CRANFIELD UNIVERSITY

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Supervisor: Dr. David Aldred

Local Supervisor: Prof. Lídia Dionísio

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Abstract

This study was concerned with a chemical and microbiological characterisation of cheese made using milk from Algarvian goat breed. Seasonal variation of the microbiota and the gross chemical composition of the raw and boiled milk and cheese during the lactation period were studied. The cardoon microbiota and the variation of microbiota during ripening were studied also. The lactic acid bacteria (LAB) were isolated, identified to genus level and their technological properties such as bacteriocin production, acidifying capacity, proteolysis and lipolysis were studied.

The results showed that boiling milk does not represent a cause of variation in its gross composition and almost all the gross components of the milk and cheese register no variation during the studied period, except for fat, which increased until the middle of the lactation period and decreased after that.

In cardoon, the microorganisms that are able to produce spores are the most important, thus analysis of yeasts and moulds was carried out which allowed the arrangement of the tested samples into three groups. Most of the identified moulds from the cardoon samples are from the genus *Aspergillus*.

During the study period, differences in the microbiota of the raw milk were not observed, with *Lactococcus* and *Lactobacillus* being the prevalent groups. All the tested microorganisms increased approximately by two orders of magnitude from milk to cheese.

Lactobacillus was the predominant group during the maturation period. Total coliforms tended to diminish in the early stages of ripening.

Isolates from *Lactobacillus* and *Pediococcus* genera showed fast acidification capacity, which could be an indicator of good potential for their use as starter bacteria. Some *Lactobacillus* produced bacteriocin which can contribute to the removal of other bacteria. *Aerococcus*, *Leuconostoc* and *Lactobacillus* presented high proteolytic activity, which mean they could be used as adjunct cultures to improve proteolysis. Only one isolate (*Pediococcus*) showed lipolytic activity. In conclusion, by their technological characteristics some isolates could be selected as starter cultures, however, further research of their pathogenesis is necessary before using them in pilot plant production.

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Chapter 1. General Introduction

1.1. Introduction

A study was published by the Coordination Commission from the Algarve Region of Portugal (CCRAlgarve) in June of 2003, in which it was intended to address the issues of desertification and depopulation in the Algarve area as well as serving as a guide for promotion of local traditional products. This study identified goat milk cheese as one of the traditional products to be supported and developed.

However, like other traditional products from the Algarve, the small scale of production, the delay in the certification processes and the weakness of the associative and commercial structures, are the more important problems that affect the goat milk cheese produced in Algarve.

The main problem results from competing production from goods with standardized characteristics in a mass-produced way, in other regions, which are much cheaper. The solution for this problem could be the product certification, with their affirmation in the market, which could result in the warranty of authenticity, safety and quality control from farm to fork. Assuring these conditions, the profits for the producers will increase; they will transform more milk in cheese and will create the need for larger breed of goats. These improvements will contribute to the economic development of the region.

The Algarve, and specially the Barrocal area, is a region of excellence for breeding of small ruminants, namely for native ones, and the necessity of the approbation of a certified goat milk cheese, justifies the importance of a deep study for this product. The technical and scientific knowledge will help in the certification of the product, but can also offer the possibility of putting at the producers' service a set of results and determinants for the success of this artisanal industry. Innovation in production processes could then permit an increase in the competitiveness of these traditional productions, whilst maintaining the crucial original organoleptic characteristics.

It is important to know the effect of the seasonal variations in the physical and chemical and microbiological characteristics of milk and cheese and if the thermal treatment influences the milk chemical characteristics, since this kind of cheese is

produced with thermal treated milk when it is for consumption in fresh and with non thermal treated milk if it is for consumption after ripening.

There is no reported description of the chemical composition of the Algarve cheese, but through the organoleptic differences sometimes seen between batches of the cheeses, it can be concluded that it could be possible to find some differences in chemical properties. These differences can be attributed to the lack of consistency of the quality of the raw milk and to the lack of standardised procedures for the manufacture and ripening of the cheese. Each cheese maker has an individualised method for all steps in the manufacture of Algarve cheese, e.g., heat treatment (boil) (when it is for consuming in fresh), clotting, pressing, salting. Hence, the composition of cheese manufactured in the same dairy plant can vary significantly, thus there is a need to study the chemical and microbiological composition of the cheese produced with goat milk from the Algarve in order to standardize its quality (Kongo & Malcata, 2007) so that it could obtain a certified product.

Another technological advance that could be used is the utilization of native microbiota in cheese production, which permits the use of heat treated milk. Boiling process would assure the safety of the milk, eliminating the pathogenic microorganisms possibly present and the native microbiota (used as starter or as non starter during ripening) would assure the maintenance of the organoleptic characteristics of the traditional products.

Thus, it will be important to isolate the lactic acid bacteria (LAB) present in the milk and in the cheese made from raw milk, during the ripening period. These LAB should be identified and their technological properties studied in order to determine if they could be used as adjunct microbiota in cheese production.

1.2. Literature review

1.2.1. Historical perspective

Milk first appears in the human diet in the “Fertile Crescent” between the Tigris and Euphrates rivers, in Iraq, during the pre-historic period, more specifically after the Neolithic period, 10 000 years B. C (Braga, 2003; Fox, 1987).

Milk has a short shelf life, especially in warm climates, as it is a rich source of nutrients for contaminating bacteria. Since 6000-7000 B. C., cheese manufacture has been used as a means of milk preservation. In this process, two of the classic principles of food preservation, lactic acid fermentation, and reduction of water activity through removal of water and addition of sodium chloride were used (Robinson, 2002).

Lactic acid fermentation is achieved by Lactic Acid Bacteria (LAB) present in the milk, and the removal of water results from the separation of the whey from the curd after coagulation. It is generally believed that animal rennet was the first enzyme coagulant used, but rennet produced from a range of plant species have been common too, mostly in the period B. C. (Fox, 1987).

According to authors such as Braga (2003), Asian nomads were the first people to discover the coagulation properties of milk when they stored it in bags made from the stomachs of herbivorous animals that contain rennet.

The Greeks and Romans are known to have appreciated cheese, although it was probably only available to the privileged classes (Braga, 2003) and the migrations of people throughout Europe after the fall of the Roman Empire, must have promoted the further spread of cheese manufacture, during the Middle Age.

The monasteries were the agents that most contributed to the development of cheese technology. Certain religions orders, which used very good hygiene rules during the production process, became widely known due the quality of their cheeses and through their monks' travels the spread of cheese varieties and consequently the development of hybrid varieties occurred. These varieties occurred due to local circumstances like the chemical composition and/or microbiota of the milk supply (Fox, 1987).

According to a chorographer from the XV century, the Algarve mountains were rich in cows and goats for milk production. The Italian Gorani, travelling from Castro Marim to Mértola, in 1765, received different gifts from countryman that he met on his way, among them milk. However, at the time, it was unusual to drink milk. The most common use was in cheese production or, occasionally to add milk to bread and cakes (Braga, 2003). Cheese has been used ever since owing to a unique capacity to preserve the nutritional properties of milk.

