM in 50 mM sodium acetate buffer 0.1 M NaCl + 9.6% v/v Me_2SO	pH 4.0 37 °C	Fluorescence intensity		
		oxidized insulin β-chain 1 mg/mL	recombinant E/ substrate 1:100 in 0.1 mM sodium acetate buffer pH 4.0, overnight 37 °C	RP-HPLC C18, Abs 220 monitored; fraction collected e submitted QTrap 4000 (linear ion trap)		
Cynara cardunculs	-flower opening; only some styles; stigmas -flowers fully open -flowers begin to dry	Mix bovine casein (α,βand k)0,5mg /mL dissolved 5mL distilled water. SDS-PAGE loading buffer (62,5mM Tris-HCl pH 6,8 20%(w/v) glycerol, 2%(w/v)SDS,5%(w/v) β-mercaptoethanol, 0,025 %(w/v) broophenol blue)	1)S+mix 2,5% extract (v/v) 2 h room T° 2) denatured adding 30 µL SDS-PAGE loading buffer 3) incubate 99°C 5 min	densitometric Electrophoresis gel analysis		Ordiales et al Food Chemistry 2012
Moringa oleifera	Dried flower	0,1M sodium phosphate pH7,5 containing 0,6%(w/v) azocasein 0,1%(v/v) Triton X-100 Trichloroacetic acid 10%(w/v) Bovine αs-β-k casein solution Sodium phosphate buffer 0,1M pH 6,5 Flower extract	1)E(100µL, 3mg of protein)+PP(100µL, 3,2mg protein) or 60% supernatant (100µL, 3mg protein) was mixed 300µL sodium phosphate 2)incubate 37°C 3h 3)stopping 200µL trichloroacetic acid 4)incubate 4° 30 min 5)centrifuged 9000g	Abs 366 nm SDS-PAGE sodium dodecyl sulphate	Amount of enzyme that promoted a 0,01 increase in abs	Pontual et al Food Chemistry 2012

		(100µL, 3mg of protein) PP(100µL, 3,2mg protein) supernatant (100µL, 3mg protein)	10 min PP (50µL,1,7mg protein) in sodium phosphate buffer 37°C Aliquots of 10 and 900 µL from the reaction mixtures were retrieved within 10,30,60,120 min and 24h of incubation Aliquots 10 µL heated 100°C 5 min (SDS-PAGE) Aliquots 900µL evaluated abs after addition trichloroacetic acid (200µL) Centrifugation 9000g 10 min 4°C			
Cynara scolymus	Dried flower	Synthetic peptide H-Pro-Thr-Glu-Phe-p-(NO2)-Phe-Arg-Leu-OH (PNPE) 50 mM sodium acetate buffer pH 5	0,2mM S in sodium acetate buffer Incubate room T°	Abs 310 nm	Amount of enzyme that hidrilyzed 1 mol/min of PNPE at 25 °C under above condition	Chazarra et al International Dairy Journal 2007
		Commercial bovine αs-β-k casein Sodium acetate buffer 100 mM pH 6,2 Sample loading buffer (tris-HCl pH 6,8 20% glycerol 4% SDS 0,1% bromophenol blu 1,5 M β-mercaptoethanol	Casein dissolved in 2 mg/mL in sodium acetate buffer Incubate 30°C After 60 min stopped with equal vol sample loading buffer Heated 100°C 5 min	SDS-PAGE		
Cynara cardunculus	Dried flower	Ovine casein 1%(w/v) Citrate buffer 10 mM (pH 6,2) Sodium azide 0,03% (w/v)	Hydrolysis 30°C in citrate buffer Start addition of 120 µL E solution + 3 mL sub solution Stopping heating 100°C 5min Evaluating peptides soluble in aqueous 5%(w/v) TCA at volumetric ratio 1:2 Settle for 10 min Centrifuged 12000 g 10 min	Abs 280		Silva et al Food Chemistry 2005
Lactuca sativa	Fresh lettuce	Casein-α-β-k Milk at different fat contents	37°C Ref: Kaneda, M. et al 1994 Except after TCA precipitation of the unhydrolyzed substrate, the surnatant was centrifugation 10000rpm		Amount of enzyme thet yielded a 0,001 absorbance change for min	Lo Piero et al Agricultural and Food Chemistry 2002

		Synthetic peptides N-Cbz-GGR-βNA, N-Cbz-LLE-βNA, N-succinil_GGF-pNa, N-Cbz-VKM-MCA, N-succinyl-AAA-pNa, N-Cbz-GGL-pNa, N-succinyl-GGG-pNa, N-succinyl-IIW-MCA, N-succinyl-YLV-pNa tris-HCl 100mM pH 8 TCA 20 % (w/v)	0,25mL mixture + 100mM tris-HCl + 0,4 mM synthetic peptide 37°C Reaction stopped 0,25 mL TCA Centrifuged 10000 rpm 5 min Released pNa,βa oe MCA			
		Total casein NaH2PO4 pH 6,8	α- β-k casein dissolved in 67 mM NaH2PO4 final volume 1,5 mg/mL + 30 pmol (Vf= 0,3 mL) incubation 37 °C 3h sample obtain as describe above were prepared SDS-PAGE by adding an equal volume loading buffer			
Cynara cardunculus	Dry flowers	0,1 M aqueous sodium phosphate buffer pH 6 Azocasein 2% w/v Cold 5% trichloroacetic acid 0,5 M aqueous NaOH	E + buffer + 250 µL azocasein Incubate 25°C 10 min Reaction stop added 0,5 mL cold TCA Centrifuged 10000 rpm 10 min 1 mL supernat + 1mL aqueous NaOH	Abs 440 nm		Silva et al Journal of Food Science 2002
		Commercial bovine αs-βcasein Phosphate buffer pH 6,5 containing sodium azide Ammonium sulfate 30% w/v and 70% w/v saturation	1 mg/mL + buffer + 0,05g/mL sodium azide Treated ammonium sulfate Incubated 30°C Ratio protein:substrate 1:400 (w/w) Predeterminate interval (0,30 min 1-2-5-8-12-24 h) added for quenched reaction double concentrated buffer 50% v/v in the case urea-PAGE Or heating 95°C 15 min FPLC			
Cynara cardunculus	Dried flowers	**Overall** Ovine and caprine Na-caseinate 200mM phosphate buffer pH 6,5 5% trichloroacetic acid	Na-caseinate + buffer Final conc 100g/L Warmed 30°C Reaction start add E solution 526 µL for 10 mL casein Aliquots 1 mL were taken at 0-20-60-120-240-360…min and add TCA 2 mL Resting 10 min	Abs 280		Tavaria et al Food Chemistry 2001

			Centrifuged 12000g			
		Specific Ovine and caprine Na-caseinate 200mM phosphate buffer pH 6,5	Na-caseinate + buffer Final conc 100g/L Warmed 30°C Reaction start add E solution 526 µL for 10 mL casein Aliquots 750 µL were taken at 6 h Mixed with an equal volume of double concentrated buffer Vortexed 30 s prior UREA SDS-PAGE Quantification of intact βcasein and αs casein was done by densitometry	UREA SDS-PAGE Densitometer		
Cynara cardunculus	Fresh flower	Synthetic peptide Leu-Ser-Phe(NO2)-Nle-Ala-Leu-oMe 50 mM sodium acetate pH 4,7 0,2 M NaCl, 4% DMSO	E preparations + S Incubate 37°C with sodium acetate, NaCl, DMSO Rate of hydrolysis of the bond Phe(NO2)-Nle for different S concentration	Abs 310 nm HPLC		Verissimo et al Biotechnology Letters 1995
Streblus asper	latex	Denatured natural substrates such as casein and azoalbumin 15µg tot volume of 5 ml 0,05 M tris-HCl buffer pH 7,5 Equal vol 1% casein solution w/v Final vol 1 mL TCA 10% Azoalbumin (0,6 % w/v) Haemoglobin 1% w/v determined in the same manner of casein	15µg E + o,5 mL buffer Incubate 37°C 10 min Add casein solution Incubate 37°C 30 min Reaction stop 0,5 mL TCA Stand for 10 min room T°C Centrifugation 10 min (tabletop centrifuge) Azoalbumin + 0,5 mL supernat (after precipitation TCA) 0,5 M NaOH equal volume Incubate 15 min	Abs 280 nm for TCA-soluble peptide Abs 440 nm	One unit of enzyme activity was defined as the amount of enzyme, under given assay condition that gave rise to an increase of 1 unit of absorbance at 280 0r 440 nm per minute of digestion	Tripathi et al Food Chemistry 2011

7.4.2. Milk-Clotting activity (MCA)

Note: specific MCA = MC Units per mg of protein

Plant	Anatomic part	Substrate	Incubation	Measure	1 Unit of milk-clotting activity =	Ref
Calotropis gigantea	stem, leaves, flowers, latex	0.25 g skim milk powder / 0.75 mL of 0.05 M Na acetate buffer pH 5.5	- equilibration substrate at 35 °C for 10 min - addition 1 mL CE	time for the milk to clot (within 40 min)	vol of milk that can be clotted by one unit vol of CE in 40 min at the T evaluated	Anusha et al, Eur Food Res Technol 2014
kiwi fruit (Actinidia deliciosa), ginger (Zingiber officinale), melon (Cucumis melo)	pulp, rhizomes (slices)	10 mL low fat (1%) pasteurized milk containing 0.02% CaCl2	- equilibration substrate at desired T - addition 1 mL CE	time for the milk to clot (within 60 min)	vol of milk that can be clotted by one unit vol of CE in 40 min at the T evaluated	Mazorra-Manzano et al, Food Chem 2013
sour orange (Citrus aurantium L.)	flowers	10 mL low fat (1%) pasteurized milk containing 0.02% CaCl2	- equilibration substrate at desired T - addition 1 mL CE	time for the milk to clot (within 60 min)	vol of milk that can be clotted by one unit vol of CE in 40 min at the T evaluated	Mazorra-Manzano et al, LWT 2013
Cynara scolymus	flowers	1mL skim milk recostituited 12% (w/v) 10 mM $CaCl_2$, 0,02%(w/v) sodium azide	- equilibration substrate at desired T - addition 100 µL CE	time for the milk to clot		Llorente et al, Food Chemistry 2014
Sideroxylon obtusifolium	latex	1 mL skim milk powder 12% (w/v) 10 µM $CaCl_2$	-preheated for 10 min 37°C -100 µL CE added	Time first milk clots appared	One milk coagulating unit for mL (U/mL) is definite 400 t^{-1}	Da Silva et al Food Science And Technology 2014
Onopordum acanthium L.	Stigmas and stiles	12 g skim milk reconstituted 100 mL of 10 mmol/L $CaCl_2$ (pH 6,5)	- 1mL milk 30°C + 100 µL CE	Time to clot	amount E that coagulates 10 mL milk at 30°C in 100 s	Brutti et al LWT 2012
Solanum elaeagnifolium var. Cavanilles	berries	pasteurized cow milk 20 mL + 8 µL $CaCl_2$ 6.6M	20 mL substrate + 0.5 mL CE or 0.5-1 mL PFs; 32°C	Changes viscosity (Rheometer)	amount E that coagulates 1 mL substrate in 40 min	Gueltièrrez-Mèndez et al J Food Sci 2012
Cynara scolymus	Different organ of globe artichoke	cow skim milk powder 10% (w/v) in 10 mM $CaCl_2$ (pH 6)	3 mL substrate + 500µL CE; Incubate at 37°C	Time initial appearance solid material	amount of E required to clot 1mL of substrate in 40 min	Llorente et al, J Agric Food Chem 2004
Actinidia chinensis	fruit	skimmed milk powder 10% in 67 mM NaH_2PO_4 pH 6.8 (+ 10 mM $CaCl_2$)	2-40 µg E + substrate (final vol 3 mL), 30 °C	time first clotting flakes appearance	amount of E to clot 10 mL of substrate in 40 min	Lo Piero et al, Eur Food Res Technol, 2011
Cirsium vulgare	flower buds	skim milk powder reconstituted 12% w/v in 10 mM $CaCl_2$, pH 6.5	incubation E/milk 1:5 v/v at 37 °C	time to curd formation		Lufrano et al, Phytochemistry 2012
Cynara cardunculs	-flower opening;only	0,5 g cardoon + 75 mL ultrapure	0,2mL E + 2 mL milk	Time clotting	Amount of E coagulate 10	Ordiales et al Food Chemistry

	some styles; stigmas -flowers fully open -flowers begin to dry	w for 1-24 h 12 g skimmed milk powder in 100 mL of 0,01 M CaCl2 pH 6,5	Incubation 30°C		mL milk at 30°C 100 s	2012
Moringa oleifera	Dried flower	10%skim milk +distilled water or 10mMCaCl2 in water pH6,5 Flower extract (0,3mL, 9 mg protein) PP (0,3 ml, 9,8 mg protein) supernatant fraction(0,3mL, 0,9 mg protein) o,3mL,	2ml milk+flower extrct ,PP or 60% supernatant fraction Incubation 37°C Milk-clotting activity was olso determined using skim milk 10% (w/v) heated at 30,50, 70°C)	Time clotting	Amount of enzyme that clots 2 ml of the substrate within 180 min	Pontual et al Food Chemistry 2012
Cynara cardunculus	Dry flower	Skim milk powder 12%(w/v) CaCl2 10 mM/L pH 6,40	Lyophilized extract diluted in distilled water to 2,18% (percentage of powdered obtained)	Coagulant units (CU)	Amount of extract required to clot 10 ml 0f milk in 100 sec	Tejada et al Journal of the science of Food and Agricolture 2008
Cynara scolymus	Dried flower	Skim milk powder + 100 mL deionized water (final protein conc. 13% w/v)	1mL E + 10 mL skim milk Incubate 10 min 35°C	Milk-clotting time		Chazarra et al International Dairy Journal 2007
Cynara cardunculus	Dried flower	Low-heat milk powder 10 mM aqueus CaCl2 (pH 6,5) final concentration 12 Kg/L	0,1 mL E + 1 mL milk	Milk-clotting time	Rennet-unit R.U. amount of protein that coagulates 10 ml of reconstituted low-heat skim milk powder at 30°C 100 s	Silva et al Food Chemistry 2005
Lactuca sativa	Fresh lettuce	E 11 nmol 10% solution in 67 mM NaH2PO4 pH8 5 mM CaCl2	E+ skim milk Vol Final 3 mL 30°C	Milk-clotting time	Rennet-unit R.U. amount of purified enzyme needed to coagulate 10 mL of skim-milk at 30 °C in 100 s	Lo Piero et al Agricultural and Food Chemistry 2002
Cynara cardunculus	Dry flowers	Low-heat skim-milk 10 mM aqueous CaCl2 ph 6,5	0,2 mL E+ 2 mL skim-milk	Time clotting	Rennet-unit R.U. amount of protein that coagulates 10 ml of reconstituted low-heat skim milk powder at 30°C 100 s	Silva et al Journal of Food Science 2002
Cynara cardunculus	Dried flowers	Low-heat bovine skim-milk powder 0,01 M CaCl2	12 g milk + 100 mL caCl2 30°C 0,1mL E+ 2	Time clotting	Rennet-unit R.U. amount of crude enzyme extract	Tavaria et al Food Chemistry 2001

		pH 6,5	mL milk		needed to coagulate 10 ml skim-milk at 30°C 100s	

7.4.3. Milk-Clotting index

Plant	Anatomic part	Definition	Ref
Calotropis gigantea	stem, leaves, flowers, latex	ratio of milk-clotting activity to caseinolytic activity	Anusha et al, Eur Food Res Technol 2014
Actinidia chinensis	fruit	ratio of milk-clotting activity to caseinolytic activity	Lo Piero et al, Eur Food Res Technol, 2011

7.5.Effect of temperature, pH, calcium ions, enzyme concentration on clotting activity

7.5.1. Temperature

Plant	Anatomic part	Substrate	Incubation	Measure	1 Unit of milk-clotting activity =	Ref
Calotropis gigantea	stem, leaves, flowers, latex	0.25 g skim milk powder / 0.75 mL of 0.05 M Na acetate buffer pH 5.5	- equilibration substrate at 30, 35, 40, 50, 60, 70 °C for 10 min - addition 1 mL CE	time for the milk to clot	vol of milk that can be clotted by one unit vol of CE within 40 min at 35 °C	Anusha et al, Eur Food Res Technol 2014
kiwi fruit (Actinidia deliciosa), ginger (Zingiber officinale), melon (Cucumis melo)	pulp, rhizomes (slices)	10 mL low fat (1%) pasteurized milk containing 0.02% CaCl2	- equilibration substrate at desired T: 35-50 °C for kiwi; 35-80 °C for melon; 40-70 °C for ginger - addition 1 mL CE	time for the milk to clot (within 60 min)	vol of milk that can be clotted by one unit vol of CE in 40 min at the T evaluated	Mazorra-Manzano et al, Food Chem 2013
sour orange (Citrus aurantium L.)	flowers	10 mL low fat (1%) pasteurized milk containing 0.02% CaCl2	- equilibration substrate at desired T: 35-80 °C - addition 1 mL CE	time for the milk to clot (within 60 min)	vol of milk that can be clotted by one unit vol of CE in 40 min at the T evaluated	Mazorra-Manzano et al, LWT 2013
Sideroxylon obtusifolium	latex	1 mL skim milk powder 12% (w/v) 10 µM $CaCl_2$	- preheated for 10 min 30-80 °C - 100 µL CE added - incubated 30-80 °C	Time first milk clots appared	One milk coagulating unit for mL (U/mL) is definite 400 t^{-1}	Da Silva et al Food Science And Technology 2014

7.5.2. pH

Plant	Anatomic part	Substrate	Incubation	Measure	1 Unit of milk-clotting activity =	Ref
Calotropis gigantea	stem, leaves, flowers, latex	0.25 g skim milk powder / 0.75 mL of 0.05 M Na acetate buffer pH 4.5, 5.0, 5.5; citrate phosphate buffer pH 6.0, 6.5; phosphate buffer pH 7.0	- equilibration substrate at 35 °C for 10 min - addition 1 mL CE	time for the milk to clot	vol of milk that can be clotted by one unit vol of CE within 40 min at 35 °C	Anusha et al, Eur Food Res Technol 2014
Cynara scolymus	Dried flower	Stigmas 15g homogenized in 50 mL different buffer 50 mM sodium citrate pH 3,4-5 50 mM sodium phosphate pH 6,5-7 50 mM tris HCl pH8	Centrifuged 50000g 30 min T= 35 °C	Clotting properties		Chazarra et al International Dairy Journal 2007

7.5.3. Calcium ions

Plant	Anatomic part	Substrate	Incubation	Measure	1 Unit of milk-clotting activity =	Ref
Calotropis gigantea	stem, leaves, flowers, latex	0.25 g skim milk powder / 0.75 mL of 0.05 M Na acetate buffer pH 5.5 + calcium chloride 5, 10, 15 mM	- equilibration substrate at 35 °C for 10 min - addition 1 mL CE	time for the milk to clot	vol of milk that can be clotted by one unit vol of CE within 40 min at 35 °C	Anusha et al, Eur Food Res Technol 2014
Sideroxylon obtusifolium	latex	1 mL skim milk powder 12% (w/v) 1, 5, 10, 15 mM $CaCl_2$	-preheated for 10 min 37°C -100 µL CE added	Time first milk clots appared	One milk coagulating unit for mL (U/mL) is definite 400 t^{-1}	Da Silva et al Food Science And Technology 2014
Cynara scolymus	Dried flower	Milk substrates CaCl2 (3-7,7-15,4-40,3-80,6 mM) Calcium lactate (3,2-6,5-8,1-12,9-16,13-25,8-32,5 mM)	pH 6,3 incubate 10 min 35°C final protein concentration 13%(w/v)	Milk-clotting capacity		Chazarra et al International Dairy Journal 2007

7.5.4. Enzyme concentration

Plant	Anatomic part	Substrate	Incubation	Measure	1 Unit of milk-clotting activity =	Ref
Onopordum acanthium L.	Stigmas and stiles	12 g skim milk reconstituted 100 mL of 10 mmol/L $CaCl_2$ (pH 6,5)	30°C 5, 10, 25, 50 : 1 (vol milk:vol E)	Time to clot	amount E that coagulates 10 mL milk at 30°C in 100 s	Brutti et al LWT 2012

7.6. Effect of temperature, pH, calcium ions on proteolytic activity - Optimum pH, T, calcium ions

7.6.1 pH

Plant	Anatomic part	Substrate	Incubation	Measure	1 Unit of protease activity =	Ref
sour orange (Citrus aurantium L.)	flowers	1 % BSAbovine serum albumin) in: - 100 mM Na citrate buffer pH 2.5, 3.0, 4.0 - 100 mM Na acetate buffer pH 4.5, 5.0, 5.5 - 100 mM Na phosphate buffer pH 6.0, 6.5 - 100 mM Tris-HCl buffer pH 7.0, 8.0 Na carbonate buffer pH 9.0, 10.0, 11.0, 12.0	- 50 µL CE + 450 µL substrate incubated at 37 °C for 60 min - reaction stopping: 500 µL of 5% w/v TCA; stand on ice for 30 min	Abs 280 nm of clear supernatant	% relative to the maximun Abs observed	Mazorra-Manzano et al, LWT 2013
Actinidia chinensis	fruit	total casein in 25 mM buffer (MES pH 5.5 and 6.0; bis-tri-propane pH 6.5; HEPES pH 7.0 and 7.5; tris pH 8.0, 8.5 and 9.0)	- 0.5 mL assay mixture (12 µg E, 2% substrate) 55 °C per 20 min - reaction stopping: 1 mL 20% TCA	Abs 280 nm of clear supernatant	amount of enzyme that yields a 0.001 Abs change per min	Lo Piero et al, Eur Food Res Technol, 2011
Onopordum acanthium L.	Stigmas and stiles	denatured haemoglobin 20 g/L 0.1 M sodium citrate/citric acid pH 2.0 - 6.0	- 0.1 mL E + 0.5 mL substrate, 37 °C - stopping: 1 mL TCA 50 g/L	Abs 280 nm of clear supernatant	amount of enzyme that yields a 1.0 Abs increase per min	Brutti et al LWT 2012
Cynara scolymus	dry stigmas	1% azocasein in in: 50 mM sodium acetate buffer (pH 3.0-5.5); 50 mM sodium phosphate buffer (pH 5.5-7.0)	- 1 mL substrate + 0.1 mL E at 35.5 °C per 15 min - stopping: 2 mL 5% TCA	Abs 345 nm of clear supernatant		Sidrach et al, Phytochemistry 2005
		syntethic peptide [H-Pro-Thr-Glu-Phe-p-(NO_2)-Phe-Arg-Leu-OH] 0.2 mM in: 50 mM sodium acetate buffer (pH 3.0-5.5); 50 mM sodium phosphate	- substrate + E at RT	Abs 310 nm	amount of E that hydrolysed 1 mol substrate/min at RT	

		buffer (pH 5.5-7.0)				
Sideroxylon obtusifolium	latex	-azocasein (1%) in: 0.1 M phosphate buffer pH 5.8-8.0; 0.1 M tris-HCl buffer pH 7.0-9.0; 0.1 M carbonate/bicarbonate buffer pH 9.5-10.7	100 µL substrate+ 60 µL CE 1h RT -reaction stopping:480 µL TCA 10% w/v -centrifugate 8000g 5min 4°C - 320 µL supernatant added to 560 µL NaOH 1 M	Abs 440 nm	Change optical density of 0.01 Abs unit/min	Da Silva et al Food Science And Technology 2014
Cirsium vulgare	flower buds	(MCA)Lys-Lys-Pro-Ala-Glu-Phe-Phe-Ala-Leu-Lys(DNP)	37 °C Buffers: 0.05 M sodium citrate (pH 2.25-2.5); 0.05 M sodium acetate buffer (pH 3.5-6.0); 0.05 M tris-HCl buffer with 0.1 M NaCl (pH 7.0)	Fluorescence intensity		Lufrano et al, Phytochemistry 2012
Cynara scolymus	Different organ of globe artichoke	- bovine haemoglobin (pH range 2.7-10) - azocasein (pH range 4-8)	Buffers: 0.1 M sodium citrate-citric acid (pH 2.7-5.5); 0.1 M potassium phosphate (pH 6-8); 0.1 M boric acid-potassium chloride-sodium hydroxide (pH 8-10)	Abs 440 nm or 280 nm (residual proteolytic activity)		Llorente et al, J Agric Food Chem 2004
Lactuca sativa	Fresh lettuce	Enzyme toward synthetic peptides N-Cbz-GGR-βNA and N-Cbz-LLE-βNa 50 mM MES pH 5.0-5.5-6.0 , 50 mM tris pH 8.0-8.5-9.0,	50 mM MES 50 mM tris (pH 5.0-9.0)	Monitoring the peptidase activity		Lo Piero et al Agricultural and Food Chemistry 2002

7.6.2. Temperature

Plant	**Anatomic part**	**Substrate**	**Incubation**	**Measure**	**1 Unit of protease activity =**	**Ref**
Actinidia chinensis	fruit	total casein in 25 mM buffer (bis-tri-propane pH 6.5; HEPES 7.5)	- 0.5 mL assay mixture (12 µg E, 2% substrate) 5-55 °C per 20 min - reaction stopping: 1 mL 20% TCA	Abs 280 nm of clear supernatant	amount of enzyme that yields a 0.001 Abs change per min	Lo Piero et al, Eur Food Res Technol, 2011
Cynara scolymus	dry stigmas	syntethic peptide [H-Pro-Thr-Glu-Phe-p-(NO_2)-Phe-Arg-Leu-OH] 0.2 mM in 50 mM sodium acetate buffer pH 5.0	- substrate + E 1.9 nM at 30-90 °C for 20 min	Abs 310 nm	amount of E that hydrolysed 1 mol substrate/min at RT	Sidrach et al, Phytochemistry 2005
Sideroxylon	latex	--azocasein	100 µL substrate+	Abs 440 nm	Change optical	Da Silva et al

obtusifolium		(1%) in tris-HCl buffer	60 µL CE 1h at 35-80 °C -reaction stopping:480 µL TCA 10% w/v -centrifugate 8000g 5min 4°C - 320 µL supernatant added to 560 µL NaOH 1 M		density of 0.01 Abs unit/min	Food Science And Technology 2014
Cirsium vulgare	flower buds	(MCA)Lys-Lys-Pro-Ala-Glu-Phe-Phe-Ala-Leu-Lys(DNP) in 0.05 M sodium acetate buffer pH 4.0 + 0.1 M NaCl	incubation T range: 10 - 65 °C	Fluorescence intensity		Lufrano et al, Phytochemistry 2012
Lactuca sativa	Fresh lettuce		T°= 5-50°C pH 6.8			Lo Piero et al Agricultural and Food Chemistry 2002

7.7. Enzyme stability

7.7.1. pH

Plant	Anatomic part	Substrate	Pre-Incubation	Measure	Results	Ref
Actinidia chinensis	fruit	total casein in 25 mM buffer (MES pH 5.5 and 6.0; bis-tri-propane pH 6.5; HEPES pH 7.0 and 7.5; tris pH 8.0, 8.5 and 9.0)	- 0.5 mL assay mixture (10 µg E) per 3 h at 45 °C	Caseinolytic activity	% relative activity	Lo Piero et al, Eur Food Res Technol, 2011
Cynara scolymus	dry stigmas	syntethic peptide [H-Pro-Thr-Glu-Phe-p-(NO_2)-Phe-Arg-Leu-OH] 0.2 mM in 50 mM sodium acetate buffer pH 5.0 pH 5.5–7.0, 50 mM sodium phosphate buffer	0.1 mL each buffer containing 0.5 mg/mL E for 150 min at RT buffers: 50 mM sodium acetate buffer (pH 3.0-5.5); 50 mM sodium phosphate buffer (pH 5.5-7.0)	Abs 310 nm (proteinase activity)	% relative activity	Sidrach et al, Phytochemistry 2005
Sideroxylon obtusifolium	latex	--azocasein (1%) in: 0.1 M phosphate buffer pH 5.8-8.0; 0.1 M tris-HCl buffer pH 7.0-9.0; 0.1 M carbonate/bicarbonate buffer pH 9.5-10.7	100 µL buffer+ 60 µL CE at RT for 30, 60, 90, 120 min	Abs 440 nm (proteinase activity)	% relative activity	Da Silva et al Food Science And Technology 2014
Lactuca sativa	Fresh lettuce	Under standard condition by assaying the remaining (Total casein, β-casein, *α*-casein, and *k* casein) ph 5,5-9 buffers used were 50 mM MES pHs 5.0,	Pre-incubation 4 h different T° Pre-incubation 10°C different pH		% relative activity	Lo Piero et al Agricultural and Food Chemistry 2002

		5.5, and 6.0, 50 mM bis-tris-propane pH 6.5, 50 mM HEPES pHs 7.0 and 7.5, 50 mM Tris pHs 8.0, 8.5, and 9.0. caseinolytic activity				

7.7.2.Temperature

Plant	Anatomic part	Substrate	Pre-Incubation	Measure	Results	Ref
Actinidia chinensis	fruit	total casein in 25 mM buffer (bis-tri-propane pH 6.5; HEPES 7.5)	- 0.5 mL assay mixture (10 µg E) per 3 h at 5-55 °C	Caseinolytic activity	% relative activity	Lo Piero et al, Eur Food Res Technol, 2011
Cynara scolymus	dry stigmas	syntethic peptide [H-Pro-Thr-Glu-Phe-p-(NO_2)-Phe-Arg-Leu-OH] 0.2 mM in 50 mM sodium acetate buffer pH 5.0	- E 1.9 nM in distilled water at 50, 60, 70, 80 °C for varying intervals of time(0-80 min)	Abs 310 nm (proteinase activity)	% relative activity	Sidrach et al, Phytochemistry 2005
Sideroxylon obtusifolium	latex	--azocasein (1% tris-HCl buffer)	100 µL buffer+ 60 µL CE per 30, 60, 90, 120, 180 min at 35-70 °C	Abs 440 nm (proteinase activity)	Change optical density of 0.01 Abs unit/min	Da Silva et al Food Science And Technology 2014
Cynara scolymus	Different organ of globe artichoke	- 1%casein (w/v) in 0,1M potassium phosphate buffer (pH 6)	- 0.1 mL CE + 1.1 mL substrate, 37-65 °C for 0-180 min - stopping: 1.8mL TCA 5% w/v - Centrifuged 4000 g 20 min	Abs 280 nm (residual caseinolytic activity)		Llorente et al, J Agric Food Chem 2004
Lactuca sativa	Fresh lettuce	Under standard condition by assaying the remaining (Total casein, β-casein, *α*-casein, and *k* casein) 5 to 50	Pre-incubation 4 h different T°			Lo Piero et al Agricultural and Food Chemistry 2002

7.8. Enzyme characterization

7.8.1. Inhibition/activation studies

Plant	Anatomic part	Substrate	Inhibitor	Incubation	Measure	1 Unit of protease activity =	Ref
Calotropis gigantea	stem, leaves, flowers, latex	1% w/v casein in 0.05 citrate phosphate buffer pH 7.5	IAA, PMSF, EDTA, 1,10-O-phenanthroline 0.01 M	- equilibration 1 mL CE + 1 mL inhibitor at RT for 30 min - addition 1 mL of pre-incubated E to casein - proceed as described in CA	Abs 280 nm of clear supernatant	amount of E that liberated 1 µg/min tyrosine under assay conditions	Anusha et al, Eur Food Res Technol 2014
sour orange (Citrus aurantium L.)	flowers	1 % casein in 100 mM phosphate buffer pH 7.0 1 % hemoglobin in 100 mM citrate buffer pH 3.4	10 µM pepstatin A 100 µM leupeptin 1 mM PMSF 5 mM EDTA 5 mM DTT 0.1 mM TLCK	450 µL of a 1 g/100 mL solution of **casein** (100 mmol/L Phosphate buffer, pH 7.0) or **hemoglobin** (100 mmol/L citrate buffer, pH 3.4) was **mixed** with **50 µL E** pre-incubated with the inhibitor for 15 min. -37 C for 60 min - reaction stopped 500 µL of 2 SDS sample buffer, heated at 100 C for 5 min	SDS-PAGE electrophoresis		Mazorra-Manzano et al, LWT 2013
Onopordum acanthium L.	Stigmas and stiles	12 g skim milk reconstituted 100 mL of 10 mmol/L $CaCl_2$ (pH 6,5)	1 mM PMSF 0.1 mM E-64 10 mM 1,10-phenantroline 100 µM pepstatine A	- pre-incubation E for 30 min at 37 °C + inhibitor	remaining MCA		Brutti et al LWT 2012
Cynara scolymus	dry stigmas	syntethic peptide [H-Pro-Thr-Glu-Phe-p-(NO_2)-Phe-Arg-Leu-OH] 0.2 mM in 50 mM sodium acetate buffer	E+ pepstatin	- pre-incubation E 26.4 nM + inhibitor for 30 min at 25 °C - reaction started by addition of	initial rates vs inhibitor concentrations		Sidrach et al, Phytochemistry 2005

		pH 5.0		substrate			
Sideroxylon obtusifolium	latex	azocasein (1% tris-HCl buffer) containing 10 mM cation	cation: $Mg2+$, Zn^{2+}, K^{+}, Mn^{2+}, Na^{2+}, Fe^{2+}	100 µL substrate+ 60 µL CE 1h RT -reaction stopping:480 µL TCA 10% w/v -centrifugate 8000g 5min 4°C - 320 µL supernatant added to 560 µL NaOH 1 M	Abs 440 nm	Change optical density of 0.01 Abs unit/min	Da Silva et al Food Science And Technology 2014
		azocasein (1% tris-HCl buffer)	100 mM EDTA 100 mM PMSF 100 mM IAA 1 mM pepstatin A	- pre-incubation 1:1 (I:CE) for 30 min at 28 °C -addition substrate	remaining protease activity		
Cirsium vulgare	flower buds	(MCA)Lys-Lys-Pro-Ala-Glu-Phe-Phe-Ala-Leu-Lys(DNP)	pepstatin A 0.001 mM EDTA 0.005 mM pefabloc 1 mM E-64 0.01 mM amastatin 0.01 mM dithiothreitol 1 mM	- pre-incubation 2.1 µg recombinant E + inhibitor for 6 min at RT in 50 mM sodium acetate buffer pH 4.0 + 0.1 mM NaCl	Fluorescence		Lufrano et al, Phytochemistry 2012
Cynara scolymus	Different organ of globe artichoke	casein 1% w/v in 0.1 M potassium phosphate buffer pH 6.0	cysteine 5 mM E-64 10 µM pepstatin 1 µM PMSF 1 mM	- pre-incubation CE + inhitor for 30 min at 37 °C, pH 6.0	residual caseinolytic activity (Abs 280 nm)		Llorente et al, J Agric Food Chem 2004
		azocasein in 0.1 M Tris-HCl buffer pH 6.0	1,10-phenanthroline 10 mM		residual caseinolytic activity (Abs 440 nm)		

7.8.2. Casein hydrolysis pattern

Plant	Anatomic part	Substrate	Hydrolysate preparation	Analysis of hydrolysate	Ref
Calotropis gigantea	stem, leaves, flowers, latex	casein 1% w/v in 0.05 M citrate phosphate buffer pH 7.5	- 0.45 mL casein + 0.45 mL CE incubated at 35 °C - reaction stopped at 1 and 24 h by addition 3 mL cold 10 % TCA - centrifugation 7,000 g for 10 min	Tricine-SDS-PAGE electrophoresis	Anusha et al, Eur Food Res Technol 2014
Cynara scolymus	Flowers	Casein α, β (0.80 mg/mL), κ (0.65 mg/mL)	- 300 µL casein + 30 µL purified enzyme -at 30-60 min stop by add 2x sample buffer for sodiumdodecyl-sulfide-	SDS-PAGE	Llorente et al,Food Chemistry 2014
Onopordum acanthium L.	Stigmas and stiles	12 g skim milk reconstituted 100 mL of 10 mmol/L $CaCl_2$ (pH 6,5)	- 100 µL partially purified E + 1 mL substrate, 30 °C - 0, 10, 30, 60, 90 min after start	Tricine-SDS-PAGE	Brutti et al LWT 2012
Cynara scolymus	dry stigmas	commercial bovine αs, β,	- incubation E/S 1/500 w/w at 30 °C for 60 min	SDS-PAGE	Sidrach et al, Phytochemistry 2005

		κ caseins 2 mg/mL in desired buffer			
Cirsium vulgare	flower buds	κ-casein 0.3 mg/mL	incubation E/S 1:250 in 50 mM sodium acetate buffer pH 5.5 at 37 °C for 18 h	SDS-PAGE	Lufrano et al, Phytochemistry 2012

7.8.3.Kinetic parameters (K_m, K_{cat})

Plant	Anatomic part	Substrate	Incubation	Measure	Conc. range	Ref
Actinidia chinensis	fruit	- α-casein, β-casein, κ-casein 2% w/v in 67 mM NaH_2PO_4 pH 7.2 and 2.5 mM DTT	- 1 mL reaction mixtures incubated 55 °C for 20 min - reaction stopping: 1.5 mL 5% TCA - centrifugation 9,000 g per 10 min	Abs 280 nm of clear supernatant	substrate 0 - 6 mM enzyme 1.6 nM	Lo Piero et al, Eur Food Res Technol, 2011
Cynara scolymus	dry stigmas	syntethic peptide [H-Pro-Thr-Glu-Phe-p-(NO_2)-Phe-Arg-Leu-OH] 0.2 mM in 50 mM sodium acetate buffer pH 5.0	25 °C	Abs 310	substrate 10 - 200 µM enzyme 23.5 nM	Sidrach et al, Phytochemistry 2005
Cirsium vulgare	flower buds	(MCA)Lys-Lys-Pro-Ala-Glu-Phe-Phe-Ala-Leu-Lys(DNP)	37 °C in 50 mM sodium acetate buffer pH 4.0 + 0.1 M NaCl	Fluorescence	substrate 0.5 - 5 µM	Lufrano et al, Phytochemistry 2012

7.8.4. Protein profile of CE/protein fractions

Plant	Anatomic part	Sample	Technique	Ref
Cynara scolymus	Flowers	Fractions from IEC	SDS-PAGE	Llorente et al,Food Chemistry 2014
Onopordum acanthium L.	Stigmas and stiles	partially purified enzyme extract concentrated by cold (-20 °C) acetone precipitation	Isoelectrofocusing (IEF) on PAGE + zymogram	Brutti et al LWT 2012
		partially purified enzyme extract	Tricine-SDS-PAGE	
Solanum elaeagnifolium var. Cavanilles	berries	protein fractions from salting-out	SDS-PAGE	Guetièrrez-Mèndez et al J Food Sci 2012
Cynara scolymus	dry stigmas	partially purified enzyme extract	SDS-PAGE IEF gel filtration (Sephacryl S-75 16/60 Hi-Prep) SDS-PAGE + electroblotting + staining glycosylated proteins	Sidrach et al, Phytochemistry 2005
Sideroxylon obtusifolium	latex	latex	PAGE	Da Silva et al Food Science And Technology 2014
Cynara scolymus	flowers	crude extract, purified extract by anion exchange	gel filtration (Sephadex G-75) SDS-PAGE IEF + zymograms	Llorente et al, J Agric Food Chem 2004

7.8.5. Peptide-mass fingerprint

Plant	Anatomic part	Sample preparation	Technique	Ref
Cynara scolymus	Flowers	- Cys-sulfhydryl alkylation (50 mM iodoacetamide) - digestion: trypsin 4 ng/mL, 12 h, 37 °C	MALDI-TOF (m/z range 1000-3500)	Llorente et al,Food Chemistry 2014
Cirsium vulgare	flower buds	- in situ tryptic digestion of an electrophoretically homogeneous band	MALDI-TOF (m/z range 1000-3500)	Lufrano et al, Phytochemistry 2012

7.9.Other studies

Plant	Anatomic part	Technique	Objectives	Ref
Cynara scolymus	dry stigmas	binding to immobilized lectins	characterization of sugar composition	Sidrach et al, Phytochemistry 2005
Cirsium vulgare	flower buds	1) total RNA isolation 2) cDNA cloning and sequence analysis 3) expression in E.coli, refolding and purification of recombinant protease	production of recombinant protease	Lufrano et al, Phytochemistry 2012
Cynara scolymus	Fresh flower	Sequence analysis by Edman degradation	N-terminal amino acid sequence	Verissimo et al Biotechnology Letters 1995

7.10. Physicochemical, sensory and textural characteristics of curds/cheeses

Plant	Anatomic part	Type of cheese	Parameter	Method	Ref
Cynara scolymus	Flower	Gouda	Total nitrogen Water soluble nitrogen Moisture Internal and external pH Casein-hydrolysis profile Sensory analysis	semimicro Kjeldhal (AOAC) semimicro Kjeldhal (AOAC) Oven drying (AOAC) direct contact with electrode urea-PAGE Panel of trained judges	Llorente et al, Food Chemistry 2014
Onopordum acanthium L.	Stigmas and stiles	semi-hard cow milk	Protein content Moisture NaCl pH Fat Sensory analysis	macro Kjeldhal (IDF) Oven drying (IDF) silver nitrate method (AOAC) direct contact with electrode Van Gulik's method Panel of trained judges	Brutti et al LWT 2012
Solanum elaeagnifolium var. Cavanilles	berries	cow milk	texture profile analysis stress relaxation curve	texture analyzer texture analyzer	Guetièrrez-Mèndez et al J Food Sci 2012
kiwi fruit (Actinidia deliciosa), ginger (Zingiber officinale), melon (Cucumis melo)	pulp, rhizomes (slices)	cow-milk	texture profile analysis (curd samples)		Mazorra-Manzano et al, Food Chem 2013

Cynara cardunculs	-flower opening;only some styles; stigmas -flowers fully open -flowers begin to dry	Torta del Casar	Sensory analysis texture profile analysis	Panelist	Ordiales et al Food Chemistry 2012

7.11. Manufacture of miniature cheese

Steps	Ref
- 300 mL milk in wide-mouth centrifuge bottle - equilibrate to coagulation temperature - add the amount of coagulant necessary to clot the milk within 40-60 min (redissolving lyophilized CE in a minimum volume of water, 3-4 mL, at 25 °C) - cut coagulum manually and allow it to rest for 10 min - centrifuge at 1700 g for 30 min at 30 °C - drain whey - store curd overnight at 4 °C	Mazorra-Manzano et al, Food Chem 2013
-pasteurized 63°C 30 min -portions 250 g in 500 mL glass beakers -cooled 32°C -dosed 100µL $CaCl_2$ (20% ammonium sulfate extract) 6,6M -afterwards, either: 1µL chymosin 20 µL rennin 500 µL plant coagulant (20% ammonium sulfate extract) -incubate 1h 32°C -coagulum was cut with stainless steel spatula into small cube -held 10 min -stirred at 150 rpm 60 min in an orbital shaker -to the curd was added 1% NaCl (w/w) -transferred into polypropylene tubes with flat bottom +-centrifuged 1700g 15 min room T°C -expelled was drained and the curd centrifuged again 1700g 30 min -remove from the tube -wiped with tissue paper -packed in hermetic polyethylene bags -kept 4°C	Gueltièrrez-Mèndez et al J Food Sci 2012

7.12. Manufacture of cheese

Steps	Ref
Gouda-type - 200 L pasteurized milk(72°C for 15s) - acidity adjusted to 16° Dornic with food-grade acetic acid - inoculated with mesophilic starter culture - incubate to 32°C for 1h before rennet addition - bovine rennet (0.6 mL for 10 L) or artichoke-flower extract (30 mL [54 mg of protein] for 10 L), incubated 35 °C - curd was cut with blades left to heal for 5 min and gently stirred mechanically for 5 min - temperature was raised gradually to 40°C over a period of 15min and the curd was then washed with chlorine-free water before a second step of cutting - stirring was performed under the same condition - the curd was packed into cylindrical molds and pressed (2 kg/cm^2) at 20°C for 1h -curd were salted by brining in NaCl solution (22°B) - vegetable curd cheese separated into two group (1- brined 30h 2-brined 40h) - rennet cheese brined 30h - finally cheese were transferred to a ripening for a 44 day storage 14°C	Llorente et al,Food Chemistry 2014
Semi-hard -milk pasteurized at 70°C for 15 sec -divided in two batches (18L)	Brutti et al Foof Sciece And Technology 2012

-cooled to 38°C -added lactic acid bacteria, increasing in acidity to 20°D after 7 min -added $CaCl_2$ (3,6g/18L of milk) and onopordosin (400mL/18L of milk) -after 180 min cur was cut with wire knives +-allowed to heal for 5 min -gently stirred mechanically for 5 min -was raised gradually to 43°C in 30 min -washed with chlorine-free water so as to reduced the acidity of the crude to 9°D -curd was packed into two cylindrical mold -press (2,5kg/cm^2) at 20°C 1h -salted by brining in saturated NaCl at 13°C 18h -stored for 12 days at 5°C UR 75% -finally transferred to a ripening chamber and stored for 6 week 15°C UR 85%	
Cacioricotta cheese Four batches produced in dairy farm -row caprine milk (pH 6,65) heated 90°C -cooled 45°C Coagulated by immersing five small branches (25 cm) of caprifig (*Ficus carica sylvestris*) in 500 L 3 min Coagulated at the same temperature by calf rennet (0,5mL/L of milk) -coagulate mass very finely cut (0,2 cm) - curd pressed and left 24h T room -salted by rubbing salt on both surface -24h later placed 11°C ventilate room RH 11% 7 days -wasched with diluted brine -packaged -stored 4°c	Faccia et al Eur Food Res Technol 2012
Ewes milk cheese Three batches with pasteurized ewes milk -powdered plant coagulant **(21g/ 100L)** -calf rennet .50:50 PPC:CR -coagulation T 29°C -pressing and salting in brine (22°brix at 12°C for 24h) -ripening chamber 10°C RH 85% -analyzed 2,30,60,120,180,240 days	Galan et al International Dairy Journal 2012
Cottage cheese -4L standardised milk -Ph brought 6,2 with 2,4%(v/v) citric acid solution -T 10°C -was raised to 30°C -added aqueus enzyme extract (*W. coagulans*) 1,5%(v/v) continuous stirring -after 30 min raised 40°C -after 25-30 min cutting curd into smaller slabs -settle 5 min -scaled gradually up to 56°C -drained -salt was added 1.75% (w/w) -pressed manually -packed -stored 8°C	Khan et al International Journal of Dairy Technology 2013
Murciano-granadina cheese -pasteurized Murciano-Granadina milk 78°C 30 sec -50 L milk + 6,5 g of powder PC *(C. cardunculus*) -(0,012 CU = 1:22000 powder) -coagulates 60 min 35°C -curd was cut (6-8 mm) -washed extracting between 15% and 20 % serum and adding water -after increasing 3-5°C -stirred slowly -pressed (2,5 bar 3h) 1kg moulds -salted with a refrigerated brine (saline conc. 17°Baumè 25w/w) 7h -stored RH 82% T12°C -analysed 2,15,30,45,60 days	Abellan et al Journal of Science of Food and Agriculture 2012
Murciano-granadina cheese -12 L pasteurized milk (78°C 30 sec) stored 4°C	Garcia et al Journal of the Science of Food and Agriculture 2014