Hundreds of different types of cheeses that can be differentiated both by the type of milk or by the animal are now produced throughout the world; representing a production of around 10^7 tonnes per annum and this is increasing at a rate near of 4 % per annum (IDF, 2007).

1.2.2. Social and economic aspects – current problems with Algarve cheese production

Regional cheeses produced in artisan conditions are part of the cultural heritage of many countries like Portugal (Cruz, 1945). Their highly valued flavour, in conjunction with long-recognized social and economic importance gives these traditional dairy products a high intrinsic value (Freitas & Malcata, 2000).

However, there are some fundamental problems with these products - most artisan cheeses obtained from goat milks are seasonal, with the peak of production in spring; which make it difficult to carry out effective milk quality improvement programs as a result of low yield per animal, rudimentary sheds, poor milking facilities, poor water supply, dirty teats and udders hand-milking and consequently, long milking time which increase the contamination from environmental bacteria (Kalantzopoulos *et al*., 2002). Besides that, this leads to logistic difficulties with supply and has led to a drastic reduction in the number of people employed in this local industry as it has become more difficult to compete with more reliable mass production from other regions (Freitas & Malcata, 2000). At the present, cheese production in the Algarve region is under threat. With negative incomes it will be almost impossible to stop rural migration, which is the principal problem from the peripheral regions and ultimately is a big loss for the entire country.

To promote sustained high quality standards, through legal protection, a few European countries have created *Denomination of Protected Origin (DPO)* (Nuñez *et al*., 1989). Council regulation (EC) No 510/2006 of 20 March 2006 on the protection of geographical indications and designations of origin for agricultural products and foodstuffs stated that *Denomination of Protected Origin* means ‘the name of a region, used to describe a food product originating in that region, with quality or characteristics that are essentially or exclusively due to a particular geographical

environment with its inherent natural and human factors, and with production, processing and preparation in the defined geographical area'.

The indication of "Protected Designation of Origin" or the Community symbols associated with them shall appear on the labelling, any operator complying with this Regulation is covered by a system of official controls assured by Member States. No direct or indirect commercial use of a registered name can be used by products not covered by the registration. No product which is an imitation or has an evocation, of the product can use the protected name, even if it is translated or accompanied by an expression such as "style", "type", "method", "as produced in", "imitation" or similar. No product can present in its packaging, material or documents relating to it any false indication to the provenance, origin, nature or essential qualities. Any other practice liable to mislead the consumer to the true origin of the product is also forbidden.

Such regions in Portugal are represented in Figure 1.1. and account for 13 traditional cheeses in Portugal (Table 1.1).

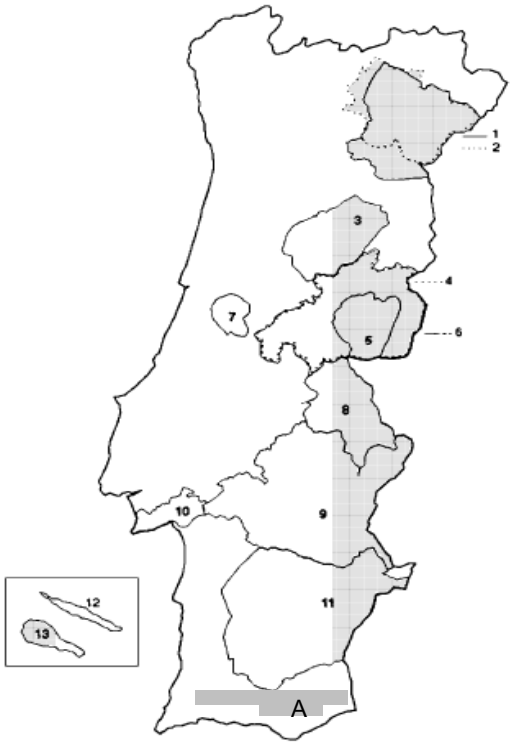

Figure 1.1. Appellation d'Origine Protegée regions in Portugal: 1. Terrincho cheese; 2. Cabra transmontano cheese; 3. Serra da Estrela cheese; 4. Picante da Beira Baixa cheese; 5. Castelo Branco cheese; 6. Amarelo da Beira Baixa cheese; 7. Rabaçal cheese; 8. Nisa cheese; 9. Évora cheese; 10.

Azeitão cheese; 11. Serpa cheese; 12. S. Jorge cheese and 13. Pico cheese (adapted from Freitas & Malcata, 2000). A – Region where algarvian goat breed cheeses are produced.

Table 1.1. Legal requirements of Portuguese cheeses with *Denomination of Protected Origin (DPO)* status (adapted from Freitas & Malcata, 2000).

Cheese variety	Milk type	Rennet type	Cheese type	Region of origin
Amarelo da Beira Baixa	Ovine and caprine	Animal	Semi hard	Castelo Branco
Azeitão	Ovine	Plant	Semi hard	Palmela, Sesimbra and Setúbal
Cabra transmontano	Caprine	Animal	Extra hard	Bragança and Vila Real
Castelo Branco	Ovine	Plant	Semi hard	Castelo Branco
Évora	Ovine	Plant	Semi hard or hard	Évora
Nisa	Ovine	Plant	Semi hard or hard	Nisa e Portalegre
Picante da Beira Baixa	Ovine and caprine	Animal	Semi hard or hard	Castelo Branco
Pico	Bovine	Animal	Soft	Pico
Rabaçal	Ovine and caprine	Animal	Semi hard or hard	Rabaçal
São Jorge	Bovine	Animal	Semi hard or hard	São Jorge
Serpa	Ovine	Plant	Soft	Serpa and Beja
Serra da Estrela	Ovine	Plant	Semi-soft	Serra da Estrela
Terrincho	Ovine	Animal	Semi hard	Trás-os-montes

Production of goat milk in Portugal is almost exclusively associated with the inland area. The milk from small ruminants (ewe and goat) represents approximately 40 % of all cheese produced in Portugal. Only 10 to 15 % of this amount is currently certified by DPO organizations (Freitas & Malcata, 2000).

The DPO cheeses manufactured at the farm level are, typically, from raw milk and are not inoculated with starter cultures. The indigenous microbiota is qualitatively and quantitatively unknown who could lead to health hazards (Freitas & Malcata, 2000). The indigenous microbiota has a significant effect on the development of cheese flavour due to more proteolysis and lipolysis (Robinson, 2002) which confers good qualities to the cheese, so it is important to isolate the milk microbiota in order to use it as adjunct cultures with heat treated milk, eliminating the referred health hazards.

The economic advantage of the certification is represented by a higher price of the product in the market. If the manufacturers loose their certification, they will loose a significant percentage of their income, which, in most cases, could be sufficient to make their farms unprofitable. To obtain the certification, all the characteristics of the cheese have to be known in order to establish the rules for all the producers to use in order to obtain a safe product, whilst maintaining its traditional organoleptic characteristics.

The Algarve goat cheese produced by traditional methods has a great social economic importance for the population living in the interior region of the Algarve

ridge of mountains; due to the large number of small producers. The certification of this product could improve the confidence in it. It is necessary to analyse the product and understand the characteristics that make this product unique among the similar ones in order to eliminate varieties that are not authentic. The practical knowledge resulting from these studies may lead to a production of higher quality which could promote certification and lead to a higher price. With higher incomes and with an established and stable market, artisan manufactures of the Algarve region will have the opportunity to develop their activity in a more profitable manner, helping to prevent the tendency for desertification of the interior by maintaining agricultural systems. This promotion on cheese production in the Algarve region will encourage decentralization by maintaining viable populations in regions that are far from the city centres, and will open the way for the establishment of integrated systems, where the manufacture of traditional products is complemented by the growth of tourism in rural areas.

One of the most important strangulations associated with the artisan cheese production in Portugal in general and in the Algarve region, in particular, has to do with the small sizes of the farms, and the consequent low volume of the milk of small ruminants processed daily. All the milk produced during the lactation period is consumed by kids' feeding and cheese production. Due to various social and historical factors, the idea of cooperativism represents great difficulties of acceptance in the poor regions of the interior of the country, which are also the regions from where the best traditional cheeses of Portugal are originated. So, it is likely that the only way that cheese production will continue in this region is to continue to process milk in low quantities in a large number of farms. Native starters permit to produce safer cheeses maintaining the traditional characteristics. If there is more information about this cheese quality the production will became easier. If the producers start to produce a more safety product and if they can use more milk (through the heat treatment (boiling) more milk can be used and their income will increase and more people will want to be a cheese producer. This will contribute to an increase in the number of producers and consequently to the development of the region.

Future production could be optimized through the use of uniform vegetable curdling (a method used in the cheese production in the Algarve region) and the use of

uniform native starter cultures with thermal treated milk. These two methods could help to promote a better characterised product, and together with controls on the quality of the milk could help to maintain a more uniform and consistent product across the region.

Typical products are highly regarded by consumers who look for originality and authenticity. Because of this, at least in Portugal, the wider agro-food industry produces imitations of the products which are available generally, and at all times of the year. It is essential that the genuine artisan products are legally protected through European legislation, if production is to continue. Of course the local producers must also adapt to today's markets, without losing their authenticity, if they wish to compete with modern large-scale producers (Boyazoglu & Morand-Fehr, 2001). This competition will never be won by quantity, but always with quality. To produce a cheese more safe through the utilization of heat treated milk with all the traditional characteristics achieved through the utilization of native starters could be a way the win this competition. In this work the native microbiota will be characterized and its technological properties will be studied in order to help in the composition of this starter.

1.2.3. Typical products

Goat products have a good ecological image and are often associated with agro-tourism, in many mountain regions like the Alps, Pyrenees, Balkans and regions of Portugal and Greece (Dubeuf *et al*., 2004).

The goat milk production in southern Europe reaches more than 80 % of the total European production and has been a traditional production for centuries; it is often less appreciated than sheep milk for cheese production in Spain, Italy, Greece or Portugal due to the strong smell and taste (Dubeuf *et al*., 2004).

A necessary condition to improve the competitiveness between dairy goats and dairy sheep in countries like Portugal is the increase of goat productivity (Dubeuf *et al*., 2004).

1.2.4. Cheese science and technology

Cheese is a fresh or ripened product, with variable consistency, obtained by milk coagulation, or from raw milk, partially skimmed milk, cream, or the mix of some or all of these components, including whey, after serum elimination (NP 1598, 1983).

A major source of cheese variation is the species from which the milk was produced. The cow is the most important of all species whose milk is used, but sheep, goat and buffalo are commercially important in certain areas (IDF, 2007). Both, significant differences in milk composition between breeds, especially fat (Raynal-Ljutovac *et al*., 2005), seasonal and nutritional variations, and lactation period and manufacturing factors could influence the cheese quality (Fox, 1987).

Each cheese is a biologically dynamic material and its production represents a series of successive biochemical phases leading to a final product with highly desirable aromas and flavours. However, if out of balance, off-flavours and odours can occur and because of this, milk is subjected to a careful manufacturing procedure with general principles which are common to most varieties, but, in spite of this, no two batches, not even two cheeses, are identical, assuring a diverse range of products (Fox, 1987).

Goat milk

Goat (*Capra hircus*) milk has been largely used for cheese production in Europe (Galina *et al*., 1995).

In 2007, the production of goat milk had risen from 12.1 million tonnes in 1997 to the estimated value of 12.5 million tonnes (IDF, 2007).

Several aspects based on major differences in composition (Table 1.2), explain the benefits of the goat milk for human use:

- small milk fat globules, which is similar to naturally homogenised milk;

- milk proteins, forming a softer curd on digestion and in cheese-making, compared with cow milk;

- milk fat in goat milk and cheese has a significantly higher contents of short chain and medium chain polyunsaturated fatty acids (Boyazoglu & Morand-Fehr, 2001).

Table 1.2. Milk composition of cow, sheep and goat breeds.

	Fat (%)	Protein (%)	Total solids (%)	Ash (%)	Source
Cow	4.0	3.25	12.7	0.7	(Walstra *et al*., 1999)
Sheep	7.92	6.18	19.3	0.91	(Boyazoglu & Morand-Fehr, 2001)
Goat	4.3	3.5	12.9	0.79	(Boyazoglu & Morand-Fehr, 2001).

In the last few years, there has been an increased interest in goat milk as an alternative milk source for people with cow milk intolerance and in the production of value-added products (Albenzio *et al*, 2006).

Goats have a lactation period up to 300 days of milking, which extends the production season for mountain farmers. Goat milk is variable in terms of composition between different breeds. Total lactation yields for different goat breeds are up to 500 kg and more per year (Boyazoglu & Morand-Fehr, 2001).

Coagulant agents

As a general rule, enzyme extracts from plants are excessively proteolytic for use in cheese making – as they cause organoleptic defects in flavour and texture of the final product. This realization is rationalised by the presence of non-specific proteases, or complex enzymatic systems – both of which are hard to control in practice. Despite this general trend, enzyme extracts from specific plants may give rise to cheeses of acceptable quality – not only from bovine milk, but mainly from small ruminant milk (Fernandez-Salguero *et al*., 1991; Vioque *et al*., 2000; Roseiro *et al*., 2003); this obviously supports the statement that a coagulant for cheese making is to be chosen depending on the source of cheese making milk.

The most often reported plants to produce proteases able to coagulate milk – and hence suitable for use in cheese making are: *Albizia julibrissin* (Silk tree) (Egito *et al*., 2007); *Lactuca sativa, Ananas comosus* (Pineapple); *Centaurea calcitrapa*; *Cirsium* sp. (thistle); plants from the genus *Ficus* (Fadýloglu, 2001); Prince melon; *Taraxacum officinale* (Dandelion); *Cynara cardunculus* and *Cynara humilis* (Veríssimo *et al*, 1995).

Although the thistle available in local markets in the Iberian Peninsula is supposedly univarietal, analyses of representative samples obtained from different pickers have shown that flowers of C*ynara cardunculus* are very often mixed with

flowers of *Cynara humilis*; *Centaurea calcitrapa* and *Silybum marianum* (Vioque *et al*., 2000).