-cooled milk 28°C -stirring slowly, 3,6 ml $CaCl_2$ at a conc. Of 510g/L -add different starter -stirred 30 min - add plant coagulant (45g flowers per 100 L milk) -cutting time(see article) -cut 20 sec is followed by a pitching step 10 min -cut 5 min after another pitching of 3 min -stirring 15min -unpressed and brined 17°Be 30-40 min -24h 4°C -transferred ripening chamber 12°C RH 85% 40 days	
Torta del Casar -unpasteurized Merino ewe's milk -each batch was clotted using different C. cardunculus L. extract -aqueous extract added 10mL/L milk (vol tot 32 L) 28-30°C -pressing 2h 2kh/cm2 -immersed salt solution (16% W/W NaCl) 4h -ripening unpacked 5-10°C RH 85-90% -analysis 2,30,60 days	Ordiales et al International Journal of Dairy Technology 2014
Torta del Casar - unpasteurized Merino ewe's milk -each batch was clotted using different C. cardunculus L. rennet -aqueous extract added 50 g dried flowers /L water -macerate 24 h -adding 10mL extract /L 28-30°C -pressing -immersed salt solution 16%(w/v) NaCl for 4 h -ripening 5-10°C UR 85-90% 60 day	Ordiales et al Food Chemistry 2012

7.13. Cheeses produced with vegetable rennet

Tipe of cheese	Origin	Vegetable material	Ref
Vino goat's cheese	goat milk	Cynara cardunculus	Tejada et al, 2008
Murcia al Vino	goat's milk	calf- plant rennet	Teiada et al, 2008
Sheep milk cheese	sheep milk	Cynara cardunculus	Galan et al, 2008
Manchego cheese		Cynara cardunculus	Padros et al, 2007
Ewe milk cheese	ewe milk	Cynara cardulnculs	Tejada et al, 2007
Vino goat milk cheese	goat milk	diff Cynara cardunculus	Tajeda et al,2006
Manchego tipe cheese		Vegetable rennet	Padros et al, 2006
Ewe's milk cheese	ewe milk	Cynara cardunculus	Fernandez et al, 2003
Serpa cheese	ewe's' milk	Cynara cardunculus	Roserio et al, 2003
Ovine milk cheese	ovine milk	Cynara L.	Roserio et al, 2003
Cheddar tipe-cheese		Cynara cardunculus	O'Mahony et al, 2003
Los Pedroches	ewe milk	Cynara cardunculus	Tejada et al, 2003
Los Pedroches	ewe milk	animal vegetable rennet	Sanjuan et al, 2002
Gouda-type cheese	Bovine milk	Cynara scolymus	Llorente et al, 2014
Burgos-type	Bovine milk	Cynara cardunculus	Timon et al, 2014
Goat's cheese	Goat milk	Cynara cardunculus	Garcia et al, 2014
Torta del Cesar	Goat milk	Cynara cardunculus	Ordiales et al, 2013
Torta del Cesar	ewe milk	Cynara cardunculus	Ordiales et al, 2013
Fresh goats cheese	goat milk	Cynara cardunculus	Garcia et al, 2012
Ewe's milk chees	ewe milk	Cynara cardunculus	Galan et al, 2012
Torta del Cesar	ewe milk	Cynara cardunculus	Ordiales et al, 2012
Goat's(vino) milk chees	goat milk	Cynara cardunculus	Abellan et al, 2012
Cacioricotta cheese	goat milk	Ficus carica sylvestris	Faccia et al, 2012
Caciotta cheese	cow's milk	Cynara cardunculus	Aquilanti et al, 2011
Ewe's milk chees	ewe milk	diff. veg. coagul	Cabezas et al, 2011
Spanish soft cheeseTC	ewe milk	vegetable rennet	Delgado et al, 2010
Goats milk cheese	goat milk	Cynara cardunculus	Pino et al, 2009
Gouda-type cheese	Bovine milk	Cynara scolymus	Llorente et al, 2014
Burgos-type	Bovine milk	Cynara cardunculus	Timon et al, 2014
Goat's cheese	Goat milk	Cynara cardunculus	Garcia et al, 2014
Torta del Cesar	ewe milk	Cynara cardunculus	Ordiales et al, 2013
Torta del Cesar	ewe milk	Cynara cardunculus	Ordiales et al, 2013
Fresh goats cheese	goat milk	Cynara cardunculus	Garcia et al, 2012
Ewe's milk chees	ewe milk	Cynara cardunculus	Galan et al, 2012
Torta del Cesar	ewe milk	Cynara cardunculus	Ordiales et al, 2012
Goat's(vino) milk chees	goat milk	Cynara cardunculus	Abellan et al, 2012
Cacioricotta cheese	goat milk	Ficus carica sylvestris	Faccia et al, 2012
Caciotta cheese	cow's milk	Cynara cardunculus	Aquilanti et al, 2011
Ewe's milk chees	ewe's milk	diff. veg. coagul	Cabezas et al, 2011
Spanish soft cheeseTC	ewe milk	vegetable rennet	Delgado et al, 2010
Goats milk cheese	goat milk	Cynara cardunculus	Pino et al, 2009
Caciofiore della Sibilla	ewe milk	*Carlina acanthifolia* All. subsp. *acanthifolia*	Cardinali et al, 2016

8. Summary of Legislation on Enzymes Used in Food

The use of coagulant enzymes in the preparation of curds intended for the production of cheese or food ingredients (casein and caseinates) are regulated in the Community by:

REGULATION (EC) No 1332/2008 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 16 December 2008 on food enzymes and amending Council Directive 83/417 / EEC, Regulation (EC) Council Directive 2000/13 / EC, Council Directive 2001/112 / EC and Council Regulation (EC) No 1493/1999. 258/97 (updated 03/12/2012)

It concerns enzymes that are added to foods to carry out a technological function in the manufacture, processing, preparation, processing, packaging, transport or storage of such foods, including enzymes used as processing aids ('food enzymes').

The definition of 'technological adjuvant' is contained in REGULATION (EC) No 1333/2008 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 16 December 2008 on food additives. This term means any substance that:

i) is not consumed as a food in itself;

(ii) is intentionally used in the processing of raw materials,

foods or their ingredients, in order to exercise a certain technological function in working or processing; is

(iii) it may give rise to the unintended but technically unavoidable presence of residues of that substance or its derivatives in the finished product, provided that such residues do not constitute a health risk and have no technological effect on the finished product.

The scope of Reg. (CE) 1332/2008 does not extend to enzymes that are not added to food to perform a technological function such as enzymes with nutritional or digestive enzymes.

It does not, however, apply to food enzymes to the extent they are used in the production of:

(a) food additives falling within the scope of Regulation (EC) No. 1333/2008 [concerning food additives];

b) technological adjuvants.

Microbial cultures traditionally used in the production of foods such as cheese and wine and which may incidentally produce enzymes but are not specifically used to produce them are not considered food enzymes.

To this end, the Regulation provides:

(a) a Community list of authorized food enzymes;

(b) conditions for the use of food enzymes in foods;

(c) the rules for the labeling of food enzymes marketed as such.

Art. 3 of Regulation (EC) 1332/2008 contains the following definitions:

(a) "food enzyme" means a product obtained from plants, animals or micro-organisms or derived products as well as a product obtained by means of a microorganism fermentation process:

(i) containing one or more enzymes capable of catalyzing a specific biochemical reaction; is

(ii) added to food for a technological purpose at any stage of manufacture, processing, preparation, treatment, packaging, transport or storage of the same;

(b) 'food enzyme preparation' means a formulation consisting of one or more food enzymes incorporating substances such as food additives and / or other food ingredients to facilitate their storage, sale, standardization, dilution or dissolution.

Food enzymes which are authorized for use in the Community must be listed on a Community list (Article 4, Reg. (CE) 1332/2008), which is still unavailable, clearly describing the enzymes and specifying the conditions of their use, including , where relevant, information on their function in the final product. This list should be supplemented by specifications, in particular on their origin, including, where relevant, information on allergenic properties and purity criteria.

By way of derogation from the procedures for inclusion in the Community list referred to in Articles 7 and 17 of Regulation (EC) 1332/2008, this list shall comprise the following food enzymes:

(a) invertate E 1103 and lysozyme E 1105, indicating the conditions of their use specified in Annex I and in Part C of Annex III to Directive 95/2 / EC;

(b) urease, beta-glucanases and lysates intended for use in wine in accordance with Regulation (EC) 1493/1999 and its implementing rules.

Food enzymes fall within the definition of 'food' of Regulation (EC) No. 178/2002 and, when used in foodstuffs, must therefore be indicated as ingredients in food labeling in accordance with Directive 2000/13 / EC:

"ingredient" means any substance, including additives and enzymes, used in the manufacture or preparation of a foodstuff still present in the finished product, possibly in modified form (Article 6 (4) (a))

When an ingredient of a food product has been processed starting from several ingredients, these are considered ingredients of the product. However, they are not considered as ingredients:

(i) components of an ingredient which, during the manufacturing process, have been temporarily removed to be subsequently introduced in quantities not exceeding the initial content;

(ii) additives and enzymes:

- whose presence in the foodstuff is due solely to the fact that they were contained in one or more ingredients of that product, provided that they no longer have any technological function in the finished product,

- which are used as technological auxiliaries;

(iii) Substances used, in the strictly necessary doses, as solvents or supplements for additives, enzymes and flavorings.

Food enzymes should be designated by their technological function in food, followed by the specific name of the food enzyme. However, Directive 2000/13 / EC introduces the possibility of derogating from labeling provisions in cases where the enzyme does not play a technological role in the final product but is present in the food product only as a residue of one or more ingredients of such foodstuff or when used as a technological adjuvant.

e use of food additives, including adjuvants, in food enzymes is regulated by

REGULATION (EC) No 1333/2008 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 16 December 2008 on food additives

PART 3: Food Additives, including Adjuvants, in Food Enzymes

General rules on the conditions of use of food additives in Part 3

1. Save as otherwise provided, the food additives listed in Part 6, Table 1 of this Annex, normally authorized in foods in accordance with the general principle "quantum satis" and included in Annex II, Part C, point 1, Group I , have been included as food additives in food enzymes according to the general principle "quantum satis".

2. For phosphates and silicates, maximum levels - when used as additives - have been established only in relation to food enzymatic preparations and not in relation to final food.

3. For all other food additives whose DGA is expressed by a numeric value, the maximum quantity has been fixed for food enzymatic preparations and for final food.

4. No food additive is authorized as a colorant, sweetener or flavor enhancer.

9. Botanical characteristics

Carlina zolfina (scientific name *Carlina acanthifolia* All., 1785) is a perennial herb plant, with large inflorescences that predominantly live on the ground, belonging to the Asteraceae family (Fig 12).

Fig. 12 *Carlina acanthifolia* All

The appearance of this plant is herbaceous and thorny. It can reach up to a maximum height of 1 to 3 dm. The biological form of the species is emicriptophite, that is, a perennial plant with gems placed at the ground level while the leaves are arranged in basal rosette.

Roots

The root is secondary from rhizome (root of the type).

Frame

Hypogeal part: It consists of a big, swollen rhizome and normally vertical and woody.

Epigenetic part: the plant is almost acaule, with very short or nonexistent stem (1 - 4 cm).

Leaves

The more or less plain leaves have an elliptical-oblanceolata elliptic-oval shape; it is also divided into various spinous segments ending in rigid acules resulting from the continuation of the central ribs. We therefore have pennatopartite or pennatifide leaves with sub - opposed lobes with spatulary contour. The lower surface of the lamina is velvety and whitish (or white-tomentosa). It is quite thick.

The lower leaves (the basal rosette ones) are pinned, while the upper ones (cauline) are arranged in a round form, they are sessile and in the final part (near the inflorescence) they turn into thorny bratheas.

Leaf size: width 5 - 15 cm; length 15 - 30 cm (Fig.13).

Fig. 13 Laves of *Carlina acanthifolia* All.

Inflorescence

The inflorescence is composed of a peacock (typical structure of the Asteraceae) supported by a brattee shell (or scales).

The cover is cylindrical or slightly ply-shaped. The brattees are coloured in pale yellow but also yellow-sulfur and always brilliant. The latter has the most visible morphological appearance of the

plant with the function of attracting insect-like insects (floating function that in the Asteraceae usually performs the flowers of the outer beam).

The receptacle (the upper and inner part of the wrapper supporting the true flowers), yellowish, is flat but deeply veiled (the pitted) and the various florals (all tubular) are inserted into these honeycombs and are surrounded by bratheal scales similar to acute fringe bristles (recipe with wadding).

The peacock, which generally opens on the ground, is yellow in colour (colour determined by tubular flowers).

Peak diameter: up to 15 cm (normally 5-8 cm). Dimensions of Yellow Stirring Squares: Width 3 - 4 cm; length 30 - 40 cm.

Flower

The flowers are all of the tubular type (the ligated type, the ray flowers, present in most Asteraceae, are absent in Carlina flower), are also hermaphrodites, tetra-cyclic (with four verticilli: goblet - corolla - androceo - gynecologic) and pentameri (that is, both the chalice and the corolla are composed of five elements).

Chalice: The cups of the goblet are reduced to a chalk of scales.

Corolla: Corolla has a cylindrical (or bell-shaped) shape and ends with 5 teeth. Corolla size: 1 mm wide; 16 - 18 mm long.

Androceo: The stamens are 5 with free filaments; the caudated anthers (with tail) are welded together and form a sleeve surrounding the stylus.

Gineceo: the ovary is sublime and unilocular with 2 carpels; the stylus is unique with a twin and glabrous stem stimulation (there is only a bunch of hairs at the apex of the stylus).

Flowering: from July to September (Fig.14).

Fruits

The fruits are oblong branches (circular section) covered by long silky hairs at the top of a feathered pappa (or rather "pennata": it is a single or double set of feathered bristles)

Fig. 14 Flower of *Carlina acanthifolia* All.

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10. Evaluation of technological properties (materials and methods)

10.1. Plant material

Carlina acanthifolia All. subsp**.** *acanthifolia* was collected from area of the Monti Sibillini National Park. The plant was identified, authenticated and divided into the different anatomic parts. (stem and flowers).

10.2. Preparation of crude enzyme

Five grams each of tender stem and flowers were homogenised with a kitchen blender in 5 ml of 0.1 M potassium phosphate buffer 1mM EDTA (pH 6). The extracts were filtered through muslin cloth. Crude extracts was stored in deep freezer (−20 °C) until further analysis or used fresh.

10.3. Milk-Clotting activity (MCA)

Clotting activity of proteases under study was evaluated by adding commercial skim milk (5mL g) with CaCl2 5% in a clean dry test tube it and placing in water bath at different temperatures for 15 min. 1 ml of the CE was added to substrate. Time taken for the milk to clot was recorded.

10.4. Effect of Temperature on MCA

To evaluate the effect of temperature, the substrate was pre-incubated with 200 µL $CaCl_2$ at different temperatures (37–60 °C) prior to addition of CE. The time taken for the milk to clot was taken as measure of enzyme activity

10.5. Effect of calcium chloride on MCA

To evaluate the effect of calcium chloride (5%), different concentrations of this (50-100-150-200 µL) were added to the milk at different temperatures (37-45-55°C), to study its effect on MCA. The time taken for the milk to clot was taken as measure of enzyme activity.

10.6. Effect of enzyme concentration on MCA

To evaluate the effect of enzyme concentration, different concentration of crude extract (0,5-1mL)were added to milk at different temperature(37-45-55°C), to study its effect on MCA. The time taken for the milk to clot was taken as measure of enzyme activity.

10.7. Effect of storage on MCA

To evaluate the effect of storage, fresh and frozen aqueous extracts were added to the milk at different temperatures to study its effect on MCA. The CE from plant parts was stored at −20°C. The time taken for the milk to clot was taken as measure of enzyme activity.

11. Results

11.1. Effect of Temperature on MCA

MCA was highest in aqueous extract by flower and in decreasing on stems. Another major difference was seen between the top of the flower and the bottom. MCA was very high for the upper portion of the flower and totally absent for the lower part (Fig.15-16)

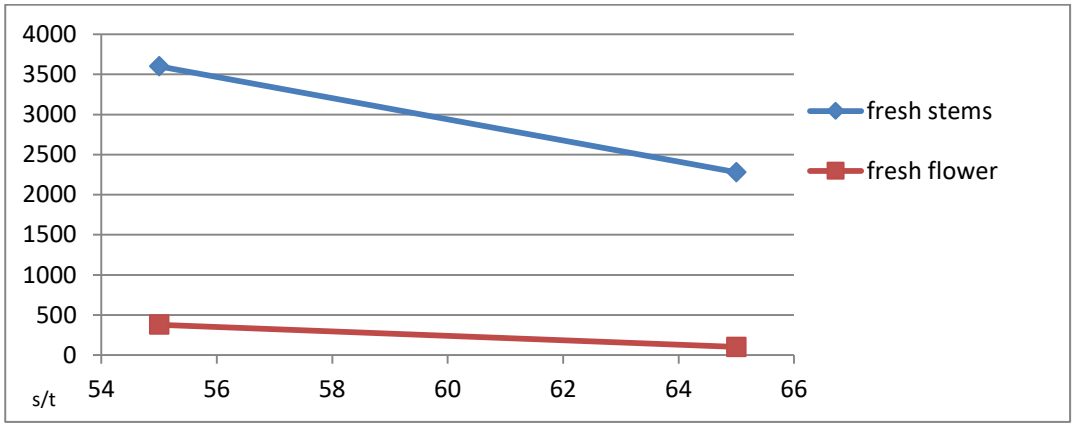

Fig. 15 Effect of temperature on MCA in a samples of aqueous extract of *Carlina acanthifolia* All. subsp. *acanthifolia* obtained from fresh stems and fresh flowers

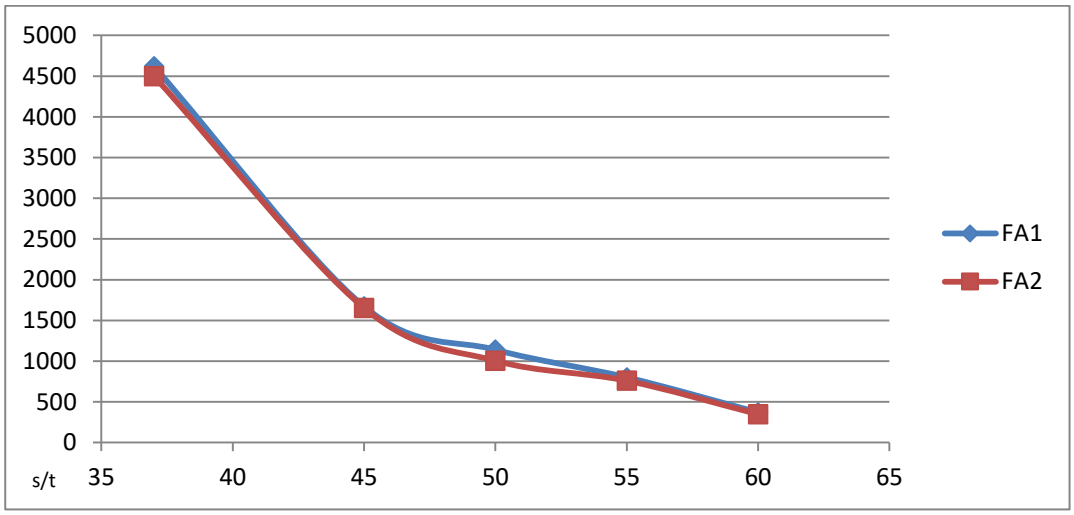

Fig. 16 Effect of temperature on MCA in a samples of aqueous extract obtained from the upper part of the *Carlina acanthifolia* All. subsp. *acanthifolia* flower

11.2. Effect of calcium chloride on MCA

MCA was higher in samples with 200 µl $CaCl_2$ and decreases in the other concentration at different temperatures (Fig.17).

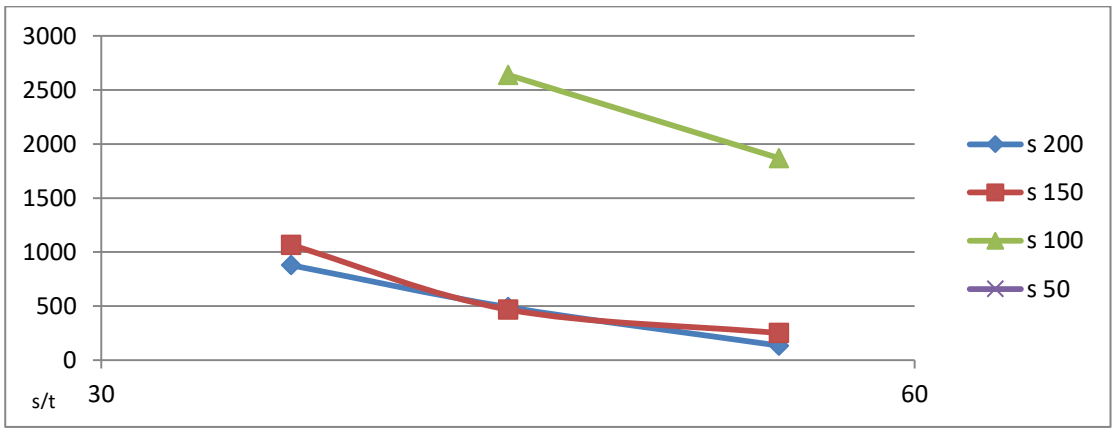

Fig. 17 Effect of different concentration of calcium chloride on MCA in crude aqueous extract obteined from *Carlina acanthifolia* All. subsp. *acanthifolia.*

11.3. Effect of enzyme concentration on MCA

The different amount of enzyme used was found to be very discriminating for MCA.
MCA was higher in samples with 1mL and decreases in the other concentration (Fig.18)

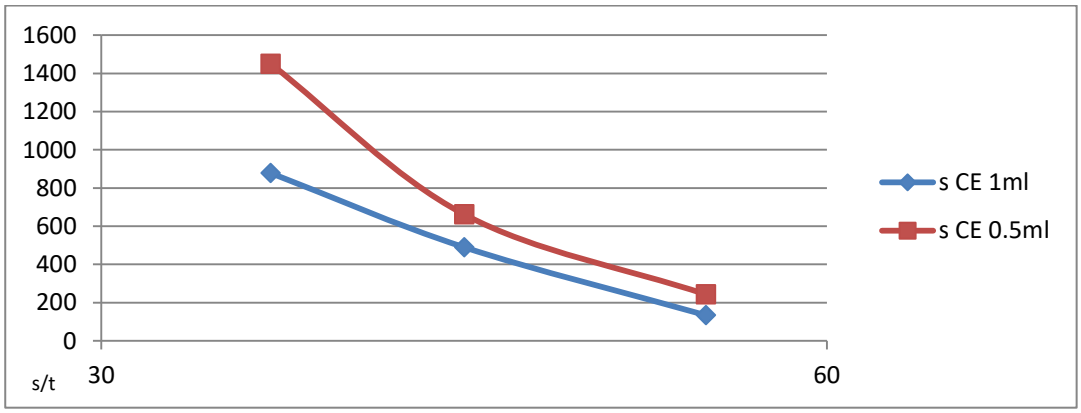

Fig. 18 Effect of different concentration of enzyme on MCA in crude aqueous extract obteined from *Carlina acanthifolia* All. subsp. *acanthifolia.*

11.4. Effect of storage on MCA

Storage at -20 had no effect on MCA. the same results were obtained on fresh samples (Fig.19)

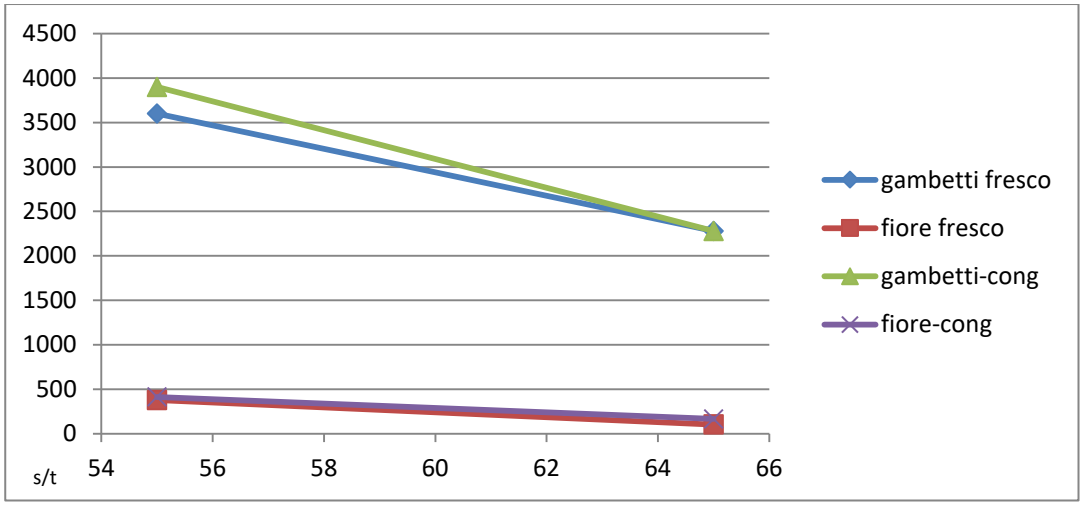

Fig. 19 Effect of storage at -20 on MCA in crude aqueous extract obteined from different anatomical parts of *Carlina acanthifolia* All. subsp. *acanthifolia.*

12. Cheese - making and sampling

Cheese-making trials were conducted in a familyrun dairy farm located in the Monti Sibillini National Park (Marche Region, Central Italy), following a traditional manufacturing method, without any addition of starter culture. The raw milk obtained from ewes of the breed Sopravissana, was coagulated with a crude extract from young fresh leaves of *C. acanthifolia All. subsp. acanthifolia*. The crude extract was prepared as follows: the petioles of fresh young leaves from four plants of *C. acanthifolia All. subsp. acanthifolia*, harvested from an uncultivated foothill area of the Monti Sibillini National Park, were carefully peeled, chopped with a mezzaluna knife and macerated in an aqueous solution of wine vinegar (1:1 ratio) for ~18 h at room temperature. The preparation was filtered through a muslin cloth and ~100 ml crude extract were used to clot ~10 l raw ewes' milk. filtered raw milk was poured into a foodgrade steel coagulation vat, heated at ~35–36°C, the coagulant added and gently stirred. After clotting (ca. 30 min), curd were manually broken into rice-sized grains, then transferred into 8×8 cm plastic perforated moulds and manually pressed to drain the whey. the cheese was held at 12–13 °C for 4 h, dry salted and ripened for 20 days at 12–13°C and 70% relative humidity.