C. humilis L, is used in Portugal by traditional cheeses makers (Pires *et al*, 1994, Vioque *et al*., 2000). The use of these plant proteinases as milk coagulants is interesting since they have natural enzymes (Fernández-Salguero *et al*., 2002) and can be used for producing cheeses intended for consumption by the vegetarian market.

Centaurea calcitrapa, a proteolytic plant widely distributed in Portugal, taxonomically included in the *Compositae* family, related to the genus *Cynara* (Reis *et al*., 2000), possess aspartic proteinases in all parts of the plant, particularly in flowers (Domingos *et al*., 2000). Its milk-clotting capacity was attributed to cenprosin, a proteinase which exhibits better proteolytic activity than commercial rennet and which also exhibits higher specificity towards ovine and caprine caseinates, which means that, besides higher activity, *C. calcitrapa* could produce high-quality cheeses from small ruminants' milks (Lourenço *et al*., 2002). The low yield of the final product and the heterogeneity in the proteinase profile of the flowers are the major limitation for the use of *C. calcitrapa* as coagulant agent (Lourenço *et al*., 2002).

The cardoon (*C. cardunculus*) also called "cardo de coalho", "cardo hortense" or "wild artichoke" is the most used in cheeses from algarvian goat breed.

Cardoon present different fenotyps, all of them with similar coagulant properties, and belongs to the *Astracea*e family (*Compositae*), a prickly variety of thistle (Figure 1.2) which also includes the artichoke, the safflower, and the sunflower (Encinar *et al*., 2000).

Figure 1.2. Cardoon flower (*Cynara cardunculus*).

The corollas, of blue violet colour, after cutting from their capitula, are utilized as coagulants substances (Christen & Virasoro, 1935). Studies by Faro (1991) had corroborated that the stylets have the greatest proteolytic activity.

The plant can be found in dry, stony, uncultivated areas of many Mediterranean regions such as Portugal, North Africa, the Canary Islands, Southern Spain and Madeira Islands (Sousa e Malcata 2002).

The cardoon is collected from wild plants growth in uncultivated areas, dried and sold to cheese producers in local markets.

The chemical composition of this product changes widely with different lots and different years. To give an idea of its composition, some values are presented in table 1.3.

Table 1.3. Chemical and microbiological characterization of *Cynara cardunculus* thistle dried flowers (adapted from Barbosa, 1983).

Parameter	Value
Humidity	5 %
Dry extract	95 %
Protein	26 %
Total sugar	42,5 %
Tanine	4,5 %
Chlorides	9,5 %
pH	5,8
Heterotrophic microbiota	98 x 10^3 cfu/g
Lactobacillaceae	51 x 10^3 cfu/g
Proteolytic bacteria	3 x 10^3 cfu/g
Lipolytic bacteria	5 x 10^3 cfu/g
Coliforms	negative/0,1 g
Enterococcus	positive/0,01 g
Staphylococcus coagulase reaction	negative/0,01 g
Spores of Clostrídia (sulphite reducers)	negative/0,1 g
Fungi	95 cfu/0,1 g

Cynara cardunculus extracts contain aspartic proteases that possess specificity for cleaving casein in the peptide bond Phe_{105}-Met_{106} like chemosin, but less specific activity that pepsin, which results in extensive breakdown of the casein network, producing a cheese with more soft, buttery texture and creamy flavour (Faro *et al*., 1993; Mecedo *et al*., 1993). In studies made by Chen *et al*. (2003), cheese manufactured with cardoon flower extract received very good sensory evaluation

from both trained and consumer panels when compared with cheeses made with rennet preparations. However, it is known that the final quality of cheeses manufactured using flowers of *C. cardunculus* varies considerably. Esteves *et al*. (2002) measured the rheological properties of milk gels made with vegetable coagulants from *C. cardunculus* and the gelation temperature was claimed to have an important role in the final properties of the gel. At low temperature, the coagulum was formed very fast and the proteolysis was not excessive, which have a favourable effect on texture and flavour of the final products.

1.2.5. Cheesemaking

Algarvian goat breed cheese

Cheese made from milk (Figure 1.3) of the algarvian goat breed is a cheese that can be consumed fresh or ripened. According to Regulation (EC) 1662/2006 from 6th November, it is forbidden to sell fresh cheese made from raw milk in Portugal. There is not a uniform processing method for goat cheese from algarvian goat breed, but in general we can say that it's made from boiled milk when it will be consumed fresh and with raw milk when it will be subject to ripening. When cheese is to be consumed fresh, the milk used is boiled (Figure 1.4) and allowed cool to around 30 ºC before the aqueous extract of coagulant agent (*Cynara cardunculus* 15 % w/v) addition (Figure 1.5). When it will be for consumption after ripening, raw milk is used and vegetable coagulant is added to milk heated to around 30 ºC.

Figure 1.3. Milk from algarvian goat breed (Dias *et al*., 2005).

Figure 1.4. Boil up of the milk (Dias *et al*., 2005).

Figure 1.5. Aquous extract of *Cynara cardunculus* (Dias *et al*., 2005).

The vegetable coagulant is collected from wild plants (Figure 1.6), dried and ground in a domestic grinder (Figure 1.7). The powder is diluted in water and rested at room temperature during more or less five hours. After that it is filtered trough a cloth and added to milk.

Figure 1.6. Collection of wild cardoon (Dias *et al*., 2005).

Figure 1.7. Grinding of cardoon (Dias *et al*., 2005).

The milk is clotted at a temperature between 29 and 32 ºC. In both raw and treated milk calcium chloride could be added (optional) in order to enhance coagulation and sodium chloride (usually 12 g/l) is added. Both concentrations of calcium chloride and sodium chloride are dependent on the producer choice.

After coagulation, the curd is cut, the whey is drained by pressing (Figure 1.8) and the curd is put in molds (Figure 1.9). At this step, fresh cheese (Figure 1.10) is ready for consumption. It should be consumed over five days if it is kept under

refrigerated conditions, since it is a very good medium for microorganisms development, namely listeria.

Figure 1.8. Drainage of the whey by pressing (Dias *et al*., 2005).

Figure 1.9. Curd in molds (Dias *et al*., 2005).

Figure 1.10. Fresh cheese (Dias *et al*., 2005).

Cheese to be ripened is placed in a cold ripening room (Figure 1.11) at around 15 ºC and 70 % humidity, for two months. This period is the minimum required by law (Reg (CE) No 1662/2006) for cheese made from raw milk and it is, at the same time, the time that traditional cheese producers from this kind of cheese used traditionally, as this is enough to achieve the ideal organoleptic characteristics for this kind of cheese.

Figure 1.11. Cheeses in the ripening room (Dias *et al*., 2005).

During the ripening period the concentration of faecal coliforms and moulds should decrease, probably because of acidic conditions in cheese and growth of non-starter lactic acid bacteria. Nevertheless it is possible that, sometimes, some pathogenic microorganisms stay in cheese during this period, especially if the initial level of contamination is high. Accordingly cheese will be safer if the milk used was treated by heat. In this case, starters must be added during the production process. In order to maintain the original (traditional) properties of the cheese, the added starters must originate from milk from the same region that is usually used (native starter), that's why it is so important to study the microbiota of the milk and the cheese. Knowing the native microbiota and its technological properties, it is possible to develop adequate starters for this kind of cheese, which will be able to maintain the traditional characteristics of the product.

Quality control is very poor, being milk and cheese tested for *E. coli* and *Listeria* once a month and the herd is regularly controlled for *Brucella*. No outbreak caused by any of these microorganisms is registered involving cheese from algarvian goat milk.

Goat cheese in general

Goat cheese is appreciated by consumers because of its strong typical flavour. It is a high added value product, since it is one of the most costly cheeses (Saldo *et al*., 2003). Goat cheese's popularity has increased, especially among ethnic groups, health food consumers and goat milk producers (Park, 1990). It is a distinctive Mediterranean product; its quality is closely associated with the territory of production and its traditions. The interaction between environmental characteristics, native genetic variations and way of living create an environment so specific that it would be extremely difficult to reproduce authentically elsewhere. Safeguarding these products also safeguards the uniqueness of their historical and cultural environment (Boyazoglu & Morand-Fehr, 2001).

Potential problems relating to the microbial safety of the milk could lead to the use of pasteurization treatments, although many goats' milk cheeses have always been traditionally made from raw milk. However, thermal treatment may induce numerous changes in milk relevant to cheese-making, like the destruction of the heat sensitive microbiota and the inactivation or the activation of enzymes and the partial denaturation of whey proteins (Buffa *et al*., 2004). These changes may have a detrimental effect on the cheese quality.

Milk composition and the influence of ripening are the two factors of major importance in cheese quality: the effect of ripening on the composition of cheese is shown in Table 1.4.

Table 1.4 Changes in composition of cheese during ripening (adapted from Bayazoglu & Morand-Fehr, 2001)

Days of ripening	Moisture (%)	Lactose (%)	Fat (%)	Total Protein (%)	Ash (%)	CaO (mg/g cheese)	NaCl (mg/g cheese)
1	61.2	3.2	20.1	18.1	1.06	0.53	0.71
35	55.0	1.3	23.8	17.6	1.21	0.59	2.53
65	55.2	0.8	23.7	17.3	1.23	0.62	2.80
100	53.8	0.0	24.8	17.1	0.91	0.65	2.83
125	51.6	0.0	25.6	17.0	0.94	0.71	2.75

In general, the manufacture of cheese can be divided in two main steps: production and ripening. Although the manufacturing procedures for individual varieties differ in detail, the basic steps are common to most varieties.

Cheese manufacture is essentially a process in which the fat and casein in milk are concentrated by loosing water. The degree of this loss is regulated by the extent and combination of technological operations, as well as the chemical composition of the milk. The biochemical changes that occur during ripening, which determine the flavour, aroma and texture of the finished cheese are regulated, besides the milk type, by the moisture level, salt content and microbiota (Fox, 1987).

Pre-treatment of cheese milk

Usually, the milk for cheese production is cooled to 4 ºC immediately after milking and may be held at this temperature for several days on the farm or at the processing facility (Fox, 1987).

Low temperature limits the development of the total bacterial count and some pathogens, but increases the growth of psychotropic microorganisms such as *Pseudomonas*. These bacteria, contribute to the progressive degradation of milk proteins and lipids through their enzymes that are heat resistant. Thus, the problems can occur not only during storage, but also after heat treatment. Besides influencing the action of bacteria, cold storage can provoke the destabilisation of the balance of the minerals and proteins in milk. Under refrigeration, the solubility of micelar calcium and β–casein increases which cause degradation of technological properties like an increase in the renneting time. However, this effect seems to be less marked in goat milk than in cow milk (Raynal & Remeuf, 2000).

A lesser propensity of milk for acidification observed in refrigeration conditions may be due to a partial inhibition of the development of the lactic acid bacteria by the endogenous microorganisms of milk, causing a reduction in the firmness of curd and favouring the increase of material losses (namely casein) in whey.

Addition of starters

It is a very common practice to add a culture (starter) of selected lactic acid-producing bacteria to the heat treated milk to guarantee uniform and a predictable rate of acid production. Acidification is the first step of cheese production and transcends

the other manufacturing operations. It occurs due to the transformation of lactose into lactic acid by lactic acid bacteria (Fox, 1987).

Coagulation

A fundamental part of cheese making is the coagulation of milk which is usually accomplished by the use of a coagulant agent under suitable conditions (Hayaloglu *et al*., 2002). Of the entire coagulant agents added to the milk during cheese making, only a minor fraction, which depends on the type of cheese, is retained within the curd, the rest is lost in the whey (Choisy *et al*., 1987). During this step, several physicochemical changes take place in the casein micelles. These changes can be due either to the clotting enzymes, milk acidification and/or increasing temperature, which eventually lead to the formation of a protein network. This network entraps fat and other solids to form a gel - coagulum (Dimassi *et al*., 2006).

Generally, milk coagulation is a consequence mainly of the action of proteolytic enzymes. After hydrolysis, the casein aggregates and the coagulum is formed (Dalgleish, 1987). The spontaneous contraction of the coagulum (syneresis) induces loss of water and water-soluble compounds (whey) and the coagulum decreases in volume via expulsion of whey (Walstra *et al*., 1987).

At the beginning of the process, caseins are hydrolyzed into large, well-defined peptides. After that, they are digested into smaller ones and even free aminoacids by enzymes from starter or non-starter microorganisms, which will contribute to the taste and texture of the cheese (Benfeldt & Sorensen, 2001). Besides this, primary proteolysis caused by the coagulant agent affects the cheese yield through the curd firming rate (Lawrence, 1991). However, the magnitude of this effect could be greatly influenced by several factors, like type and concentration of enzyme, temperature and pH (Kowalchyk & Olson, 1977). According to Barbosa (1983), cheeses are organoleptically better when the coagulation temperature is maintained between 27 and 29 ºC; higher temperatures usually lead to losses of the softness of the final cheese.

Post-coagulation operations

A coagulated gel is very stable before cutting operation occur but after cutting synereses occurs rapidly, expelling whey. The rate and extent of this are influenced by

heating temperature, rate of stirring of the curd-whey mixture, time, concentration of Ca^{2+} and casein and pH of the whey.

To produce different cheese varieties, the cheese maker can control the syneresis (which controls the final composition of the cheese) through the amount and type of starter and the amount and type of coagulant (Fox, 1987). This knowledge can contribute to the development of new varieties of cheese made from algarvian goat milk. A better knowledge of the traditional cheese production steps will contribute to the enhancement of its quality, and development of new products to increase their consumption.

Salting

The different salting methods that can be employed are milk salting (before coagulation) as done in cheese from algarvian goat breed; salting of curd (before pressing) or immersion in brine (after pressing).

The level and method of salting influences the pH variation, physical, chemical (proteolysis, lipolysis), biological, organoleptic and rheological properties of cheese (Hayaloglu *et al*., 2002), playing an important role in cheese ripening (Guinee & Fox, 1987; Tzanetakis & Litopoulou-Tzanetaki, 1992 and Turhan & Kaletunc, 1992).