During cheese making and ripening, the following samples were collected and transported to the laboratory under refrigerated conditions (+4°C): cheeses after 1, 3, 6, 10 and 20 days of ripening.

13. Chemical Analyses

Dry matter was assessed by a gravimetric method. The samples of cheeses at different ripening days was drying by lyophilisation after being weighed with the analytical balance. After drying it in thermoregulated stove (105°), the sample was weighed again and dry matter was calculated. Crude fat content was determined by Manual solvent extraction with diethyl ether. After solvent evaporation, the extracted fat was weighed and the fat content calculated. Total nitrogen was determined by the Kjeldahl method (AOAC, 1990), with conversion to total protein content using a factor of 6.38. Ash contents of milk, curd and cheese samples were assayed according to AOAC (1990)(Tab.1). Raw ewe's milk, curd and cheese samples underwent pH measurements with a model 300 pH-meter equipped with an HI2031 solid electrode. (Tab 2)(Fig20-21).

Ripening days	Dry Matter (DM) [g/100 g cheese]	Fat [g/100 g DM]	Protein [g/100 g DM]	Ash [g/100 g DM]
0 (milk)	20.5	7.1	5.5	1.1
0 (curd)	37.9	47.9	39.0	3.9
1	40.1	48.8	39.7	5.4
3	42.1	48.0	40.1	5.6
6	64.8	51.1	39.9	6.0
10	67.1	51.5	40.4	6.6
20	52.5	53.4	39.5	5.6

Tab. 1 Changes in proximate composition of ewe's milk cheeses made with plant coagulant during ripening

Sample	pH
Carlina acanthifolia All. (stem and leaves)	n.d.
Raw ewe's milk	6.39 ± 0.02
Aqueous extract	3.79 ± 0.03
Curd	5.14 ± 0.02
Caciofiore della Sibilla	
1 d	5.22 ± 0.01
3 d	5.31 ± 0.02
6 d	5.38 ± 0.01
10 d	5.23 ± 0.01
20 d	5.02 ± 0.03

Tab. 2. pH determination of ewe's milk cheeses made with plant coagulant during ripening

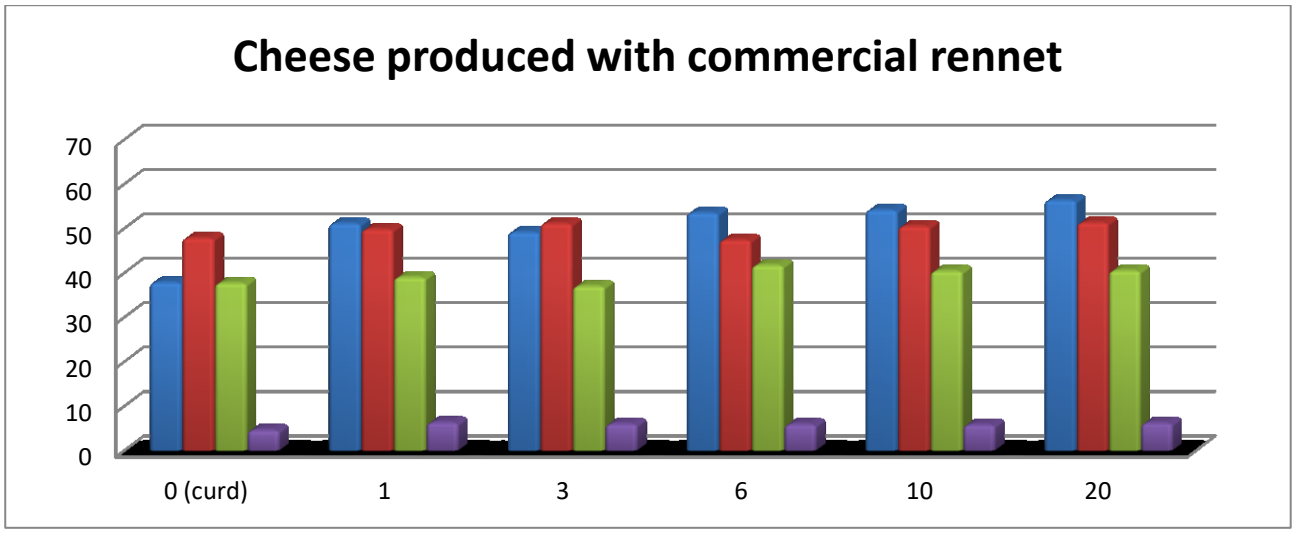

Fig. 20. Change in dry matter content (%t.q.) Fat (%ss) protein (%ss) in cheese produced with commercial rennet.

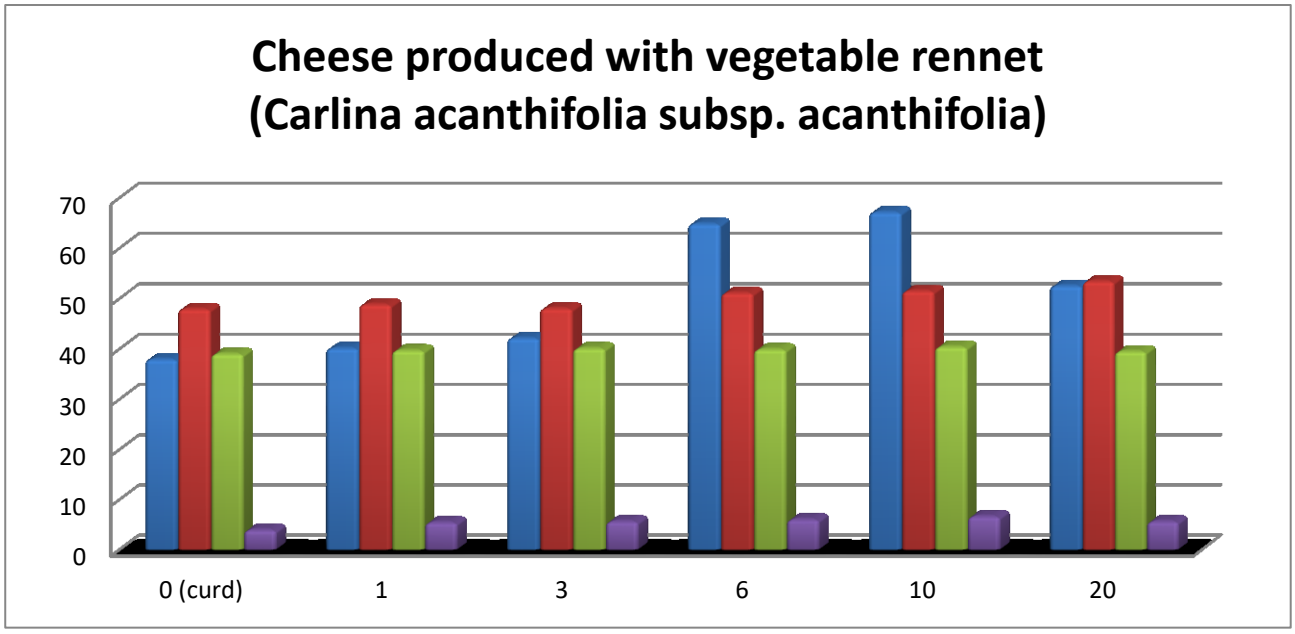

Fig. 21 Change in dry matter content (%t.q.) Fat (%ss) protein (%ss) in cheese produced with vegetable rennet.

14. Determination of volatile aroma profile: water crude extract of leaf flower and stems from *Carlina acanthifolia* All. Subspecies *acanthifolia* and "Caciofiore" cheese

14.1 Volatile aroma profile

SPME-GC/MS was used to characterize volatile profile of crude extract c from C. *acanthifolia* All. subsp. *acanthifolia* and "Caciofiore della Sibilla" cheese.

Crude aqueous extract (2ml) homogenate and cheese (2 gr for each sampling) ground in small pieces with knife were introduced on 10 mL vials respectively and closed with a screw cap equipment with elastomeric septum. The vials was placed in a heating bath at 40°C for 5 min. After this time, the SPME fiber (divinylbenzene/carboxen/polydimethylsiloxane) from Supelco/Sigma-Aldrich was introduced onto the vial and exposed to the head space of the sample for 15 min. Thermal desorption of the compounds from the fiber took place in the GC injector at 220°C for 3 min. The injection was performed in the splitless mod (splitless time 0,3 min) at 220°C. The GC-MS runs were performed with a Varian 3900 gas chromatograph coupled to a Saturn 2100Tion trap mass spectrometer. The chromatographic separation was performed on TG-5MS capillary column. The oven temperature program started at 40°C during 10 min then was raised to 180°C at a rate of 3 °C/min and reached 250°C at a rate of 15 min °C/min. The transfer line and trap temperatures were set at 200°C. The mass spectra were recorded in full scan mode (mass range 31-250 *m/z*) with a scan rate of 1 scan/s. The volatile compounds were analyzed with the Varian Workstation software. The identification was confirmed with the chromatographic retention index and the NIST library mass spectra of pure standard substances.

14.2 Results

The main identified volatile compounds found in flower of Carlina acanthifolia All. Subspecies acanthifolia samples were shown in the table 3.

RT	Compound	Abundance
1,779	1-carbahexaborane(7)	1,59E+06
1,884	thiazolidin.4-one 5 ethil-2-2imino	6,40E+06
2,005	isobutylamine	3,39E+06
2,255	hexane 3,4 dimethyl	6,51E+07
2,437	1,1dichloropentane	2,42E+07
2,543	3methyl1penten3ol	8,82E+06
2,832	butanal3methyl	8,82E+06
3,534	hexene3methyl	7,07E+06
4,532	2chloro2methylhexane	1,33E+06
6,532	hexanal	4,22E+06
8,693	2hexanal	1,62E+06
8,933	3hexanaol	1,84E+07
9,424	butanoicacid3 methyl	2,22E+06
9,677	butanoicacid32methyl	1,34E+06
13,218	phenylglioxal	3,13E+06
13,587	benzoicacid3formyl	1,51E+06
16,546	benzylalchol	1,38E+06
16,697	toluene	4,51E+06

Tab. 3 The main identified volatile compounds found in the flower of *Carlina acanthifolia All. Subspecies acanthifolia*

The main identified volatile compounds found in stems of *Carlina acanthifolia All. Subspecies acanthifolia* samples were shown in the table 4

RT	Compound	Abundance
1,819	1propanon2methyl	6,70E+06
1,954	methylene chloride	2,31E+06
2,07	1,1diisobutoxy isobutane	1,57E+06
2,624	hexanoicacid	2,90E+06
2,914	Butanal3methyl	1,39E+06
3,353	3,3dimethylbutane	1,38E+06
6,672	hexanal	3,02E+06
8,82	2hexanal	4,15E+06
9,065	3hexen1ol	7,62E+06

Tab. 4 The main identified volatile compounds found in the stems of *Carlina acanthifolia* All. Subspecies *acanthifolia*

RT	Compound	Abundance
1,929	propane1,1dimethoxy	1,19E+06
2,016	3pentanol2,4dimethyl	3,67E+06
2,153	hydroperoxyde1methylhexyl	1,32E+06
2,402	furanmethanol	2,06E+07
2,497	pentane2,4dimethyl	2,02E+07
2,906	benzene	2,18E+07
3,487	hexane3methyl	1,69E+06
10,163	2hexanone4methyl	1,69E+06
12,62	4methyl2oxavaleric acid	1,37E+06
18,574	2nonanone	1,23E+06

Tab. 5.The main identified volatile compounds found in the "Caciofiore" cheeses after 1 day of ripening.

RT	Compound	Abundance
1,727	pyrrolidine1nitroso	2,51E+06
2,014	oxetane	1,50E+06
2,157	3,6octadecadyonic acid methyl	2,23E+07
2,503	pentane2,4dimethyl	2,32E+06
2,91	benzene	2,36E+07
3,494	hexane3methyl	2,09E+06
4,369	1hexane4,5dimethyl	2,90E+06
4,454	pentafluoruro propinoicacid	1,05E+06
10,179	2ehexanone4methyl	6,45E+06
12,623	4methyl2oxavaleric acid	1,05E+06
18,592	2nonanone	6,28E+06

Tab. 6 The main identified volatile compounds found in the "Caciofiore" cheeses after 3 days of ripening

RT	Compound	Abundance
1,736	1butanol2,2,dimethyl	9,84E+06
2,0121	oxetane2methyl4propxy	2,66E+06
2,233	piperidine1,1dithiobis	5,37E+07

2,41	2furanmethanol	1,99E+07
2,504	2,5dimethyl5hexan3ol	1,76E+07
3,192	benzene	2,90E+06
3,499	hexane3emethyl	1,86E+06
3,813	ethanol2methyEthoxy	2,01E+06
4,427	1butanol3methyl	1,44E+06
6,553	butanoicacid ethylester	2,36E+06
10,177	2hexanone4methyl	4,65E+06
14.865	hexanoicacidethylester	1,57E+06
18,589	2nonanone	4,73E+06

Tab. 7 The main identified volatile compounds found in the "Caciofiore" cheeses after 6 days of ripening.

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RT	Compound	Abundance
1,73	1heptanol	3,20E+06
1,859	thiazolidin4ine5ethyl	3,78E+06
2,017	3pentanol2,4dimethyl	3,78E+06
2,101	oxetane2methylpropyl	1,47E+06
2,146	3,6,octapentanoicacid methylester	1,20E+06
2,229	piperidine1,1dithiobis	8,35E+07
2,404	hexane1,1oxybis	2,05E+07
2,499	2,5dimethyl5hexen3ol	2,44E+07
2,903	benzene	1,90E+07
3,481	hexane3methil	2,11E+06
3,71	4amio1butanol	1,01E+06
4	butanoic acid	1,10E+06
4,349	acetic acid	2,24E+06
4,433	1butanol3methyl	2,01E+06
5,249	toluene	4,87E+07
6,539	butanoic acid ethylestere	2,27E+06
10,171	2hexanone4methyl	3,66E+06
12,636	4methyl2oxovaleric acid	1,51E+06
14,87	hexanoicacidiethyleste	1,75E+06

Tab. 8 The main identified volatile compounds found in the "Caciofiore" cheeses after 10 days of ripening

RT	Compound	Abundance
1,741	1butanol2,2,dimethyl	1,76E+07
2,025	3pentanol2,4dimethyl	4,52E+06
2,111	oxetane2methyl4propyl	3,41E+06
2,239	piperodine1,1dithiobis	9,67E+07
2,408	2furanmethanol	2,78E+07
2,511	2,5dimethyl5hexen3ol	2,91E+07
2,912	benzene	2,20E+07
3,499	hexane3methyl	2,42E+06
5,281	toluene	4,60E+07
6,562	butanoicacid ethylester	1,22E+06

7,727	butanoic acid	1,57E+06
10,714	2heptanol	1,16E+06
11,691	hexanoicacid methylester	1,17E+06
12,634	4methyl2oxovalericacid	1,26E+06
14,873	hexanoicacid ethylester	3,50E+06
15,616	pentanoic acid	4,35E+06

Tab. 9 The main identified volatile compounds found in the "Caciofiore" cheeses after 20 days of ripening.

The volatile compounds identified for the flower were numerically greater than the stems. some compounds have been identified in both samples (butanal3methyl, hexanal, 2hexanal, 3hexenol). butanal3methyl and 2hexanal they were much abundant in flowers while 2hexanal; 3hexenol had a higher concentration in the stems.

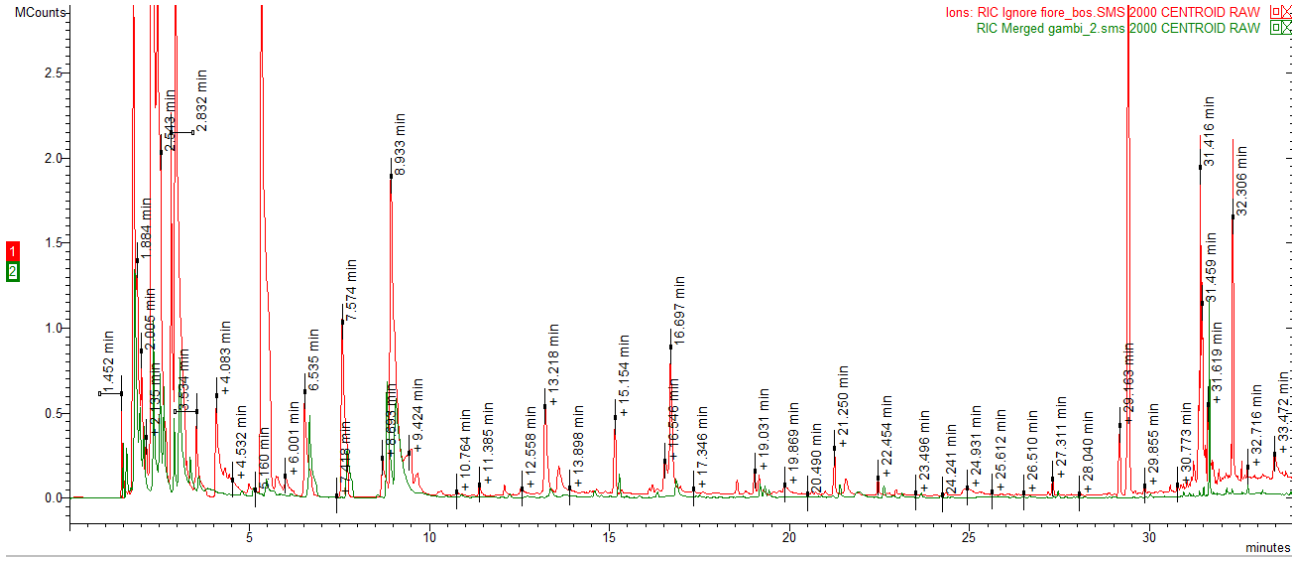

Fig. 22 Chromatogram of volatile compounds of flowers and stems in overlaid plot.

Identification of the profile of volatile organic compounds during the ripening of cheese has shown, on the first day of ripening, many compounds (2hexanone4methyl, 2nonanone, furanmethanol, hydroperoxyde1methylhexyl, pentane2,4dimethyl, propane1,1dimethoxy) were found, which were absent in the cheese analyzed for 20 days of ripening. Other compounds (3pentanol2,4dimethyl, hexane3methyl) were identified in both samples (T1-T20) but exhibited a higher concentration in the cheese sample at 20 days of ripening. The other compounds present in both samples (T1-T20) didn't undergo any variation in concentration (4methyl2oxavaleric acid, benzene).

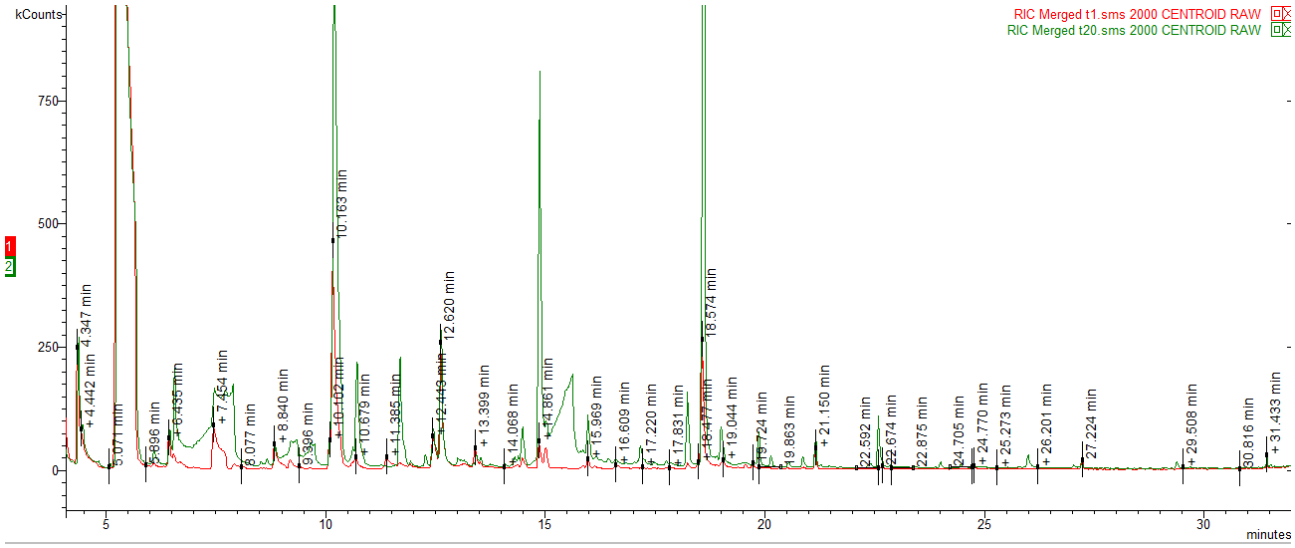

Fig. 23 Chromatogram of volatile compounds of T1 and T20 in overlaid plot.

15. Yeast and mould dynamics in Caciofiore della Sibilla cheese coagulated with an aqueous extract of Carlina acanthifolia All.