Salt acts not only as a flavour enhancer but as a preservative also, which permits the cheese to be preserved at ambient temperature in warm climates. It can control microbial growth and activity; control various enzyme activities and reduce moisture content; promote physical changes in cheese proteins which influence cheese texture, protein solubility and protein conformation and, consequently, influence the flavour development (Hayaloglu *et al*., 2002).

Ripening

Most cheese varieties need to undergo a period of ripening which varies from 4 weeks to more than 2 years before consumption. During this period, environmental conditions in the cheese production can change sufficiently to allow growth of initially inhibited microorganisms, or conditions may become even more inhospitable for microbial growth. The cheese environment is dynamic and the microbiota in cheese can be considered to be a dynamic ecological system (Marth & Steele, 2001).

This ripening time is inversely related to the moisture content of the cheese. Depending on the flavour preferences of consumers, several varieties may be consumed at different stages of maturity (Fox, 1987).

The biochemical changes that will occur during ripening, which confer the flavour, aroma and texture of the mature cheese, are largely pre-determined by the type and quality of the milk, especially moisture, NaCl, pH, type of starter and other inocula added to the cheese milk or curd (Fox, 1987).

Microorganisms and enzymes largely determine the final characteristics of the cheese, thus it must be maintained under favourable conditions for the desired microbial growth and enzyme activity.

If improper manufacturing procedures are used, the ageing conditions can result in undesirable changes. Thus, it is desirable to have a good knowledge of the main physicochemical, biochemical, and microbiological characteristics at various stages of ripening for the development of an acceptable product (Guizani *et al*., 2006).

During cheese ripening, several biochemical processes including proteolysis, lipolysis, and glycolysis occur. Proteolysis is the principal and most complex biochemical event occurring during the ripening process (Grappin & Beuvier, 1997) and contributes to cheese ripening through a direct contribution to flavour. This contribution is expressed by the formation of peptides and amino acids, and by changing the texture of cheese due to the breakdown of the protein network (Fox, 1989). At room temperature, milk proteins can contribute to firmness and milk fat can provide smoothness to cheese.

Lipolysis is another process that can play an important role in cheese flavour. Most of the free fatty acids generated from lipolysis are precursors of volatile compounds and contributed by themselves to the "goaty" flavour of cheese (Rahmet & Richter, 1996; Le Quéré *et al*., 1998).

For safety reasons, most commercial cheeses are produced from pasteurized milk, but raw milk tends to develop stronger flavours and to accelerate the ripening process (Guizani *et al*., 2006).

Sometimes microorganisms such as total coliforms and the faecal coliform E. coli, indicators of a low level of hygiene, are present in high numbers, even above the maximum permitted by legislation. However, during ripening, these numbers usually

undergo reductions, suggesting that they could be inhibited by lactic acid bacteria, which are expected to be very competitive during ripening. This process can act as a natural selector. The presence of yeasts could give important and positive sensorial effects via the development of aromas through the action of their proteolytic and lipolytic enzymes (Rohm *et al*., 1992), producing ethanol, acetaldehyde ethyl acetate and ethyl butyrate (Lenoir, 1984; Honvood *et al*., 1987). Others microorganisms can play an important role in the formation of precursors of aroma such as amino acids, fatty acids and esters, not only through their proteolytic and lipolytic activity but by stimulating the growth of other microorganisms due to the excretion of growth factors like B-vitamins, pantothenic acid, niacin, riboflavin and biotin (Purko *et al*., 1951; Lenoir, 1984).

They could also have a synergistic role by metabolizing the lactic acid present in the curd, leading to a pH level near to neutrality, which favours bacterial growth (Purko *et al*., 1951; Szumski & Cone, 1962; Fernandez Del Pozo *et al*., 1988).

1.2.6. Product quality

Both yield and characteristics of all cheese such as flavour, aroma, taste and texture are determined by the quality of milk used for their production (Summer *et al.*, 2003; Scott, 1981).

The great variation of the physical, chemical and biochemical characteristics of the cheeses are considerably influenced both by the absence of a uniform processing method and for the lack of constancy on the microbiota present. Although the Algarve goat cheese is usually manufactured without the addition of starter cultures, its safety and quality could improve significantly with the use of boiled milk and starters based on the native microbiota. Boiled milk assures that there are not any pathogenic microorganisms present. Starters based on native microbiota assure the traditional characteristics of the cheese. Knowing exactly the correct microorganisms to add permits the control of the final flavour of the product. The addition of uncontrolled native microbiota could include some microorganisms that can confer properties other than the expected ones.

Organoleptic quality and texture of cheese

The typical organoleptic properties and particular flavour of raw milk cheeses are associated with specific attributes of raw milk, related to the breed and nutrition of dairy goats, the cheese-making process and the natural microbiota responsible for the fermentation process and ripening, especially the metabolic activities of indigenous LAB (Corroler *et al*., 1998; Beresford *et al*., 2001 and Perez *et al*., 2003).

The aroma of cheese develops mainly from the volatile components released by the curd and during the ripening processes. These components include, ketones, alcohols, amines, hydrogen sulphide, ammonia, esters, fatty acids and aldehydes, but the last three are probably the most important (Scott, 1981).

Cheese made from raw milk is generally considered as having a much more intense flavour than the same kind of cheese made from pasteurized milk, which is an important marketing advantage for raw milk cheeses. The use of native starters permits to obtain cheese with similar characteristics to those produced with raw milk.

Tunick (2000) stated that the texture of a cheese could be as important as its flavour, and that most of the rheological research on solid dairy foods has dealt with cheese. The texture is closely related to the casein and its quantity and distribution, as well as the manufacturing steps, which also determine the structure of the cheese matrix (Van Hekken *et al*., 2004).

The cheese texture is a result of structure and consistency. The structure changes during the ripening period. When observing a freshly made cross-section of an algarvian goat cheese, one perceives a white colour and certain heterogeneities, such the tendency to some areas to be crumbly and not very firm. It feels rather hard and brittle generally. Upon maturation, it becomes a hard cheese.

The characteristics of cheeses change widely from rigid, almost stony, to nearly pourable; or from rubber-like to crumbly or spreadable. Even within a cheese, the consistency may vary: e.g. between the rind and centre, presence of holes, and acid spots or grains (crystals). Factors like content of fat, ripening temperature, content of calcium phosphate, content of water, content of salt and protein degradation affect the cheese consistency in different ways (Walstra *et al*., 1999).

Chemical Quality

The quality of milk affects both cheese yield and cheese characteristics, playing a very important role in the production of all types of cheeses. The efficiency of the transformation from milk to cheese depends on the loss of fat and casein to the whey, while the yield depends on the milk casein content and on the ratio between fat and casein (Summer *et al*., 2003).

In many countries, quality criteria are established according to hygienic, technological and sensorial requirements, in order to answer the needs of milk processors and consumers. These criteria are to ensure better quality final products from the hygienic and sensorial point of view (Kalantzopoulos *et al*., 2004). The above mentioned criteria have been improved over many years, according to knowledge and consumers' expectations. Some of these criteria, now applied to goat milk, some of them with adaptation, were initially established for cow milk. A better understanding of the effect of all these parameters on the final goat milk product quality is necessary in order to justify the validity of each criterion (Raynal-Ljutovac *et al*., 2005).

Milk composition has a direct impact on the acidification properties.

The chemical composition of milk varies considerably between species and even between individual animals (it is affected by breed and genetics of the animal, feed, environmental conditions, stage of lactation, and animal health (Jensen, 2002; Summer *et al*., 2003).

Milk solids are composed mainly of casein and whey protein, milk fat, lactose, citric acid, and mineral salts (usually associated with the casein). All these factors, especially a high percentage of minerals and proteins, favour the activity of the lactic acid bacteria which can influence cheese making processes and cheese characteristics. In addition, milk with high protein and especially with high casein content has high buffering properties (Raynal-Ljutovac *et al*., 2005).

Chemical analysis of the major components of milk curd and whey are usually done for processing purposes, and the estimation of minor components is sometimes necessary to rectify faults (calcium) or for the control of same processing steps, like salt addition (Scott, 1981). However, the production of cheese from algarvian goat milk is currently made in so small quantities that this kind of analysis is not made.

The pH of the milk or curd often permits the evaluation of the biological and biochemical conditions of the milk (Scott, 1981), since microbial growth changes the pH.

Microbiological quality

In many traditional cheeses to get the DPO certification, most of the specifications prescribe or advise the use of raw milk. This is due to the fact that specific native microbiota is probably the most important factor that contributes to the sensory properties of the final product (Kalantzopoulos *et al.*, 2004). Thus, it is very important to conserve this microbiota and avoid the contamination by spoilage organisms to achieve a good and consistent product. An improvement in the cheese production quality will probably rely on the exclusion of undesirable organisms, perhaps by heat treatment, and then the addition of a desirable native microbiota via a starter culture.

However, raw milk cheese represents a significant proportion of the ripened goat cheese produced in Mediterranean countries and cheese produced from raw milk is highly valued for its organoleptic characteristics (Buffa *et al*., 2001).

The criteria of hygienic and bacteriological quality in goats' milk are outlined in the Regulation (EC) nº 1662/2006 of the European Parliament of the Council of 6 November laying down specific hygiene rules on the hygiene of foodstuffs. These limits are bacterial count at 30 ºC less than 1 500 000 cfu/ml for products based on thermally treated milk and less than 500 000 cfu/ml for products based on non-thermally treated milk. Obviously if the milk will be boiled during processing it could have more microorganisms when raw, since most of them will be killed during treatment.

Cheese is very complex in respect to microbiology because of the diversity of manufacturing protocols, ripening regimens and composition of the original microbiota in the milk. Each individual cheese (not type) has its own unique microbiota depending on many factors including the starter or any deliberately added secondary ripening microorganisms, like moulds or yeasts (Marth & Steele, 2001).

The chemical composition and the microbiological quality of milk play an integral part in the quality of the cheese made from it (Marth & Steele, 2001) since, by its nature, it is a natural growth medium for microorganisms (Robinson, 2002).

The characterization of the microbial population in raw milk is important to dairy farmers and processors for several reasons. Once the milk has high bacterial counts, thermal treatment can still result in treated milk with high bacterial numbers that may be unsafe to consume and that may have reduced quality and shelf life. It may also contain high levels of enzymes, produced by bacteria that may adversely affect the quality of any processed products made from it (Marth & Steele, 2001).

The total microbial count is useful information for satisfying legal regulations, but it is of less use for assessing risks to the milk quality due to a particular bacterial population or for identifying specific sources of high bacterial counts.

Detection and quantification of a specific type or group of bacteria using selective and/or differential tests can prove to be more useful. Through these tests the dominant organism(s) in a given bacterial population can be identified. This can usually suggest a possible contamination route or source and thus aid in focusing future contamination prevention efforts. It can also help to assess bacterial problems with respect to milk quality and safety, as many spore-formers and thermoduric organisms, which can survive thermal treatments, can also grow in the processed product, diminishing product quality and its shelf life (Marth & Steele, 2001). Also bacteria with importance in the development of good properties of cheese will be detected by these methods.

This characterization, however must always consider the limitation inherent in any analytical technique, which is that no one test can detect all bacteria. Selective media may be used to promote the recovery and growth of injured or stressed cells. Further limitations are that certain microorganisms could be in very small numbers in a competitive environment or may be viable and metabolically active but not cultivable with current methods (Marth & Steele, 2001). Further, some organisms are extremely fastidious; they require additional nutrients, more time to form visible colonies, or complex selective media, to ensure sufficient nutrient access (Boor *et al*., 1998).

Deciding which tests will provide the most useful information about the microbial population of a particular product being examined is an important step to obtain an economically and logistically feasible overall picture of the product (Marth & Steele, 2001).

Spoilage and pathogenic microorganisms

There are different levels of risk from the pathogenic microorganisms eventually present in cheese (high, medium, low). The industry considers as high risk three species: *Salmonella* spp., *Listeria monocytogenes*, and enteropathogenic *Escherichia coli*. As low risk, they consider *Staphylococcus aureus* as its growth and toxin production can be readily suppressed by modern lactic culture technology and pH control in cheese (Park *et al*., 2004). However, this is not the case of cheese from algarvian goat milk.

A large number of other different organisms can be found in raw milk. This include psychrotrophs (growth at 7 ºC or less); thermoduric bacteria (which can survive pasteurisation conditions), sporeformers (which produce spores); coliforms (aerobic and facultative anaerobic); non sporeformers, Gram-negative rods (that ferment lactose with acid and gas production within 48 h between 32 and 35 ºC) and other Gram-negative bacteria commonly found in milk; pathogens that cause mastitis (infected udders), and various yeasts and moulds (Robinson, 2002).

Among the psychrotrophs we can find *Pseudomonas*, *Enterobacter*, *Flavobacterium*, *Klebsiella*, *Aeromonas*, *Acinetobacter*, *Alcaligenes* and *Achromobacter*. Examples of thermoduric bacteria could be Gram-positive *Bacillus*, *Clostridium*, *Micobacterium*, *Micrococcus*, and *Corynebacterium.* Gram-negative rods could be represented by the genera *Escherichia*, *Enterobacter*, *Citrobacter* and *Klebsiella* and Gram-negative bacteria commonly found in milk could be *Pseudomonas flourescens*, *P. putida*, *P. fragi* and *P. putrefaciens* for example (Robinson, 2002).

The most frequently isolated yeasts are *Candida* spp., *Yarrowia lipolytica*, *Kluyveromyces marxianus*, *Geotrichum candidum*, *Debaryomyces hansenii* and *Pichia* spp. (Marth & Steele, 2001).

Although the presence of some microorganisms can improve the cheese flavour, it is known that the metabolism of the above mentioned contaminating microorganisms can cause poor flavour quality in cheese (Marth & Steele, 2001).

Lactose present in milk could be fermented by spoilage bacteria producing numerous volatile compounds, including acetic and butyric acids, carbon dioxide and hydrogen gas, and various alcohols that can adversely affect milk flavour. Bacterial

degradation of proteins and their secreted enzymes can create bitter-tasting peptides; cause curdling and clotting of the milk; resulting in production of ammonia and hydrogen sulphide; or even cause jellification of the milk (Cousin, 1982).