The term 'Caciofiore' refers to Italian soft cheeses traditionally manufactured with raw ewes'milk and a vegetable coagulant mostly obtained from dried flowers of herbaceous perennial plants, native to the western and central Mediterranean area, which are commonly known as 'thistles' and scientifically ascribed to different genera within the composite family Asteraceae, viz. *Cynara* (artichoke or cardoon), *Scolymus* (golden thistle or oyster thistle), *Carlina* (carline thistle) or *Silybum* (milk thistle or Marian thistle).
In the Mediterranean area, the exploitation of thistle rennet, especially obtained from *Cynara cardunculus* L., *Cynara scolymus* L. and *Cynara humilis* L., is not confined to Italy, where renowned speciality cheeses, such as Caciofiore Aquilano and Caciofiore di Columella, are traditionally manufactured in Abruzzo and Lazio, respectively. The use of this type of vegetable coagulant is also particularly widespread in western Africa (García *et al*., 2012) and the Iberian Peninsula, the latter boasting a large number of Protected Designation of Origin (PDO) cheeses (Roseiro *et al*., 2003). Even in Latin-American countries, such as Argentina and Chile, where 'cardo de Castilla' (*C. cardunculus* L.) grows vigorously, thistle rennet is used for the manufacture of a variety of ewes' milk cheeses (Fox, 1999).
Crude extracts from flowers or even leaves of thistles are thermostable and characterized by a high proteolytic activity (Fox, 1999) for the occurrence of aspartic proteases, known as cardonsins (or cynarases and cyprosins), with a high specificity for caseins (Roseiro *et al*., 2003).
As the number of flowers used for the preparation of the coagulant largely depends on the cheesemaker's experience, the amount of coagulant to be added to the milk is also often empirical.
The impact of vegetable coagulants derived from thistles on milk clotting and the physicochemical, sensory and textural traits of cheese has been thoroughly investigated (Sanjuán *et al*., 2002; Tejada *et al*., 2007; García *et al*., 2012; Llorente *et al.*, 2014; Ordiales *et al*., 2014) whereas, to date, the contribution of these plant extracts to cheese microbial diversity and dynamics has undoubtedly been less explored, with only a few studies carried out for this specific purpose (Sousa and Malcata, 1997; Vioque et al., 2000; Gómez et al., 2001; Roseiro et al., 2003; Tejada and Fernández-Salguero, 2003; Aquilanti *et al*., 2011; Galán *et al*., 2012; Ordiales *et al.*, 2013). This is especially true for yeasts and moulds, whose occurrence and diversity in vegetable coagulants have been very poorly explored (Roseiro *et al*., 2003).
The importance of these microorganisms in cheese manufacturing is being increasingly acknowledged, due to the many beneficial activities of these microorganisms, including the excretion
of growth factors (especially vitamins), the metabolization of lactate/lactose, which enhances the growth of a secondary microbiota constituted by acid-sensitive bacteria, the production of alkaline metabolites and aromatic compounds, the inhibition of spoilage microorganisms, the enhancement of cheese flavour and aroma through proteolysis and lipolysis, and even probiosis (Beresford *et al*., 2001; Jacques and Casaregola, 2008; Panelli *et al.*, 2013; Padilla *et al*., 2014; Cardoso *et al*., 2015).
On the other hand, fungi can also act as spoilage microorganisms, causing cheese defects such as cheese softening, superficial discoloration, early blowing and the development of off-flavours (Fleet, 1990; Jakobsen and Narvhus, 1996; Carreira *et al.*, 1998; Wyder et al., 1999). Finally, toxicogenic moulds can produce secondary metabolites, generally referred to as 'mycotoxins', which, once ingested, have serious consequences on consumers' health (Creppy, 2002; Sengun *et al*., 2008).
The fungal biota is widely distributed in dairy environments, originating from multiple sources, including the raw milk, the indoor air, the processing equipment, brine, starter cultures (Banjara *et al*., 2015) as well as deliberately added ingredients, such as milk coagulants.

Based on these premises, the aim of the present study was to investigate the fungal diversity and dynamics of a central Italian speciality raw ewes' milk cheese, locally known as 'Caciofiore della Sibilla', throughout its manufacturing and ripening, as affected by milk coagulation with a crude extract of *Carlina acanthifolia* All. subspecies acanthifolia. The ancient procedure for the preparation of the vegetable coagulant has been handed down from father to son for generations; it is based on the use of new fresh leale containing a considerable quantity of latex – of carline thistle spontaneously grown in the high-altitude pastures (>1000m above sea level) of the Monti Sibillini National Park. The availability of young leale within a very short period of time, spanning late spring to early summer, limits the manufacture of
this niche cheese to this time window. If on the one hand Caciofiore della Sibilla represents a small-batch cheese, produced and consumed in a constricted geographical area, on the other hand it represents a magnificent model of Mediterranean cheese made with carline thistle rennet, whose exploitation in dairy transformations has never yet been documented. Accordingly, the fungal communities of both Caciofiore della Sibilla and a control ewes' milk cheese manufactured using commercial animal rennet were analysed through a combined culture-dependent and –independent (PCR–DGGE) approach, relying on the analysis of the fungal DNA extracted directly from samples and the bulk of cells harvested from selected agar dilution plates.

15.1 Materials and methods

15.1.1. Yeast and mould reference strains

Two DGGE ladders, referred to as Mix′ and Mix″, were constructed using five yeast and three mould reference strains, respectively. These were *Wickerhamomyces anomalus* DBVPG 6613, *Starmerella bombicola* DBVPG 3827, *Candida humilis* CBS 6897T, *Saccharomyces cerevisiae* CBS 1171T, *Kazachstania exigua* DBVPG 6481, *Alternaria alternata* M9, *Cladosporium* spp. M5 and *Mucor racemosus* M12. The yeasts were purchased from the Industrial Yeasts Collection (DBVPG; University of Perugia, Italy; http://www.dbvpg.unipg.it/index.php/en/) and the Centraalbureau voor Schimmelcultures (CBS; Utrecht, The Netherlands; http://www.cbs.knaw.nl/index), whereas moulds had previously been isolated and molecularly identified in our laboratory (Garofalo *et al*., 2012).

15.1.2. Cheese - making and sampling

Cheese-making trials were conducted in a familyrun dairy farm located in the Monti Sibillini National Park (Marche Region, Central Italy), following a traditional manufacturing method (for a flow chart, without any addition of starter culture. The raw milk obtained from ewes of the breed *Sopravissana* during one milking day was split into two batches. The first batch, labelled 'Cf', was coagulated with a crude extract from young fresh leaves of *C. acanthifolia* All. subsp. *acanthifolia*. The crude extract was prepared as follows: the petioles of fresh young leaves from four plants of *C. acanthifolia* All. subsp. *acanthifolia*, harvested from an uncultivated foothill area of the Monti Sibillini National Park, were carefully peeled, chopped with a mezzaluna knife and macerated in an aqueous solution of wine vinegar (1:1 ratio) for ~18 h at room temperature. The preparation was filtered through a muslin cloth and ~100 ml crude extract were used to clot ~10 l raw ewes' milk. The second batch, labelled 'C', was coagulated
with commercial powdered calf rennet (Caglificio Clerici, Cadorago, CO, Italy; 1:10000). For each batch, filtered raw milk was poured into a foodgrade steel coagulation vat, heated at ~35–36°C, the coagulant added and gently stirred.
After clotting (ca. 30 min), both curds were manually broken into rice-sized grains, then transferred into 8×8 cm plastic perforated moulds and manually pressed to drain the whey. Moulded cheeses were held at 12–13 °C for 4 h, dry salted and ripened for 20 days at 12–13°C and 70% relative

humidity. For each batch of milk, three cheese wheels were produced. During cheese making and ripening, the following samples were collected in triplicate and transported to the laboratori under refrigerated conditions (+4°C): raw ewes' milk; fresh young leaves of *C. acanthifolia* All. subsp. *acanthifolia*; aqueous crude extract; curds; and cheeses after 1, 3, 6, 10 and 20 days of ripening. Prior to viable counting and PCR–DGGE analyses, triplicate samples were pooled and processed within 24 h.

15.1.3. pH measurements

All the collected samples, except for the fresh young leaves of *C. acanthifolia* All. subsp. *acanthifolia*, underwent pH measurement using a pH meter (Model 300, Hanna Instruments, Padova, Italy) equipped with a solid electrode (Model HI2031).
For each sample, three independent measurements were performed and means were compared using the Least Significant Differences (LSDs).

15.1.4. Viable cell counting and bulk formation

Aliquots of solid samples (10 g) were homogenized in 90 ml sterile peptone water (0.1% peptone) with a Stomacher apparatus (400 Circulator, International PBI, Milan, Italy) for 2 min at 260 rpm (Osimani et al., 2009). Liquid samples (raw ewes' milk and crude extract) and the homogenates were serially diluted and an aliquot (100µl) of each dilution was inoculated in duplicate on a Rose Bengal Agar (RBA; Oxoid) base, supplemented with chloramphenicol (0.1 g/l) and incubated at 25°C for 48 h. Viable counts were expressed as means of the Log colony-forming units (CFU)/g or /ml of sample.
After viable counting, bulk cells were prepared using the method described by Ercolini et al. (2001) and a saline solution with added glycerol for cell suspension (Garofalo et al., 2015). For all the samples except cheeses, colonies were harvested from selected RBA plates showing confluent colonies (low sample dilution) and a number of colonies in the range 30–300 (high sample dilution); for cheeses, the sole colonies grown on countable plates were suspended in 2ml saline solution with added glycerol, harvested with a sterile pipette and stored at –20°C.

15.1.5. DNA extraction

Direct extraction of fungal DNA from samples collected during cheese making and ripening was performed using a commercial kit (PowerFood™ Microbial DNA Isolation Kit,Mo Bio Laboratories, Carlsbad, USA). Briefly, 1.5 ml of raw milk, carline thistle rennet, curd and cheese homogenates were transferred into sterile 2ml tubes and centrifuged at 12 000×g for 10 min. For milk and cheese samples, after centrifugation the fat layer on the top was removed with a sterile cotton tip before discarding the supernatant. Extraction of fungal DNA from bulk cells and from the pure reference strains was performed using the method proposed by Makimura *et al*. (1999). All the DNA suspensions were subjected to optical readings at 260, 280 and 234nm using a UV-Vis spectrophotometer (Shimadzu, Kyoto, Japan) for the assessment of quantity and purity.

15.1.6. PCR–DGGE analysis

Amplification of the D1–D2 regions of the 26S rRNA gene was performed using primers NL1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′), with the GC clamp described by Sheffield *et al*. (1989) added at the 5′ end, and LS2 (5′-ATT CCC AAA CAA CTC GAC TC-3′) (Cocolin *et al*., 2000); 2µl fungal DNA (aduste to 50 ng/µl) were amplified, as reported by Osimani *et al*. (2015), in the thermal cycler My Cycler (Bio-Rad Laboratories, Hercules, CA, USA); aliquots (5µl) of the PCR products were routinely checked for positive amplification on 1.5% w/v agarose gels prior to

further DGGE analysis. PCR products were then separated in 8% polyacrylamide (acrylamide/bis-acrylamide mix 37.5:1 w/v) gels, with a denaturant gradient made of urea/formamide 30–60% w/v, using the DGGE Bio-Rad D-code™ apparatus (Bio-Rad Laboratories). The gels were subjected to a constant voltage of 130V for 4 h at 60°C. After electrophoresis, the gels were stained in 1× TAE buffer containing SYBR Green I Stain 1X (Lonza, Walkersville, MD, USA) and photographed under UV transillumination, using the Complete Photo XT101 system (Explera, Jesi, Italy). DGGE bands (labelled with unique and progressive numerical codes), selected among those migrating at the same position in the acrilamide gels, were excised from the gels with sterile pipette tips and transferred into microtubes
containing 50µl sterile deionized water for elution of DNA. After overnight incubation at 4°C, 5µl DNA suspension was re-amplified with the same primer set (the forward primer deprived of the GC clamp) and sent to Beckman Coulter Genomics (Hope End, Takeley, UK) for purification and sequencing.
The sequences obtained in FASTA format were compared with those deposited in the GenBank DNA database (http://www.ncbi.nlm. nih.gov/), using basic BLAST search tools (Altschul *et al*., 1990). A sequence identity ≥97% was chosen as a threshold for unambiguous assignment into species. Sequences>200 bp have been deposited in the NCBI GenBank data library under Accession Nos KU196966–KU197012.

15.1.7. Statistical analysis

Viable counts and pH data recorded during cheese ripening at five selected time points (1, 3, 6, 10 and 20 days of maturation) were analysed by a multivariate analysis of variance (MANOVA) for repeated measures. For each sampling time, viable counts and pHs of both batches were compared according to Student's t-test (α=0.05). All statistical analyses were performed using JMP statistical software v. 11.0.0 (SAS Institute, Cary, NC, USA).

15.2. Results

15.2.1. pH measurement and viable counting of fungi

Results from MANOVA demonstrated a significant effect of the cheese batch, Cf or C ($p<0.0001$) and ripening time ($p<0.0001$) on fungal viable counts.
A significant effect of both cheese batches ($p=0.0410$) and ripening time ($p=0.0319$) on pH was also seen, although for the latter parameter the variation during ripening was not consistent over time ($p=0.0295$) (Table 10). The results of pH measurement and viable counting are reported in Table 11. With regard to pH, a drop was seen throughout the two cheese-making processes, with significant differences occurring between curds and cheeses from batches Cf and C, until day 10 of ripening. By contrast, after 20 days of maturation, Caciofiore della Sibilla and the control ewes' milk cheeses reached comparable pH values. Regarding the aqueous extract from carline thistle, a very low pH value (3.79), feasibly due to the high concentration of acetic acid, was recorded.
Regarding the microbiological analyses, in both cheese manufactures the fungi progressively increased from the raw milk (2.42 log CFU/ml) to cheese until day 10 of maturation, whereas after 20 days the load of this microbial group decreased in both cheese manufactures. Fungal viable counts were consistently higher on Cf compared to C throughout the whole process. Relatively high fungal loads were also found in the phyllosphere of *C. acanthifolia* All. (5.35 log CFU/g) and in the crude extract from crushed fresh young leaves (3.54 log CFU/g) (Table 11).

Effect	DF	F	Fungal viable counts *	F	pH
Cheese batch	1	61592.9	<0.0001*	8.85	0.0410*
Ripening time	4	1088.8	<0.0001*	550.73	0.0319*
Cheese batch × ripening time	4	84.2	0.0815	647.17	0.0295

Tab. 10 Multivariate analysis of variation (MANOVA) on repeated measures for fungal viable counts and pH values testing the effect of cheese batch, ripening time and their interaction.
DF, Degree of freedom F, F-Value of the test

	pH		
Sample	Caciofiore della Sibilla (Cf)	Control Cheese (C)	p
Raw milk	6.39		
Carline thistle rennet	3.79	n.a.	
Curd	5.14 *	6.43	<0.0001
1-day ripened cheese	5.22_{c}	5.84_{a}	<0.0001*
3-day ripened cheese	5.31_{b}	5.04_{b}	<0.0001*
6-day ripened cheese	5.38_{a}	5.08_{b}	<0.0001*
10-day ripened cheese	5.23_{c}	4.97_{b}	0.0002*
20-day ripened cheese	5.02_{d}	4.94_{b}	0.4169
LSD	0.048	0.177	
	Fungal viable counts (log CFU/g or CFU/ml)		
	Caciofiore della Sibilla (Cf)	Control cheese (C)	
Raw milk	2.42		
Phyllosphere of Carlina acanthifolia All.	5.35	n.a	
Carline thistle rennet	3.54.	n.a	
Curd	3.50	3.51	0.8026
1-day ripened cheese	5.13_{d}	4.65_{e}	<0.0001*
3-day ripened cheese	7.12_{c} *	6.03_{c}	<0.0001
6-day ripened cheese	7.41_{b}	6.71_{b}	0.0003*
10-day ripened cheese	7.89_{a}	6.83_{a}	<0.0001*
20-day ripened cheese	7.43_{b}	5.42_{d}	<0.0001*
LSD	0.156	0.098	

Tabella 11 pH and viable counts of fungi, expressed as mean values of samples collected during cheese-making and ripening of Caciofiore della Sibilla and control ewes' milk cheese. Least significant differences (LSDs) are also reported. Mean separation test of pH and viable counts of fungi throughout ripening: different letters on the same

column indicate significant differences over time according to Tukey's test (α = 0.05). *Occurrence of significant differences due to cheese batch (C or Cf) according to Student's t-test (α = 0.05). n.a., not applicable.

15.2.2. PCR–DGGE analyses

The results of taxa identified are reported are listed in Table 12-13. The DGGE profiles obtained from analysis of the fungal DNA extracted from the samples and the bulk cells are shown in Figures 24 and 25. The analysis of the fungal microbiota characterizing the phyllosphere of *C. acanthifolia* All. and the vegetable coagulant revealed the occurrence of *Debaryomyces hansenii* and *Candida zeylanoides*, respectively.

Other yeast species were also detected in these samples: *Candida parapsilosis* plus *Galactomyces candidus*, which is better known by the anamorphic name *Geotrichum candidum*, in the vegetable coagulant; and *Rhodotorula mucilaginosa* plus *Cladosporium coralloides* on the phyllosphere of *C. acanthifolia* All., respectively; in the latter sample, the mould species *Fusarium oxysporium* was also found.

As regards the raw milk, curds and cheeses sampled during ripening, a higher biodiversity of fungi was seen with respect to milk, with numerous yeasts and moulds detected. A few taxa were exclusively found in the raw milk (*Saccharomyces cerevisiae*) or in the curd obtained by milk coagulation with commercial animal rennet (*Cladosporium cladosporioides*, *Cryptococcus wieringae*), whereas others occurred in different samples, as was the case for *Candida membranifaciens*, *C. parapsilosis*, *C. zeylanoides*, *D. hansenii*, *G. candidum*, *Kluyveromyces marxianus*, *Meyerozyma guilliermondii*, *Penicillium* sp., *C. incospicua* (teleomorph *Pichia cactophila*), *Pichia kluyveri*, *P. kudriavzevii*, *Rhizophus stolonifer*, *R. Mucilaginosa* and *Yarrowia lypolitica*. Among these, some taxa could be detected exclusively in the raw milk and during the early stage of maturation (*C. parapsilosis*, *R. mucilaginosa*), whereas others occurred occasionally (e.g. *C. zelanoydes*, *G. candidum*) or stably (*D. hansenii*, *P. kudriavzevii*, *R. stolonifer*) throughout the cheesemaking and ripening period. Furthermore, species apparently associated to one cheese manufacture rather than the other were also encountered, such as *M. guilliermondii* and *K. marxianus*, which were found exclusively in the Caciofiore della Sibilla and control ewes' milk cheese, respectively.

		Candida inconspicua	*Candida membranifaciens*	*Candida parapsilosis*	*Candida zeylanoides*	*Cladosporium coralloides*	*Cladosporium cladosporioides*	*Cryptococcus wieringae*	*Debaryomyces hansenii*	*Fusarium oxysporium*	*Geotrichum candidum*
	Raw milk			•	•				•		
	Phyllosphere of Carlina acanthifolia All.				○	●■			•	●■	
	Carline thistle rennet			•	○□				●■		■
	Curd	•		•	○				●■		■
	Cheese t1	•	•		•				●■		
Caciofiore della Sibilla cheese	Cheese t3	•							■		●■
	Cheese t6								●■		
	Cheese t10								●■		
	Cheese t20	•			■				■		●■
	Curd		•	•	•		■	•	•		
	Cheese t1		•		•				●■		
Control cheese	Cheese t3	•			•				●■		
	Cheese t6								●■		•
	Cheese t10		•		●■				●■		•
	Cheese t20				•				●■		•

Tab. 12 **Yeasts and moulds identified by PCR-DGGE analysis during the manufacture and ripening of Caciofiore della Sibilla and control ewes' milk cheeses**

●DGGE bands resulting from analysis of the DNA extracted from the bulk of colonies harvested from selected Rose Bengal chloramphenicol agar (RBA) plates, showing>97% of similarity with the sequences of the closest relatives found by a BLAST search in GenBank.

○DGGE bands resulting from analysis of the DNA extracted from the bulk of colonies harvested from selected RBA plates, showing<97% of similarity with the sequences of the closest relatives found by a BLAST search in GenBank.

■ DGGE bands resulting from analysis of the DNA extracted directly from samples collected during cheese making, showing ≥ 97% of similarity with the sequences of the closest relatives found by a BLAST search in GenBank.
□DGGE bands resulting from analysis of the DNA extracted directly from samples collected during cheese making, showing<97% of similarity with the sequences of the closest relatives found by a BLAST search in GenBank. t1, cheese sampled after 1 day of maturation; t3, cheese sampled after 3 days of maturation; t6, cheese sampled after 6 days of maturation; t10, cheese sampled after 10 days of maturation; t20, cheese sampled after 20 days of maturation.

		Kluyveromyces marxianus	*Meyerozyma guilliermondii*	*Penicillium sp.*	*Candida inconspicua*	*Pichia kluyveri*	*Pichia kudriavzevii*	*Rhizophus stolonifer*	*Rhodotorula mucilaginosa*	*Yarrowia lipolytica*	*Saccharomyces cerevisiae*
	Raw milk	•							•		□
	Phyllosphere of Carlina acanthifolia All.								•		
	Carline thistle rennet										
	Curd		•		•				•		
	Cheese t1				•	•	•	■	•		
Caciofiore della Sibilla cheese	Cheese t3		•	■	•	•	•	■		•	
	Cheese t6			■			•	■			
	Cheese t10			■				■			
	Cheese t20		•		•	•	•	■		•	
	Curd	•							•		
	Cheese t1	•				•	•	■	•	•	
Control cheese	Cheese t3	•		■	•		•	■			
	Cheese t6			•			•				
	Cheese t10			■				■			
	Cheese t20	•		■			•	■			

Tab. 13 Yeasts and moulds identified by PCR–DGGE analysis during the manufacture and ripening of Caciofiore della Sibilla and control ewes' milk cheeses.

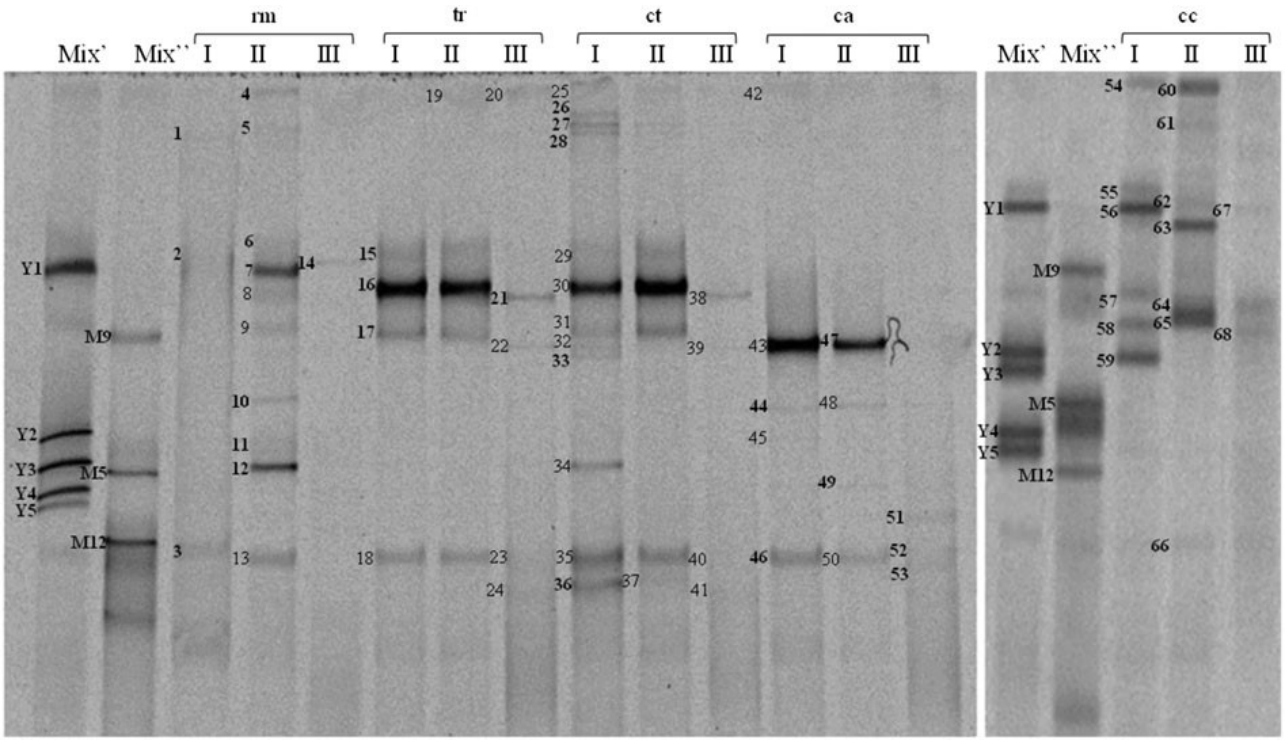

Fig. 24. Fungal DGGE profiles of DNA extracted directly from the bulk of colonies harvested from Rose Bengal chloramphenicol agar (RBA) plates spiked with the highest and lowest dilutions (lanes I and II) and of DNA extracted directly from samples (lane III) of raw ewes'milk (rm), carline thistle rennet (tr), curd obtained by milk coagulation with carline thistle rennet (ct), fresh young leaves of Carlina acanthifolia All. (ca) and curd obtained by milk coagulation with commercial animal rennet (cc). Ladder (Mix'): Wickerhamomyces anomalus DBVPG 6613 (Y1), Starmerella bombicola DBVPG 3827 (Y2), Saccharomyces cerevisiae CBS 1171T (Y3), Kazachstania exigua DBVPG 6481 (Y4) and Candida humilis CBS 6897T (Y5). Ladder (Mix"): Alternaria alternata (M9), Cladosporium spp. (M5) and Mucor racemosus (M12).

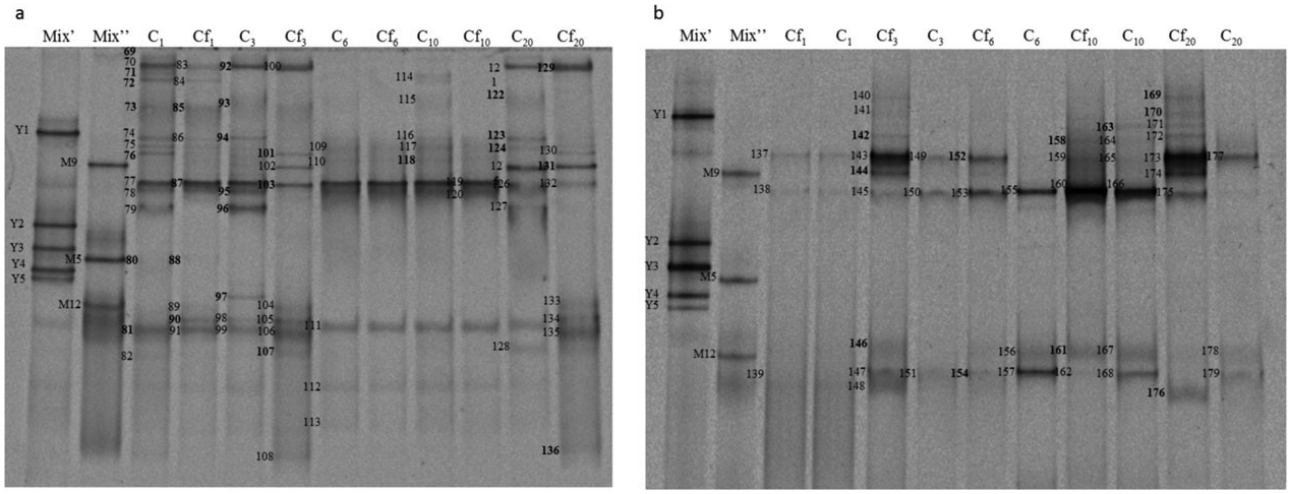

Fig. 25 Fungal DGGE profiles of the DNA extracted from (a) the bulk of colonies harvested from selected RBA plates and (b) samples of Caciofiore della Sibilla cheese (batch Cf) and control raw ewes' milk cheese (batch C) collected at days 1, 3, 6, 10 and 20 of ripening (Cf1, Cf3, Cf6, Cf10, Cf20 and C1, C3, C6, C10, C20, respectively). Ladder (Mix'): Wickerhamomyces anomalus DBVPG 6613 (Y1), Starmerella bombicola DBVPG 3827 (Y2), Saccharomyces cerevisiae CBS 1171T (Y3), Kazachstania exigua DBVPG 6481 (Y4) and Candida humilis CBS 6897T (Y5). Ladder ("Mix"): Alternaria alternata (M9), Cladosporium spp. (M5), Mucor racemosus (M12).

15.3. Discussion

Numerous studies have previously been focused on the effect of thistle coagulants, especially made from *C. cardunculus*, *C. scolymus* or *C. humilis*, on the microbiological properties of traditional cheeses (Vioque *et al*., 2000; Roseiro et al., 2003; Fernández-Salguero *et al*., 2002) but, to the author's knowledge, this is the first attempt to shed light on the contribution of thistle coagulants to the fungal diversity and dynamics of the cheese.

Furthermore, this is the first report describing the exploitation of the botanical species *C. acanthifolia* All. in cheese-making. This research also contributes to the assessment of the fungal load and diversity in thistle phyllosphere and raw ewes' milk, which are two poorly investigated ecosystems.

Concerning the latter ecosystem, comparable or slightly lower viable counts were seen compared with those reported by other authors for raw milk (Vioque *et al*., 2000; Roseiro *et al*., 2003; Pangallo *et al*., 2014; Gardini *et al*., 2006; Tejada and Fernández-Salguero, 2003). As has previously been elucidated, the relatively low contamination of raw milk with fungi can be mainly ascribed to the competitive interaction for growth substrates by psychrotrophic bacteria or the excretion of inhibiting metabolites by these microorganisms (Viljoen, 2001).

By contrast, the phyllosphere of *C. Acanthifolia* All. was found to be colonized by a relatively high load of yeasts and moulds, thus supporting the assumption of an effective role of the vegetable coagulant in the contamination of the cheese milk with these microorganisms. However, no significant differences ($p=0.8026$) were observed in the curds made with vegetable and animal rennet, respectively, thus denying such an assumption.

As regards cheeses in the early stage of maturation, after 1 day of ripening, viable counts were almost 3 and 2 Log units higher than those initially found in the raw milk, respectively; in both cheese manufactures (batches Cf and C), yeasts and moulds reached the highest load after 10 days of maturation. However, a significantly higher evolution of the fungal community was seen throughout the ripening of Caciofiore della Sibilla compared with the control ewes' milk cheese. In other studies, carried out on traditional Iberian cheeses manufactured with vegetable coagulants from thistle flowers, a relatively poorer occurrence of fungi was seen in cheeses after 15 days of maturation, with viable counts approximately two or three orders of magnitude lower (Gómez *et al*.,

2001; Vioque *et al*., 2000; Tejada and Fernández-Salguero, 2003). Although this represents interesting evidence, is not possible to determine whether it was associated with the specific cheese batch or to this type of cheese.

The PCR–DGGE fingerprinting of samples collected during cheese-making and ripening allowed the evolution of the fungal microbiota to be traced and the potential sources of curd and cheese contamination with yeasts and moulds to be identified. These microorganisms can grow either in the cheese core or on the rind, being favoured by the peculiar physicochemical properties characterizing the cheese environment, such as the low pH, the reduced water activity, the high salt concentration and the low storage temperature (Padilla *et al*., 2014; Cheong *et al*.,2014). The composition of the cheese fungal biota has been found to change among cheeses of different varieties, cheeses of the same variety from different producers and even cheese batches of the same variety from the same producer (Banjara *et al*., 2015).

In the present study, a wide range of yeast species was identified with the double PCR– DGGE approach; most of these taxa had previously been found in raw milk, curd or cheese, including *K. marxianus* (Macedo *et al*., 1995; Lavoie *et al*., 2012; Quigley *et al*., 2013), *D. hansenii*, *S. cerevisiae* and *Rhodotorula* spp. (Capece and Romano, 2009; Cosentino *et al*., 2001; Fadda *et al*., 2001). Of these, *K. Marxianus* is known to have a positive impact on cheese quality, either as a result of the development of flavour compounds during maturation or because of partial de acidification due to lactate/lactose consumption, the latter enhancing the growth of acid-sensitive bacteria (Corsetti *et al*., 2001). *D. hansenii* has also been reported to play a key role during cheese ripening, for the ability of this yeast, growing in high salt concentrations and low pH (Beresford *et al*., 2001; Büchl and Seiler, 2011; Padilla *et al.,* 2014), to utilize several carbon and nitrogen sources and to produce proteolytic and lipolytic enzymes capable of metabolizing milk proteins and fat (Banjara *et al*., 2015; Cardoso *et al*., 2015). A high proteolytic and lipolytic activity also characterizes the majority of strains ascribed to *R. Mucilaginosa* (Fadda *et al*., 2010; Cardoso *et al*., 2015), a further yeast species frequently occurring in raw milk and cheese (Fadda *et al*., 2010; Pereira-Dias *et al*., 2000; Corbo *et al*., 2001; Cocolin *et al.,* 2002; Borelli *et al*., 2006; Quigley *et al*., 2013; Lavoie *et al*., 2012).

Other species, such as *C. membranifaciens*, *G. candidum* and *P. inconspicua* have been more rarely identified in milk-based products, whereas, to the authors' knowledge, this is the first report of *M. guilliermondii* in raw ewes' milk cheese.

To date, this yeast, which is commonly isolated from fruit surfaces (Pelliccia *et al*., 2011), where it shows a great potential for the postharvest control of spoilage fungi (Corte *et al*., 2015), has been detected in goats' and cows' milk cheeses, as well as in butter milk, yogurt and the dairy environment (Büchl and Seiler, 2011; Giannino *et al*., 2011; Callon *et al*., 2006). Its capacity to produce flavour compounds in fermented food products is acknowledged (Wah *et al*., 2013).

Other interesting evidence emerged by comparing the fungal dynamics in the Caciofiore della Sibilla and the control ewes' milk cheeses.

First, the fingerprinting of the fungal biota occurring in samples collected at different steps during the two cheese-making processes allowed a microbial succession to be observed in both cheese manufactures, with some taxa occurring in the sole raw milk (*S. cerevisiae*), others in the early (*C. parapsilopsis* and *R. mucilaginosa*) or late (Penicillium sp.) stages of fermentation and maturation, and further species occurring throughout the whole process (*C. Zeylanoides* and *D. hansenii*). In addition to the species that are well adapted to the cheese environment and stably detected in cheeses during maturation, such as *C. membranifaciens*, *P. kluyveri*, *P. kudriavzevii*, *R. stolonifer* and *Y. lipolytica*, other species occurring as occasional contaminants were also sporadically found, such as *C. cladosporioides* and *C. wieringae*.

Among the overall taxa identified, only *M. guilliermondii* and *K. marxianus* were exclusively found in the Caciofiore della Sibilla and control ewes' milk cheese, respectively.

As regards cheese contamination, multiple sources, such as the vegetable coagulant, the raw milk and the dairy environment, can be assumed to have played a role; in more detail, *C. parapsilosis*, *C. zeylanoides*, *K. marxianus* and *R. Mucillaginosa* feasibly originated from the raw milk, whereas for *C. membranifaciens*, *C. incospicua*, *P. kluyveri*, *P. kudriavzevii*, *R. stolonifera* and *Y. lipolytica*, cheese contamination from the dairy environment can be hypothesized.
Although no species occurring in the vegetable coagulant were found to distinctively characterize the Caciofiore della Sibilla cheese, the joint contribution of thistle rennet together with the raw milk and the dairy environment to the fungal
diversity of this speciality cheese cannot be denied.

16. Impact of thistle rennet from *Carlina acanthifolia* All. subsp. *acanthifolia* on bacterial diversity and dynamics of a specialty Italian raw ewes' milk cheese

Caciofiore della Sibilla is a specialty soft cheese manufactured in a restricted central Italian geographical area using *Sopravissana* raw ewes' milk and thistle rennet obtained from young fresh leaves and stems of *Carlina acanthifolia* All. subsp. *acanthifolia* according to an ancient local tradition.
The term "thistle" refers to plants belonging to the tribe Cardueae Cass. = Cynareae Less. (Cassini, 1819) especially ascribed to the genera *Carduus*, *Cirsium*, *Onopordum*, *Cynara*, *Scolymus*, *Silybum*, *Onopordum,* and *Carlina*. In the Mediterranean area, the exploitation of thistle rennet, especially that obtained from *Cynara* spp., is particularly widespread in western Africa (Cardinali *et al*., 2016; García *et al*., 2012), Italy, and the Iberian peninsula, the latter boasting a large number of Protected Designation of Origin (PDO) cheeses manufactured with this peculiar coagulant (Aquilanti *et al*., 2011; Cardinali *et al*., 2016). Cheeses coagulated with thistle rennet are generally manufactured at family-run or artisan dairy farms, most often located in marginal areas (e.g., high altitude pastures, dry lands or islands) using raw ewes' or goats' milk, or a mixture of both. Though these cheeses are greatly appreciated by consumers for their unique, distinctive flavor, their manufacturing is generally seasonal, mainly due to limitations on the availability of young leaves or flowers from spontaneously growing thistles.
Caciofiore della Sibilla cheese undoubtedly falls within this cheese category. The manufacturing technology of this cheese, which had been lost for> 50 years in the original area of production, has very recently been revived by two local family-run dairies located in Pieve Torina and Belforte del Chienti (Macerata district), respectively. The cheese produced by these local dairies from late spring to early summer is 3–4 cm tall, has an average weight of 0.2 to 0.8 kg and is characterized by a very thin straw-white outer rind and a cream-white soft core, with a sweetish buttery smell, a delicate but incisive flavor with a scent of wild herbs, and a slightly acidulous, pleasant taste.
To date, few studies have been conducted to identify the bacterial biota harboured by thistle-rennet cheeses (Aquilanti *et al*., 2011; Fernández-Salguero *et al*., 2002; Galán *et al*., 2012; Gómez *et al*., 2001; Ordiales *et al.*, 2013; Roseiro *et al*., 2003; Sousa and Malcata, 1997; Tejada and Fernández-Salguero, 2003; Vioque *et al*., 2000) and almost all of these investigations have been focused on Protected Designation of Origin (PDO) or specialty cheeses manufactured with thistle coagulants obtained from *Cynara* spp.
Based on these premises, this study uses a polyphasic molecular approach based on culture and DNA-based techniques to assess the impact of an unexplored milk coagulant obtained from *Carlina acanthifolia* All. subsp. *acanthifolia* on the bacterial dynamics and diversity of *Caciofiore della Sibilla* cheese. To this end, two cheese manufactures produced with the same batch of *Sopravissana* raw ewes' milk and coagulated with either thistle rennet or commercial powdered animal rennet were analyzed in parallel and the results comparatively evaluated.

16.1. Materials and methods

16.1.1. Cheese-making process and sampling

Caciofiore della Sibilla cheese was made in a family-run dairy farm located in Pieve Torina (Italy) following an ancient local manufacturing method without any addition of starter cultures.
The sole *Caciofiore della Sibilla* cheese manufacture produced by the dairy farm during spring and summer 2015 was sampled and analyzed. Raw milk obtained from Sopravissana ewes during one milking day was filtered and separated into two batches; three cheese wheels were produced from each batch. The first batch (labelled "C") was coagulated with commercial powdered calf rennet (Caglificio Clerici, Cadorago, CO, Italy; 1:10,000), whereas the second batch (labelled "Cf") was coagulated with a crude aqueous extract prepared from *C. acanthifolia* All. subsp. *acanthifolia*. The

traditional preparation of the crude aqueous extract based on the use of fresh young leaves and stems - containing a considerable quantity of latex – of plants spontaneously growing in the high altitude pastures (> 1000 m a.s.l.) of Monti Sibillini National Park has previously been detailed by Cardinali *et al*. (2016). Milk coagulants were added to raw milk pre-heated at 35 °C and gently stirred. After clotting (ca. 30′), the curds were manually broken into rice-sized grains, transferred into plastic perforated molds (8 ×8 cm) and manually pressed to remove the whey. Molded cheeses were held at 12–13 °C for 4 h, dry salted and ripened for 20 days under controlled conditions (12–13 °C and 70% relative humidity).
Ewes' raw milk, fresh young leaves and stems of *C. acanthifolia* All. subsp. *acanthifolia*, thistle rennet, curds and cheeses (after 1, 3, 6, 10 and 20 days of ripening) were collected in triplicate. Samples were transported to the laboratori under controlled temperature (4 °C) and processed within 24 h. Triplicate samples were pooled before viable counting and molecular analysis.

16.1.2. pH measurements

The pH of samples collected during cheese-making was measured using the pH meter 300 (Hanna Instruments, Padova, Italy), equipped with a solid electrode (HI2031, Hanna Instruments). Three independent measurements were performed for each sample, and the mean values ± standard deviations were calculated.

16.1.3. Microbial counts and bulk formation

For the microbial counts, 10 g of each solid sample were accurately homogenized in 90 mL of sterile aqueous citrate (2% w/v) for 2 min at 260 rpm using a Stomacher apparatus (400 Circulator, VWR International PBI, Milan, Italy) (Osimani *et al*., 2009). Aliquots of decimal dilutions of raw milk, thistle rennet and the homogenates were inoculated in duplicate on opportune solid media to determine the load of (i) presumptive lactococci, thermophilic cocci and lactobacilli; (ii) coagulase-negative cocci; (iii) total mesophilic aerobes; (iv) enterococci; (v) and finally *Enterobacteriaceae* (Garofalo *et al*., 2017). Viable counts were expressed as log colony forming units (log cfu) per gram or mL of sample ± standard deviations. For all samples but cheeses, to evaluate the profile of the cultivable bacterial community, bulk cells were prepared by harvesting confluent colonies from both the lowest MRSA and M17 dilution plates and the countable plates with a number of colonies ranging from 30 to 300 (Garofalo *et al*., 2015). For cheeses, the sole colonies grown on the countable plates were harvested.
Harvested cells were suspended in 2 mL of sterile saline solution added with glycerol, and stored at −20 °C prior to DNA extraction (Garofalo *et al*., 2015).

16.1.4. . DNA extraction from cheese samples and bulks

For raw milk, thistle rennet, curd and cheese homogenates, total microbial DNA was extracted using a commercial kit (PowerFood™ Microbial DNA Isolation Kit, Mo Bio Laboratories, Carlsbad, USA), as previously elucidated (Cardinali *et al*., 2016). For bulk cells, DNA extraction was performed using the method proposed by Hynes et al. (1992) with some modifications as reported by Osimani *et al.* (2015).
Assessment of quantity and purity of DNA extract was carried out as described by Osimani *et al*. (2016).

16.1.5. PCR amplification and DGGE analysis

The universal primers 338F (5′-ACT CCT ACG GGA GGC AGC AGC AG-3′), added with the GC clamp at the 5′ end (Ampe *et al*., 1999), and 518R (5′-ATTACC GCG GCT GCT GG-3′) were used to amplify the V3 region of the 16S rRNA gene (Alessandria *et al*., 2010).
Approximately 100 ng of bacterial DNA were amplified as previously reported by Osimani *et al*. (2015) using the thermal cycler My Cycler (BioeRad Laboratories, Segrate, Italy). DGGE runs were performer as reported by the same authors. Sequencing of DNA eluted from selected DGGE bands was performed in accordance to Taccari *et al*. (2016). Sequences were compared with those deposited in the GenBank DNA database (http://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (BLAST). A sequence identity ≥97% was chosen as a threshold for unambiguous assignment into species.

16.1.6. DNA amplification and Illumina sequencing

The extracted DNA was used to study the bacterial diversity of the samples (raw milk, thistle rennet, curds and cheeses) by 16S rRNA amplicon Illumina sequencing. A 464-nucleotide sequence of the V3-V4 region of the 16S rRNA gene was analyzed as previously described by Alfonzo *et al*. (2017).

16.1.7. Illumina data analysis and sequence identification by QIIME

Sequences obtained from Illumina sequencing were processed using Quantitative Insights Into Microbial Ecology (QIIME) software package version 1.9 (Caporaso *et al.*, 2010) as previously detailed by Alfonzo *et al*. (2017). The data generated by Illumina sequencing were deposited in the NCBI Sequence Read Archive (SRA) and are available under Ac. No. PRJNA340351.

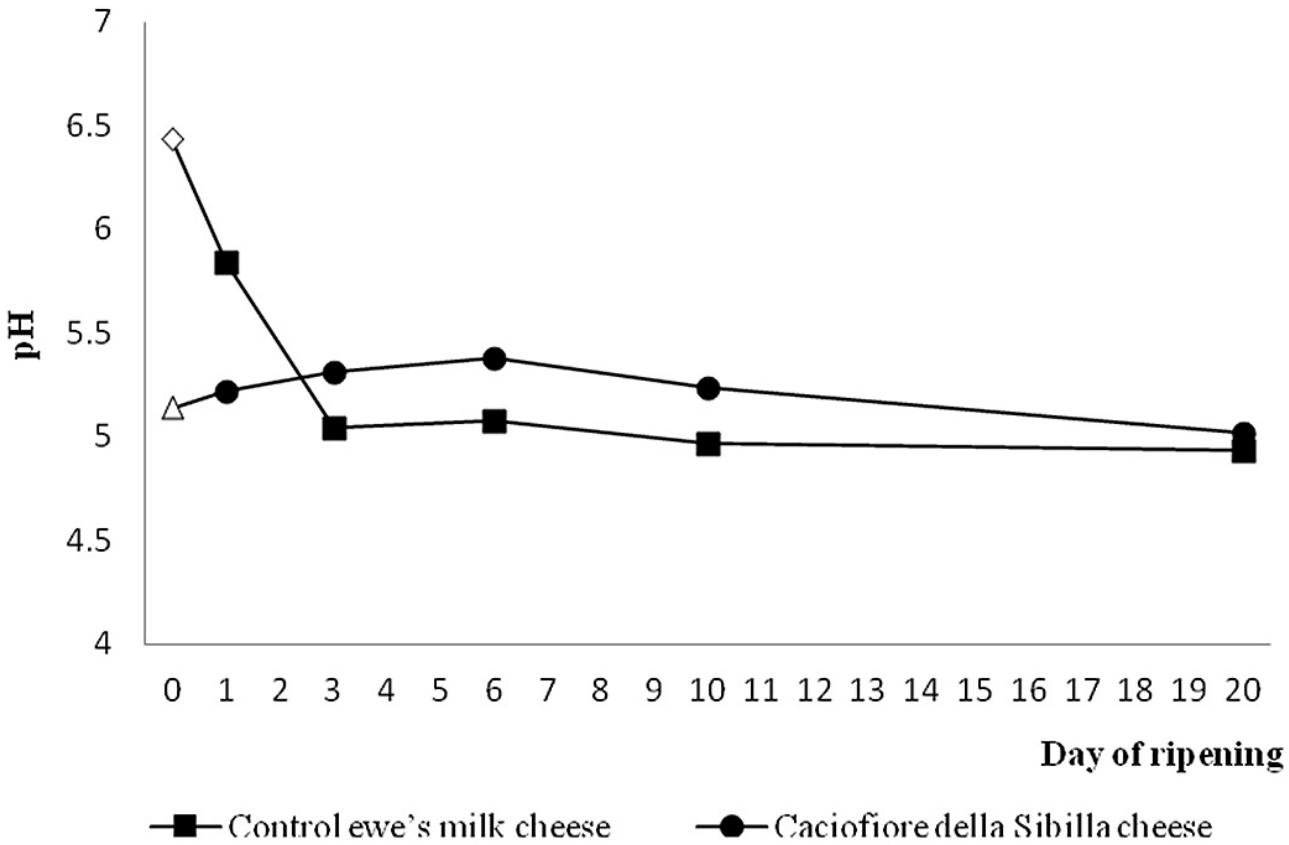

Figura 26 Results of pH measurements of curd obtained from milk coagulation with commercial rennet, curd obtained from milk coagulation with thistle rennet, Caciofiore della Sibilla and control ewes' milk cheese during cheese-making and ripening. * pH values of curd obtained from milk coagulation with commercial rennet (◊) and from milk coagulation with thistle rennet (Δ).

16.1.8. Statistical analysis

For each cheese batch, viable counts recorded after 1, 3, 6, 10 and 20 days of maturation were analyzed by an ANOVA and a Tukey's test (α =0.05) for means separation. At each sampling time, data from the two batches "C" and Cf" were compared using the Student's t-test (α =0.05). A principal component analysis (PCA) with a correlation matrix was carried out on standardized data to visually identify bacterial dynamics over time. All the statistical analyses were performer using JMP statistical software v. 11.0 (SAS Institute, Cary, NC, USA).

16.2. Results

16.2.1. pH measurement and viable counting

Raw ewes' milk and thistle rennet were characterized by pH mean values of 6.39 ± 0.10 and 3.79 ± 0.05, respectively; the results of pH measurements carried out in curds and cheeses sampled at different time points are reported in Fig. 26. As revealed by ANOVA, curds and cheeses coagulated with thistle and animal rennet significantly differed in their pH values up until the 10th day of ripening, whereas after 20 days of maturation, both cheeses reached similar pH values.
The results of viable counting are shown in Table 14. The phyllosphere of *C. acanthifolia* All. was characterized by high levels of total mesophilic aerobes, lactococci, *Enterobacteriaceae* and coagulase-negative cocci and, conversely, by low levels of presumptive thermophilic cocci, enterococci and lactobacilli. Low bacterial loads were seen in thistle rennet.

No significant differences were observed between raw ewes' milk and curds in the viable counts of presumptive lactobacilli, total mesophilic aerobes and enterococci, whereas significant differences were observe in the loads of presumptive lactococci, termophilic cocci and coagulase-negative cocci.
Viable counts of lactobacilli, total mesophilic aerobes, enterococco and *Enterobacteriaceae* increased progressively during the ripening of *Caciofiore della Sibilla* cheese; moreover, at the end of the maturation period (20 days), the two cheeses differed significantly in the load of all the bacterial groups assayed, except for lactobacilli and *Enterobacteriaceae*.
When PCA was computed, the first component accounted for 85.7% of total variability whereas the second component accounted for an additional 5.3% (Fig. 27). As clearly evidenced by the plot, the *Caciofiore della Sibilla* cheese had a greater range of variation in the bacterial loads than the control cheese, especially up until the 3rd day of maturation.

16.2.2. PCR-DGGE analyses

The DGGE profiles obtained by analyzing the DNA extracted from both the samples and the bulk cells harvested from selected dilution plates are shown in Fig. S1 and S2, whereas the results of multiple alignments of DNA sequences from selected DGGE bands. These results are summarized in Fig. 28 and 29. In the phyllosphere of *C. acanthifolia* All., the closest relatives to *Pantoea agglomerans*, *Pseudomonas alcaliphila*, *Acinetobacter baumannii*, the *Lact. alimentarius/paralimentarius* group, the *Lactobacillus graminis/curvatus* group, *Enterobacter* spp., *Pseudomonas* spp., and *Bacillus* spp. were found.
Members of the latter genus were also detected in thistle rennet, along with members of the *Lact. plantarum/paraplantarum/pentosus* group. In raw ewes' milk, the following taxa were detected: *Acinetobacter johnsonii*, *Enterobacter cloacae, Enterobacter hormaechei*, *Pseudomonas* spp. and *Lactococcus lactis*. The latter species was detected at both the lowest and highest dilution.
Completely different bacterial compositions were seen in the two curds: curd obtained with commercial animal rennet was dominated by *Enterobacteriaceae,* while curd obtained from thistle rennet was dominated by lactic acid bacteria. In general, the bacterial diversity of cheeses during ripening was higher than that of raw milk, thistle rennet and curds. Furthermore, if the two cheeses were comparatively evaluated, a few taxa, such as *E. faecium*, *Lact. brevis, Leuc. mesenteroides, Lact. delbrueckii* and *Weissella* spp., were exclusively found in *Caciofiore della Sibilla* cheese, whereas other taxa, such as *Bifidobacterium dentium*, *Citrobacter* spp., *E. hormaechei, Erwinia chrysanthemi* and the *Lact. alimentarius/paralimentarius* group, were found only in the control cheese. By contrast, the following bacteria were identified in both cheeses, namely, *Enterococcus feacalis*, *L. lactis*, the *Lactobacillus casei/paracasei/rhamnosus* group, the *Lact. plantarum/ paraplantarum/pentosus group and Staphylococcus sciuri*. With the exception of *Bacillus subtilis*, *A. johnsonii* and *P. alcaliphila*, all of the remaining taxa were detected in the highest dilution plates, thereby suggesting a load of at least 105–108 cfu/g.

16.2.3. Illumina sequencing

The DNA extracted from ewes' raw milk, curds and cheeses was successfully amplified in the bacterial V3-V4 16S rRNA gene region. After splitting and quality trimming of raw data, 62,772 reads remained for subsequent analysis. After alignment, Operational Taxonomic Units (OTUs) were clustered at a 3% distance and the doubles and singletons (OTUs counting only two or one reads, respectively) were discarded by a filter script implemented in QIIME. Chao1 estimator predicted an average of 688 and 231 ± 43 OTUs in milk and cheese samples, respectively, and the average observed counts were 661 and 176 ± 40 OTUs, respectively, suggesting that we were able

to capture approximately 96% and 76% of the OTUs estimated as present in the milk and cheese bacterial populations, respectively.
In most samples, a number of OTUs belonged to chloroplasts. These OTUs were not very abundant in raw milk, curd produced with animal rennet, and *Caciofiore della Sibilla* cheese at 3 and 20 days of ripening, accounting for< 1.2% of total reads number, with the exception of *Caciofiore della Sibilla* cheese at 3 days of ripening, where they constituted 10.6% of the total reads number. Conversely, chloroplast OTUs constituted the majority of the reads number in curd obtained with thistle rennet and *Caciofiore della Sibilla* cheese collected after 1 day of ripening, accounting for 81.9% and 78.5% of the total reads number, respectively. The relative abundance (%) of the different bacterial groups identified in raw milk, curds and cheeses are reported in Fig. 30. Only the groups with an incidence of 0.1% were considered. The majority of milk OTUs belonged to three different groups: *Lactobacillales, Enterobacteriaceae* and *Clostridiales*, which together accounted for 60% of the total number of bacterial OTUs in raw milk. Various minority taxa were also identified, including *Staphylococcus,* P*lanococcaceae and Bacteroidales*.
As the bacterial dynamics were comparatively evaluated along with the two cheese making processes, the following evidence emerged. In curd obtained with commercial animal rennet, *Planococcaceae* and *Enterobacteriaceae* prevailed, followed by *Lactobacillales, Staphylococcus* and *Macrococcus*. *Enterobacteriaceae*, *Lactobacillales* and *Staphylococcus* were also found in curd obtained with thistle rennet. At the end of the maturation period, both cheeses were dominated by *Lactobacillales,* which accounted for approximately 65% of the total number of bacterial OTUs; however, different minority taxa were found, namely, *Enterobacteriaceae* in control ewes' milk cheese, and both *Macrococcus* and *Acidobacteria* in *Caciofiore della Sibilla* cheese.
In Fig. 31, a finer taxonomic composition within the order *Lactobacillales* is shown. In raw ewes' milk, OTUs corresponding to *Lactobacillus, Enterococcus*, *Lactococcus*, *Streptococcus*, *Carnobacterium*, *Leuconostoc* and *Weissella* were found, though for the latter two taxa, a relative abundance lower than 0.1% was seen. In curd and cheese obtained with thistle rennet, *Lactobacillus* and *Leuconostoc* co-dominated, whereas *Lactococcus* prevailed in curd and cheese produced with commercial animal rennet, followed by *Lactobacillus*. The two cheese manufactures also differed in the composition of minority taxa, with L*euconostocs* and *Pediococcus* occurring at low levels in both the control and *Caciofiore della Sibilla* cheese, respectively.

Sample	Caciofiore della Sibilla cheese Lactococci (M17 22 °C)	Control cheese		Caciofiore della Sibilla cheese Thermophilic cocci (M17 45 °C)	Control cheese		Caciofiore della Sibilla cheese Lactobacilli (MRSA)	Control cheese	
Raw milk	5.00 ± 0.06			4.08 ± 0.06			4.78 ± 0.02		
Carlina acanthifolia All.	6.68 ± 0.03			4.88 ± 0.04			3.54 ± 0.10		
Thistle rennet	3.13 ± 0.14			2.10 ± 0.28			4.01 ± 0.00		
Curd 4.11 ±	0.00 4.82 ±	0.12 4.67 ±		0.00 4.28 ±	0.02 4.90 ±		0.06 4.74 ±	0.11	
1 day of ripening	5.56 ± 0.02$_{d}$	8.57 ± 0. 01$_{c}$	< 0.0001*	4.68 ± 0.00$_{e}$	7.50 ± 0.06$_{b}$	0.0002*	5.06 ± 0.05$_{d}$	8.57 ± 0.00$_{b}$	0.0001
3 days of ripening	7.30 ± 0.00$_{c}$	7.98 ± 0.04$_{d}$	0.0016*	6.32 ± 0.01$_{d}$	7.37 ± 0.08$_{bc}$	0.0028*	8.08 ± 0.00$_{c}$	8.89 ± 0.01$_{ab}$	< 0.0001
6 days of ripening	8.62 ± 0.13$_{b}$	9.00 ± 0.14$_{b}$	0.1098	8.26 ± 0.00$_{b}$ *	8.18 ± 0.08$_{a}$	0.3251	8.66 ± 0.09$_{b}$	9.03 ± 0.01$_{a}$	0.0312
10 days of ripening	9.12 ± 0.18$_{a}$	9.14 ± 0.06$_{b}$	0.9113	8.31 ± 0.00$_{a}$	8.00 ± 0.02$_{a}$	0.0012*	8.68 ± 0.01$_{b}$	8.96 ± 0.01$_{a}$	0.0009*
20 days of ripening	8.90 ± 0.05$_{ab}$	9.52 ± 0.06$_{a}$	0.0079*	7.42 ± 0.03$_{c}$	7.24 ± 0.05$_{c}$	0.0450*	9.05 ± 0.03$_{a}$	8.87 ± 0.22$_{ab}$	0.3557

Sample	Caciofiore della Sibilla cheese	Control cheese	Caciofiore della Sibilla cheese	Control cheese	Caciofiore della Sibilla cheese	Control cheese	Caciofiore della Sibilla cheese	Control cheese

	Coagulase-negative cocci (MSA)		Total mesophilic aerobes (PCA)		Enterococci (Slanetz-Bartley Agar)		Enterobacteriaceae (VRBGA)	
Raw milk	4.01 ± 0.03		5.14 ± 0.16		4.30 ± 0.06		3.93 ± 0.06	
Carlina acanthifolia All.	5.24 ± 0.01		7.27 ± 0.04		3.97 ± 0.16		5.34 ± 0.03	
Thistle rennet	3.06 ± 0.03		4.07 ± 0.04		3.60 ± 0.01		1.00 ± 0.00	
Curd	5.06 ± 0.08	4.52 ± 0.04	4.99 ± 0.01	5.13 ± 0.07	4.37 ± 0.01	4.23 ± 0.05	1.10 ± 0.00	3.39 ± 0.16
1 day of ripening	5.16 ± 0.02$_{d}$	6.75 ± 0.10$_{e}$ 0.0022$_{*}$	5.37 ± 0.02$_{e}$	8.45 ± 0.03$_{a}$ < 0.0001$_{*}$	4.60 ± 0.05$_{e}$	6.09 ± 0.05$_{e}$ 0.0011$_{*}$	1.74 ± 0.06$_{e}$	7.55 ± 0.05$_{a}$ < 0.0001$_{*}$
3 days of ripening	6.34 ± 0.02$_{c}$	7.97 ± 0.04$_{d}$ 0.0003	$_{*}$ 8.15 ± 0.08$_{d}$	8.87 ± 0.02$_{a}$ 0.0056$_{*}$	5.14 ± 0.08$_{d}$	7.12 ± 0.02$_{c}$ 0.0099$_{*}$	2.19 ± 0.02$_{d}$	7.30 ± 0.01$_{b}$ < 0.0001$_{*}$
6 days of ripening	7.47 ± 0.04$_{b}$	8.91 ± 0.19$_{a}$ 0.0086$_{*}$	8.58 ± 0.00$_{c}$	8.71 ± 0.73$_{a}$ 0.8181	7.78 ± 0.00$_{c}$	7.55 ± 0.01$_{b}$ 0.0007$_{*}$	5.41 ± 0.02$_{c}$	7.13 ± 0.07 $_{b}$ 0.0009$_{*}$
10 days of ripening	8.72 ± 0.02$_{a}$	8.40 ± 0.03$_{b}$ 0.0059$_{*}$	8.82 ± 0.01$_{b}$	9.10 ± 0.03$_{a}$ 0.0073$_{*}$	8.26 ± 0.01$_{b}$	7.90 ± 0.01$_{a}$ 0.0004$_{*}$	6.17 ± 0.05$_{b}$	7.07 ± 0.09 $_{b}$ 0.0073$_{*}$
20 days of ripening	7.44 ± 0.01$_{b}$	7.34 ± 0.01$_{d}$ 0.0074$_{*}$	9.20 ± 0.02$_{a}$	8.87 ± 0.00$_{a}$ 0.0017$_{*}$	8.51 ± 0.07$_{a}$	6.94 ± 0.04$_{d}$ 0.0012$_{*}$	6.40 ± 0.00$_{a}$	6.32 ± 0.03 $_{c}$ 0.0597

Tab. 14 Results of viable counting of bacteria (log cfu per gram or mL) in Caciofiore della Sibilla and control ewes' milk cheese. cfu colony forming units.Viable counts of lactococci, thermophilic cocci, lactobacilli, coagulase-negative cocci, total mesophilic aerobes, enterococci and Enterobacteriaceae expressed as mean values ± st. dev. of samples collected during ripening of Caciofiore della Sibilla and control ewes' milk cheese. Mean separation test throughout ripening: different letters on the same column indicate significant differences over time according to Tukey's test (α =0.05). Least significant difference (LSD) is reported. ∗ Occurrence of significant differences due to cheese batch (C or Cf) according to Student's t-test (α =0.05).

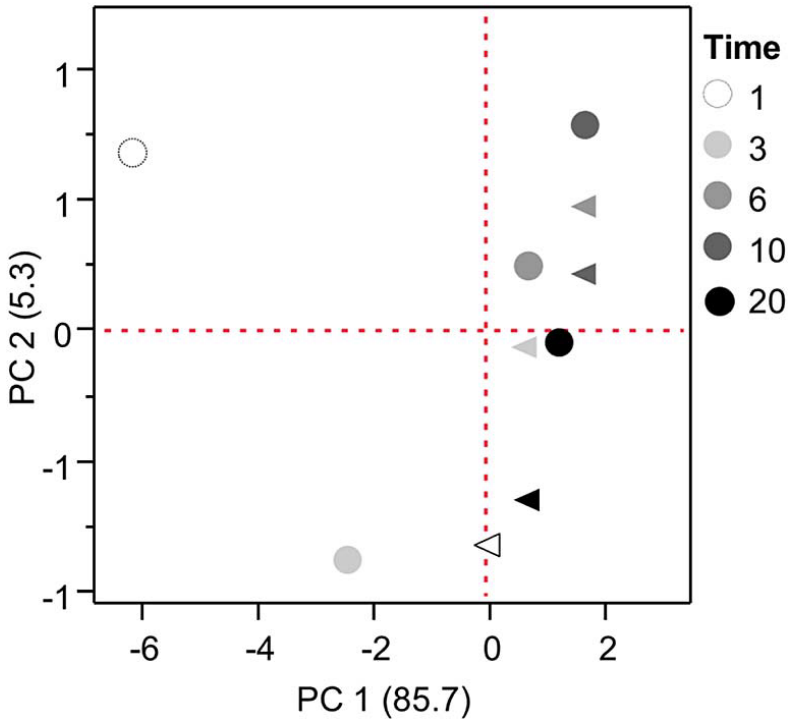

Figura 27 Loading and score plot of principal components analysis (PCA) based on viablecounts evolution over time of Caciofiore della Sibilla (dots) and control ewes' milk cheese (triangles).

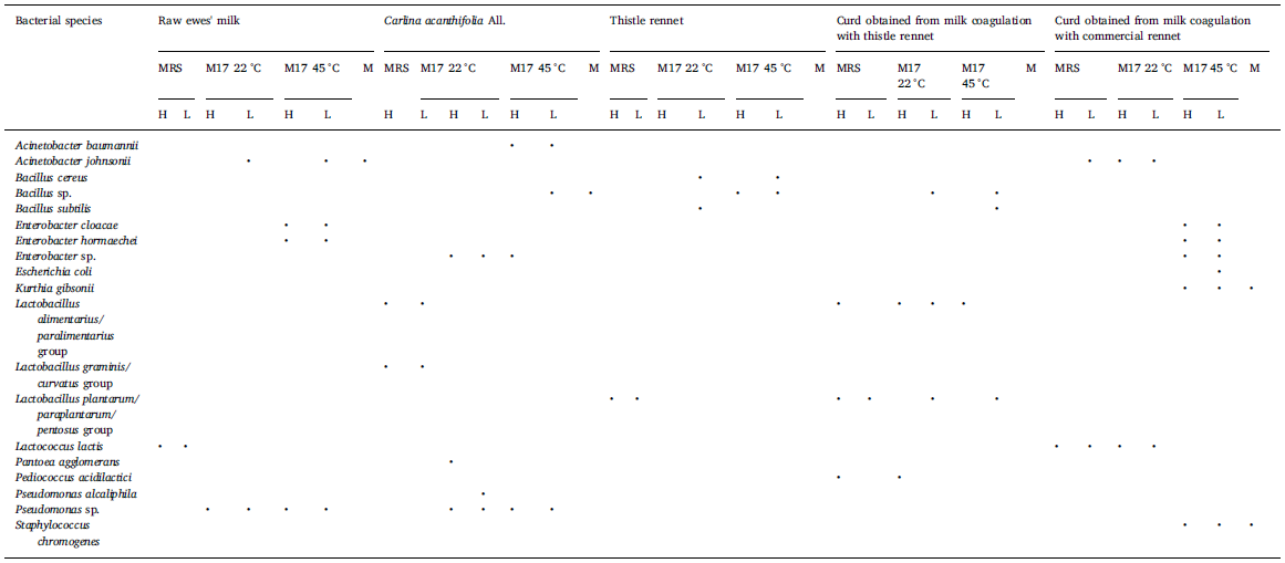

Figura 28 Bacterial species identified by PCR-DGGE analysis in raw ewe's milk, Carlina acanthifolia All. subsp. acanthifolia, thistle rennet, curd obtained from milk coagulation with thistle rennet and curd obtained from milk coagulation with commercial rennet. DGGE bands resulting from the analysis of the DNA extracted from the bulk of colonies harvested from high (H) and low (L) dilution agar plates used for viable counting from selected plates or the DNA extracted directly from samples showing ≥97% of similarity with the sequences of the closest relatives found by a BLAST search in the GenBank. M indicate the DNA extracted directly from a cheese samples matrix.

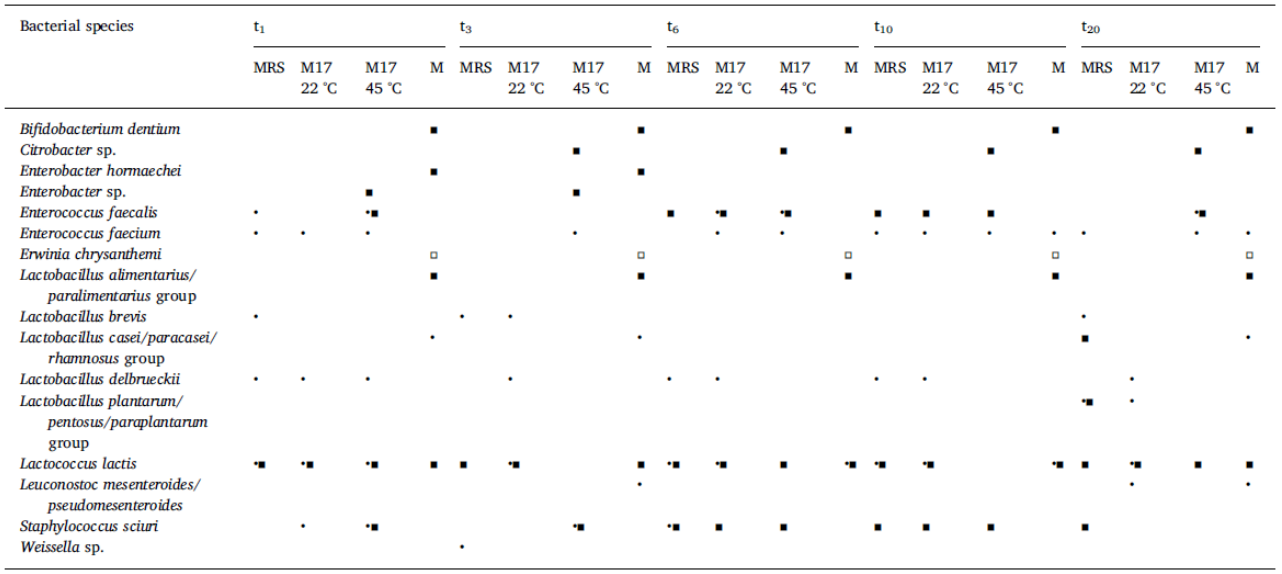

Fig. 29 Bacterial species identified during the manufacture and ripening of control ewe's milk cheeses and Caciofiore della Sibilla by PCR-DGGE analysis. • DGGE bands resulting from the analysis of the DNA extracted from the bulk of colonies harvested from selected plates or the DNA extracted directly from Caciofiore della Sibilla cheese samples showing ≥97% of similarity with the sequences of the closest relatives found by a BLAST search in the GenBank; ▪ DGGE bands resulting from the analysis of the DNA entracte from the bulk of colonies harvested from selected plates or the DNA extracted directly from control ewe's milk cheese samples showing ≥97% of similarity with the sequences of the closest relatives found by a BLAST search in the GenBank; ▫ DGGE bands resulting from the analysis of the DNA extracted from the bulk of colonies harvested from selected plates or the DNA extracted directly from control ewe's milk cheese samples showing ≤97% of similarity with the sequences of the closest relatives found by a BLAST search in the GenBank; t1: cheese sampled after 1 day of maturation; t3: cheese sampled after 3 days of maturation; t6: cheese sampled after 6 days of maturation; t10: cheese sampled after 10 days of maturation; t20: cheese sampled after 20 days of maturation. M: DNA extracted directly from a cheese samples matrix.

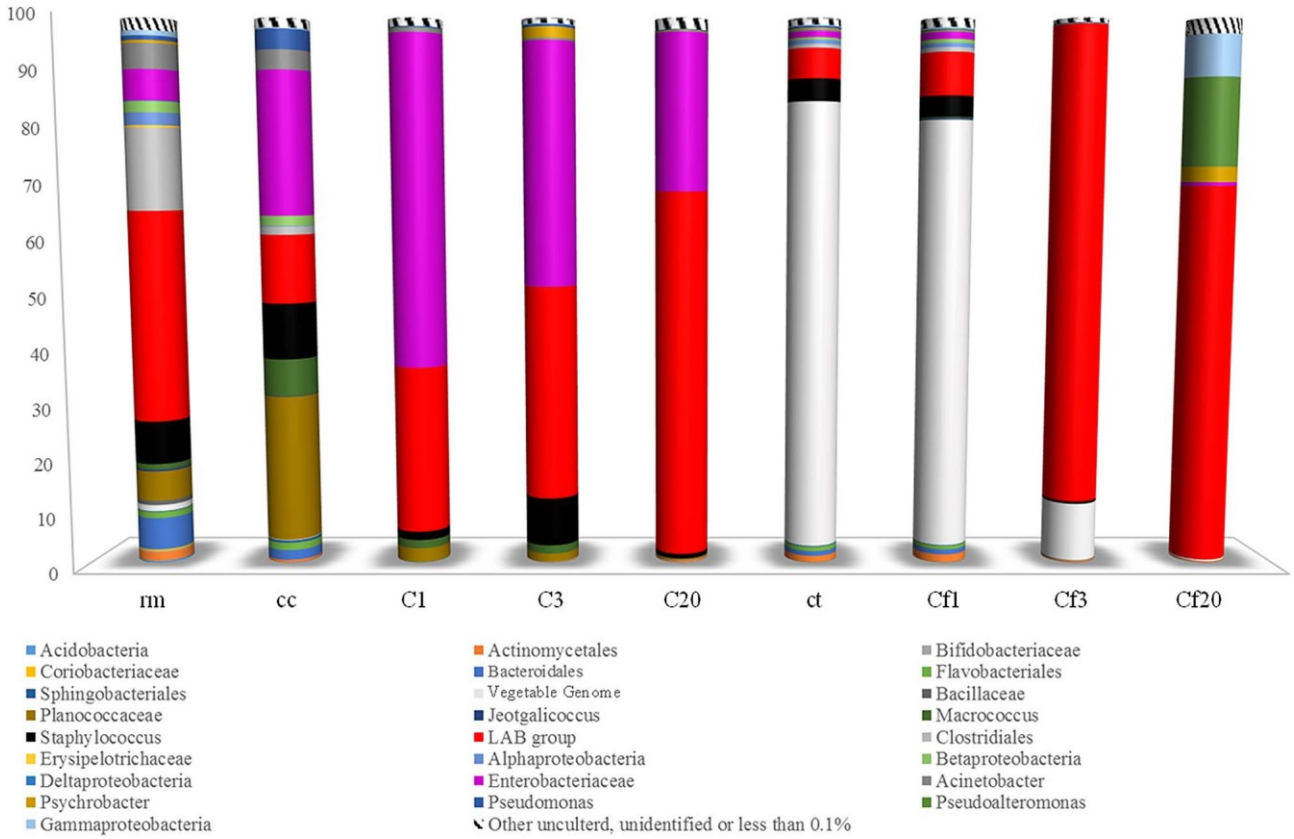

Fig. 30 Relative abundances (%) of bacterial groups and vegetable genome identified by MySeq Illumina in raw ewes' milk (rm); curd obtained by milk coagulation with commercial animal rennet (cc); curd obtained by milk coagulation with carline thistle rennet (ct); control raw ewe's milk cheese and Caciofiore della Sibilla cheese collected at 1, 3, and 20 days of ripening (C1, C3, C20 and Cf1, Cf3, Cf20 respectively). "Other" represents all genera that were unidentified, belonged to uncultured or whose abundance was less than 0.1 %.

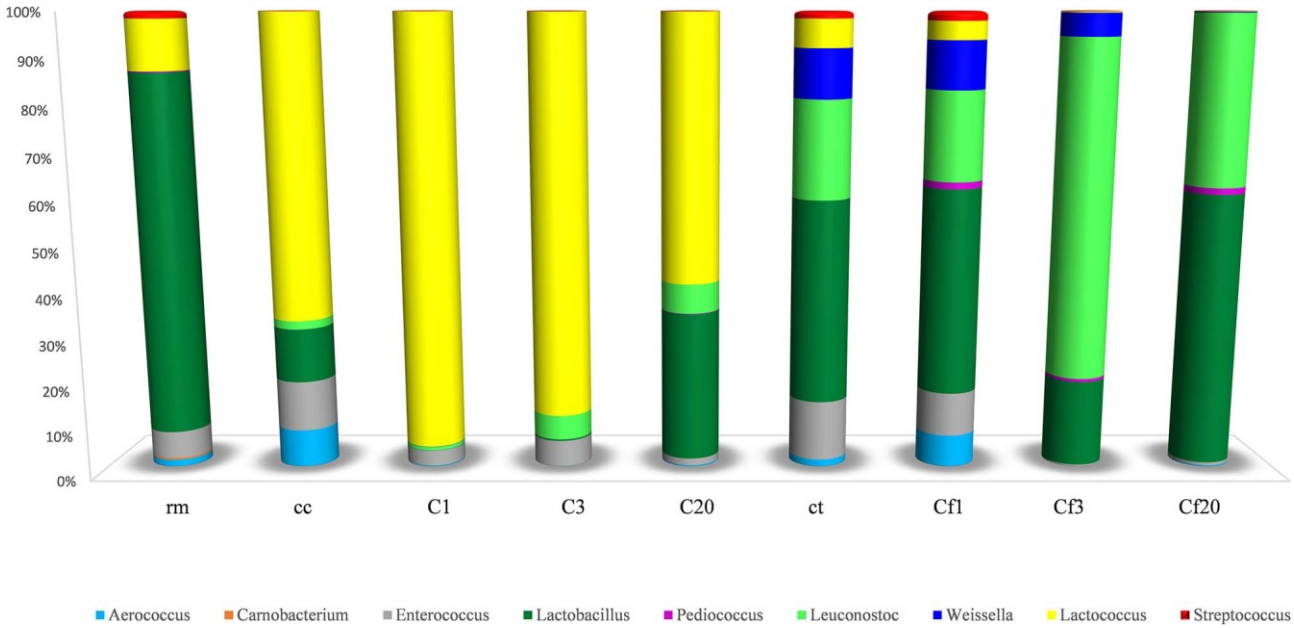

Fig. 31 Relative abundances (%) of the different LAB genera identified by MySeq Illumina in raw ewes' milk (rm); curd obtained by milk coagulation with commercial animal rennet (cc); curd obtained by milk coagulation with carline thistle rennet (ct); control raw ewe's milk cheese and Caciofiore della Sibilla cheese collected at 1, 3, and 20 days of ripening (C_1, C_3, C_{20} and Cf_1, Cf_3, Cf_{20} respectively). Each percentage is related to the total amount of LAB found in each sample.

16.3. Discussion

To the best of the authors' knowledge, this report is the first to describe the contribution of thistle rennet obtained from *C. Acanthifolia* All. subsp. *acanthifolia* to cheese bacterial composition and dynamics.
Moreover, no previous investigations have been conducted on *Caciofiore della Sibilla* cheese, a Mediterranean soft ewes' raw milk cheese manufactured according to a revived local ancient technology.
As far as raw *Sopravissana* ewes' milk was analyzed, viable counts of presumptive lactobacilli, lactococci, thermophilic cocci, total mesophilic aerobes, *Enterobacteriaceae* and coagulase-negative cocci were within the range reported by other authors for raw ewes' milk (Quigley *et al*., 2013; Pangallo *et al*., 2014).
Regarding thistle rennet, viable counts of total mesophilic aerobes and presumptive lactobacilli were comparable to those found by other authors (Aquilanti *et a*l., 2011; Barbosa *et al*., 1981) in thistle rennets obtained from *Cynara* spp. dried flowers; by contrast, slightly lower counts of presumptive lactococci and thermophilic cocci were found compared to those reported by the same authors (Aquilanti *et al*., 2011; Barbosa *et al*., 1981).
At the end of ripening (20 days) *Caciofiore della Sibilla* cheese showed viable counts of total mesophilic aerobes and presumptive lactobacilli comparable with those reported by Aquilanti *et al*. (2013) in a raw cow's milk Caciotta cheese manufactured with an aqueous extract of *C. cardunculus*, whereas the counts of coagulase-negative cocci and *Enterobacteriaceae* were higher. High counts of *Enterobacteriaceae* in cheeses at the end of their ripening might suggest a lack of hygiene during milking and cheese-making. However, the metabolic activity of members of this bacterial family has been positively correlated to high proteolysis and lipolysis in artisanal cheeses, resulting in the production of volatile aroma compounds (Tabla *et al*., 2016). OTUs obtained by Illumina sequencing are derived from clustering 16S rRNA gene rDNA sequences. Each of these clusters is intended to represent a taxonomic unit of bacterial species or genus depending on the sequence similarity threshold, thus providing relative but not absolute abundances of single microbial taxa. PCR-DGGE method is capable of separating unrelated DNAs based on the differences in their sequences and GC contents allowing a qualitative or, at most semiquantitative, overview of heterogeneous microbial communities.
When DNA-based techniques were applied to the profiling of the bacterial biota, numerous taxa were identified, including the starter lactic acid bacteria (LAB) species L. lactis, which is known to lower the pH and control fermentation rapidly, and a wide variety of adventitious microorganisms, such as environmental, spoilage and non-starter lactic acid bacteria (NSLAB), which gain access to the cheese at any stage of the manufacturing process. NSLAB are mainly responsible for key physic chemical transformations, e.g., proteolysis and lipolysis, which greatly impact basic cheese characteristics, such as flavor, appearance and texture (Gobbetti *et al*., 2015). Illumina sequencing allowed both the majority and minority taxa to be identified, whereas single bacterial species were unambiguously recognized by PCR-DGGE. When the latter technique was applied, discrepancies were seen in the fingerprints obtained by the bulk and the direct approach, in terms of both the number of taxa identified and their relative abundance; this finding was in agreement with a recent finding by the same authors on semi-hard cheese models (Aquilanti *et al*., 2016).
In *Sopravissana* raw ewes' milk, PCR-DGGE allowed the sole species *L. lactis* to be identified among LAB. Illumina sequencing confirmed the occurrence of *Lactococcus* spp. in raw ewes' milk, though as a minority taxon with respect to the members of the genus *Lactobacillus*. The presence of both lactococci and lactobacilli in raw ewes' milk has previously been documented, as reviewed by Aquilanti et al. (2007), Quigley *et al*. (2013) and Pangallo *et al*. (2014). The presence of *L. Lactis* was also evidenced in two previous studies (Aquilanti *et al*., 2011, 2013) carried out onto raw cow's milk Caciotta cheeses manufactured with aqueous extract of *C. cardunculus* dried flowers. The

occurrence of additional genera, including *Aerococcus, Leuconostoc*, *Streptococcus* and *Enterococcus*, has previously been found in raw ewes' milk by nextgeneration sequencing (Quigley *et al*., 2013).

In both the phyllosphere of *C. acanthifolia* All. and thistle rennet, culture-dependent PCR-DGGE identified various LAB and NSLAB species, as well as both spoilage and environmental microorganisms. To date, there is a paucity of data on the bacterial biota colonizing the aerial surfaces of thistles and thistle rennets. In 1968, Mundt and

Hammer (1968) first enumerated and identified lactobacilli on plant surfaces; in such a pioneering systematic study, *Lact. plantarum*, *Lactobacillus fermenti* and *Lact. brevis* were the species most frequently isolated, whereas *Lact. casei*, *Lactobacillus viridescens*, *Lactobacillus cellobiosus*, *Lactobacillus salivarius*, and *Lactobacillus buchneri* occurred at lower frequencies. A widespread but sporadic distribution of low counts of lactobacilli in the phyllosphere of higher plants was also highlighted by the same authors. In accordance with data collected in the present study, Widnyana and Javandira (2016) recently reported the presence of Bacillus spp. and Pseudomonas spp. on plant surfaces, where these microorganisms exert a plant-growth stimulating activity as well as an inhibitory activity towards plant pathogens. Lactobacilli ascribed to the *Lact. alimentarius/paralimentarius* group and the *Lact. plantarum/paraplantarum/pentosus* found by PCR-DGGE in both the phyllosphere of *C. acanthifolia* All. and thistle rennet were also detected in curd obtained by milk coagulation with thistle rennet, thus strongly suggesting a role of the coagulating agent in the early bacterial dynamics of *Caciofiore della Sibilla* cheese. By contrast, the occurrence of *Pseudomonas acidilactici* in the sole curd obtained by milk coagulation with thistle rennet is more likely related to an environmental contamination, since this species was apparently absent in ewes' raw milk, thistle phyllosphere and thistle rennet.

A different picture emerged in curd obtained by milk coagulation with commercial animal rennet. In this case, LAB and NSLAB were found to be the minority by Illumina sequencing; among these microorganisms, *Lactococcus*, likely derived from raw ewes' milk, was predominant. PCR-DGGE confirmed the occurrence of L. lactis as the sole pro-technological species, along with various spoilage and pathogenic

bacteria.

As far as the bacterial dynamics are considered, the stable presence of *L. lactis* in both the *Caciofiore della Sibilla* and control cheeses suggested by the results of PCR-DGGE might be, at least in part, ascribed to the contribution of raw ewes' milk, where lactococci and *L. lactis* have been revealed by llumina sequencing and culture-dependent PCR-DGGE, respectively. It is worth noting that when this latter technique was applied, closest relatives to *L. lactis* were found in the bulk cells harvested from both the lowest and the highest dilution plates, thus suggesting a load of this species ≥ 105 ufc/mL. *L. lactis* has a wide ecological distribution and is mostly associated with the milk environment and dairy products (Cavanagh *et al*., 2015; Šaková *et al*., 2015; Sádecká *et al*., 2016). As recently elucidated by Ruggirello *et al*. (2016), this species can persist in late ripening of cheese in both the viable and the viable but nonculturable (VNC) states, shifting its catabolism to peptide and amino acid consumption. Besides *Lactococcus*, bacteria ascribed to *Leuconostoc* and Lactobacillus dominated during ripening of *Caciofiore della Sibilla* cheese; both genera have been detected from t1 to t20 by both Illumina sequencing and PCR-DGGE analysis.

The dominance of *Leuconostoc* during the maturation of *Caciofiore della Sibilla* cheese might be explained by the higher initial pH of the curd obtained with thistle rennet compared to that produced with commercial animal rennet. Indeed, as reported by Hemme and Foucaud-Scheunemann (2004), *Leuconostoc* can be affected by the acidification of the growth medium. Interestingly, *Leuconostoc* was indicated by Illumina sequencing as a minority bacterial taxon in control ewes' milk cheese; however, no closest relatives to this genus were identified by PCR-DGGE in this

cheese manufacture in either of the two approaches, thus strongly emphasizing the usefulness of a combined analytical strategy. Moreover, since apparently neither raw ewes' milk nor thistle and

thistle rennet harboured leuconostocs, as revealed by PCR-DGGE, the occurrence of these microorganisms in *Caciofiore della Sibilla* cheese might be tentatively ascribed to a contamination from the dairy environment. However, members of the genus *Leuconostoc* have previously been detected in a raw cow's milk Caciotta cheese manufactured with thistle rennet from *C. Cardunculus* dried flowers (Aquilanti *et al.*, 2011), as well as in other cheeses produced with vegetable coagulants, such as *Serra da Estrela* PDO cheese and *La Serena* PDO cheese (Macedo *et al*., 2004). Based on these latter evidences, it might be also hypothesized that in thistle, thistle rennet and curd obtained with thistle rennet, leuconostcocs occurred at levels below the limit of detection of the PCR-DGGE technique. In addition, Lact. delbrueckii, Lact. brevis, and E. faecium were retrieved by PCR-DGGE exclusively in *Caciofiore della Sibilla* cheese throughout its maturation. Since these species could not be detected by PCR-DGGE before t1, again it might be hypothesized that they originate from the vegetable coagulant, where they occur at very low levels. *Lact. delbrueckii* includes a subspecies that can be positively affected by the addition of vegetable extracts to the dairy matrix (Joung *et al*., 2016); even *Lact. brevis* has been retrieved in vegetable-based matrices (Ruiz Rodríguez *et al.*, 2016). Finally, enterococci are NSLAB that are naturally present in the gastrointestinal tract of humans and animals; they can enter the dairy environment via cross-contaminations, where they play a crucial role during cheese ripening, due to their high adaptation to the cheese environment (e.g., high salt concentration and low pH) (Giraffa, 2003) and to acknowledged technological traits, such as the production of bacteriocins (İspirli *et al*., 2017).
Finally, members of two additional bacterial groups, namely, *Lact. casei/paracasei/rhamnosus and Lact. plantarum/pentosus/paraplantarum*, were identified by PCR-DGGE in both cheese-manufactures; since these microorganisms could not be detected in either raw ewes' milk or thistle rennet, once again, a contamination from the dairy environment might be hypothesized. An important piece of evidence addresses the presence of *Enterobacteriaceae*, with a higher occurrence of this microbial group in control ewes' milk cheese, as revealed by both DNA-based techniques.
However, this finding was not supported by viable counts; indeed, at the end of the ripening period, comparable loads of *Enterobacteriaceae* were found in the two cheeses by culturing in selective VRBA medium, thus suggesting the need for an integration between culture-dependent and different culture-independent techniques.

16.4. Conclusions

Overall, the combined analytical approach enabled differences and similarities in the bacterial biota and dynamics of the two cheeses under study to be defined. The integration of data from viable counting, Illumina sequencing and PCR-DGGE provided the detection of major and minor bacterial components (Illumina sequencing and viable counting), as well as the identification of single bacterial species (PCR-DGGE) that could not be revealed with the first two techniques. The primary result confirmed the association of lactobacilli, lactococci and leuconostocs with cheese and the dairy environment; moreover, the vegetable coagulant was demonstrated to affect the early bacterial dynamics of *Caciofiore della Sibilla* cheese, whose bacterial biota significantly differed from the control ewes' milk cheese, at the end of the maturation period.

17. Discussion

This dissertation has enabled first to evaluate the technological properties of raw extracts obtained from Carlina acanthifolia All. subsp. acanthifolia collected from the area of the Monti Sibillini National Park. Evaluating these properties has allowed us to identify the optimal parameters for this extract. MCA was the highest in aqueous extract by flower and in decreasing on stems. Another major difference was seen between the top of the flower and the bottom. MCA was very high for the upper part of the flower and completely absent for the lower part. MCA was higher in samples with 200 µl CaCl2 and decreased in the other concentration at different temperatures. The different amount of enzyme used was found to be very discriminating for MCA. MCA was higher in samples with 1mL and decreases in the other concentration. Storage at -20 had no effect on MCA. the same results were obtained on fresh samples. With respect to pH, drop was seen throughout the two cheese-making processes, with significant differences occurring between curds and cheeses from batches until the 10th ripening day. By contrast, after 20 days of maturation, the Caciofiore della Sibilla and the control ewes' milk cheeses reached comparable pH values. Regarding the aqueous extract from carine thistle, a very low pH value (3.79), due to the high concentration of acetic acid, was recorded. The main identified volatile compounds found in the flower of Carlina acanthifolia All. Subspecies acanthifolia have been: 1-carbahexaborane(7)/thiazolidin.4-one 5 ethil-2-2imino-isobutylamine/hexane 3,4 dimethyl/1,1dichloropentane/3methyl1penten3ol/butanal3methyl /hexene3methyl/2chloro2methylhexane/hexanal/2hexanal/3hexanaol/butanoicacid3/methyl /butanoicacid32methyl/phenylglioxal/benzoicacid3formyl/benzylalchol/toluene The main identified volatile compounds found in the “Caciofiore” cheeses after 20 days of ripening have been : 1butanol2,2,dimethyl/3pentanol2,4dimethyl/oxetane2methyl4propyl/piperodine1,1dithiobis/2furan methanol/2,5dimethyl5hexen3ol/benzene/hexane3methyl/toluene/butanoi/acid ethylester/butanoic acid/2heptanol/hexanoicacid/methylester/4methyl2oxovalericacid/hexanoicacidethylester /pentanoic acid. By comparing the results obtained by determining the volatile compounds during the maturation of the cheese "Caciofiore della Sibilla" and the determination of the volatile component of the *C. acanthifolia* All extract, we can state that the extract is not decisive for the final volatile aroma of the product but this is due to neoformation compounds generated during the maturation process Microorganisms are an essential component of all cheese varieties and have an essential role during both cheese manufacture and ripening. Fungi are an important constituent of the cheese microbiota, whose multiplication is encouraged by the peculiar physico-chemical properties of cheese such as a low pH, a low water activity, a high salt content and refrigeration during ripening and storage. This study provides a clear evidence of the dominance of D. hansenii in both the cardoon aqueous extract used as coagulating agent and Caciofiore della Sibilla cheese. This yeast is known to positively affect cheese ripening through proteolysis and lipolysis and to protect cheese against contaminants. Other yeast species such as C. zeylanoides, P. kudriavzevii, G. candidus/G. candidum have also been identified. As the whole manufacturing process is considered, a succession in the yeast community was found to take place with an evolution of the entire microbial ecosystem because the gradual and orderly replacement of species during cheese-making and ripening. A direct contribution of the aqueous extract to the cheese diversity and dynamics also emerged, thus suggesting a strict connection of the microbiota of Caciofiore della Sibilla with the peculiar manufacturing process. Caciofiore della Sibilla is a speciality ewes’ milk cheese traditionally manufactured in a foothill area of the Marche region (Central Italy) with a crude

extract of fresh young leaves of Carlina acanthifolia All. subsp. acanthifolia as a coagulating agent. The fungal dynamics and diversity of this speciality cheese were investigated throughout the manufacturing and 20-day ripening process, using a combined PCR–DGGE approach. The fungal biota of a control ewes' milk cheese, manufactured with the same batch of milk coagulated with a commercial animal rennet, was also monitored by PCR–DGGE, in order to investigate the contribution of the peculiar vegetable coagulant to the fungal diversity and dynamics of the cheese. Based on the overall results collected, the raw milk and the dairy environment represented the main sources of fungal contamination, with a marginal or null contribution of thistle rennet to the fungal diversity and dynamics of Caciofiore della Sibilla cheese. In the other study, the impact of thistle rennet on the bacterial dynamics and diversity of Caciofiore della Sibilla cheese was investigated by applying a polyphasic approach based on culture and DNAbased techniques (Illumina sequencing and PCR-DGGE). A control cheese manufactured with the same batch of ewes' raw milk and commercial animal rennet was analyzed in parallel. Overall, a large number of bacterial taxa were identified, including spoilage, environmental and pro technological bacteria, primarily ascribed to Lactobacillales. Thistle rennet was observed clearly to affect the early bacterial dynamics of Caciofiore della Sibilla cheese with Lactobacillus alimentarius/paralimentarius and Lactobacillus plantarum/paraplantarum/pentosus being detected in the phyllosphere of C. acanthifolia All., thistle rennet and curd obtained with thistle rennet. Other bacterial taxa, hypothetically originating from the vegetable coagulant (Enterococcus faecium, Lactobacillus brevis, Lactobacillus delbrueckii, Leuconostoc mesenteroides/pseudomesenteroides), were exclusively found in Caciofiore della Sibilla cheese by PCR-DGGE. At the end of the maturation period, Illumina sequencing demonstrated that both cheeses were dominated by Lactobacillales; however curd and cheese produced with thistle rennet were co-dominated by Lactobacillus and Leuconostoc, whereas Lactoccous prevailed in curd and cheese produced with commercial animal rennet followed by Lactobacillus. Differences in the bacterial composition between the two cheeses at the end of their maturation period were confirmed by PCR-DGGE analysis. Overall, the combined analytical approach enabled differences and similarities in the bacterial biota and dynamics of the two cheeses under study to be defined. The integration of data from viable counting, Illumina sequencing and PCR-DGGE provided the detection of major and minor bacterial components (Illumina sequencing and viable counting), as well as the identification of single bacterial species (PCR-DGGE) that could not be revealed with the first two techniques. The primary result confirmed the association of lactobacilli, lactococci and leuconostocs with cheese and the dairy environment; moreover, the vegetable coagulant was demonstrated to affect the early bacterial dynamics of Caciofiore della Sibilla cheese, whose bacterial biota significantly.

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