It must be kept in mind that not all undesirable attributes of a cheese result from contaminating microorganisms. Some cheese defects may be caused by the lack of milk quality due to late lactation milk, milk from mastitic animals that is high in enzymes of animal origin, inappropriate rates of acid development by the starter, or poor manufacturing and storage procedures (Marth & Steele, 2001).

Preventive measures on the farm and processing plant can avoid or reduce many sources for microbial contamination of raw milk and milk products. Milk quality and safety of processed products are ensured by processing strategies designed to reduce and control bacterial numbers. Some of these strategies consist of minimising the time between collection and thermal treatment or efficient cooling of milk to 4 ºC immediately following milking (Marth & Steele, 2001).

Thermal treatments aim to eliminate the non-sporeforming pathogens most resistant to thermal destruction, however, the effectiveness of this treatment in killing bacteria in milk depends on initial microbial counts, composition (fat and sugar), and thermoresistance of each microorganism. Although most of these microorganisms are easily killed by pasteurisation, some of them could produce lipases and proteases, which are not totally inactivated by this heat treatment (Griffiths *et al*., 1981).

Usually growth of yeasts is not desirable in most cheeses; however it is desirable in surface-ripened and some mould-ripened varieties. Its presence as spoilage organisms is particularly easy to identify by its heterofermentative metabolic activity (production of alcohol and CO_2) even if visible colonies are not observed. The yeasts can confer a yeasty taste to the cheese like raw fermented bread dough, but not all contaminating yeasts produce this typical smell. Some very proteolytic yeast produces 'stinker' cheeses, resembling that of rotten eggs and this is often associated with white spots on the cheese surface. Nevertheless, lipolysis can affect negatively the flavour by giving the cheese a rancid flavour, through the production of an excessive amount of volatile free fatty acids resulting from the action of lipases (Grappin & Beuvier, 1997). When present in undesirable situations, yeasts are commonly associated with

slimy surface defects, but other putrefactive organisms like *Pseudomonas* spp. and *Enterococcus* spp. can contribute to this defect too (Robinson, 2002).

Sources of contamination

Cheese is a complex entity in a constant state of variation, due to an ecological community of living organisms in which microbiological activities affect and are influenced by chemical changes (Marth & Steele, 2001).

Types and strains of spoilage bacteria present in cheese are dependent on initial numbers in milk (especially if raw milk is used), biofilm formation on equipment and subsequent contamination, and ability of individual strains to grow in the extrinsic factors of the environment (pH, salt, a_w, acidity, temperature, availability of nutrients) (Marth & Steele, 2001).

There are three basic sources of microbial contamination of milk: within the udder; the environment and subsequent handling (Robinson, 2002).

From the udder, pathogenic bacteria to humans and which commonly produce mastitis can be isolated such as *Streptococcus agalactiae*, *Staphylococcus aureus*, and enteropathogenic *Escherichia coli* (Robinson, 2002).

Environmental sources (air, water, soil, vegetation, and bedding material), could introduce microorganisms to the raw milk in varying types and numbers. Bedding material, untreated water, soil and vegetation are usually associated with psychotropic microbiota contamination; coliform are associated with soil; and spore formers with bedding material. Poor pre-milking udder hygiene can result in the contamination of milk by vegetation, soil, and bedding material and their associated microorganisms. Cleaning and drying of the udder, immediately before milking, can lower total bacterial loads (Cousin, 1982). In the Algarvian goat cheese case, an insufficient cleaning process of the udder, using moisture clothe for instance, can be a hygienic problem. Sensitization of the producers'to this problem is in progress.

The presence of coliforms in raw milk, nowadays, is not considered evidence of direct faecal contamination and should not be relied upon to detect inadequate udder cleaning prior to milking. Equipment could become a major source for contamination of the milk being collected, since coliforms can rapidly proliferate in moist residues of milk (forming biofilms). A sporadic high coliform count could be related to an unrecognized coliform mastitis in the dairy herd and relatively low coliform counts in

milk do not necessarily indicate effective cleaning and disinfecting of the equipment (Robinson, 2002).

1.2.7. Added microbiota

Both starter and non starter bacteria could be added to the milk during cheese production process.

The principal role of starter bacteria (native or added) is to produce acid at a consistent rate, but it is not the only one. The starter has practically no impact on flavour in cheese consumed fresh. As cheese matures, the direct contribution of starter bacteria to the flavour decrease at the same time as non-starter microbiota grows. So, typically, the starter and non starter bacteria can both influence cheese maturation processes (Marth & Steele, 2001).

The rate of acid development by the starter influences the speed of coagulation, being of a great importance to the moisture content of cheese. Thus, the control over the rate of acid development is the key to successful cheese manufacture characteristics. Body (soft to firm), texture (grainy to smooth), melt, stretch, chewiness, oil release during heating, casein hydration, and colour of cheese are directly controlled by pH. But also flavour development is strongly influenced by pH because it is a determinant of the metabolism of the microorganisms (Marth & Steele, 2001).

Cheeses made from pasteurized milk tend to have a milder flavour (the flavour takes longer to develop or the flavour is atypical of raw-milk cheese) (Marth & Steele, 2001). However, the utilization of starters from indigenous microbiota could solve this problem.

The balance of strains of bacteria used in the starter formulation is often dictated by tradition and by the manufacturing protocol in order to obtain the desired cheese characteristics. The choice of starter depends on the desired properties to be given to the cheese. These include the rate and extent of acid development (pH) during manufacture, proteolytic activity of the strains and conditions encountered during manufacture and storage (pH, acidity, salt and temperature). The amount of starter used is dictated by cheese variety, but it is based on the rate of acid development

desired by the manufacturer, and is also influenced by the method of culture propagation (Marth & Steele, 2001).

1.2.8. Lactic Acid Bacteria

Lactic acid bacteria (LAB) have been used in food processing, namely in dairy and meat fermented products for their contribution to shelf life, texture, and organoleptic properties (Cogan & Hill, 1993). This capacity of food preservation is due to the production of a variety of antimicrobial agents, including organic acids like lactic and acetic acid, ethanol, carbon dioxide, diacetyl, and hydrogen peroxide (De Vuyst & Vandamme, 1994). Some LAB are also able to produce bacteriocins. These substances, from a proteic origin, have bactericidal activity against microorganisms closely related to the bacteriocin producer strains (Klaenhammer, 1988; Jack *et al*., 1995, Daeschel, 1989; Piard & Desmazeaud, 1992 and Klaenhammer, 1993). LAB are 'generally regarded as safe' (GRAS) microorganisms and so are their bacteriocins. This condition potentially permits the bacteriocins to be used to control the growth of spoilage and pathogenic organisms in food (Hoover & Steenson, 1993; De Vuyst & Vandamme, 1994).

Lactobacillus, *Lactococcus*, *Enterococcus* and *Leuconostoc* are some of the LAB genera involved in cheese manufacture and ripening. Some strains of bacteriocin-producers *Lactococcus* species have been used successfully as part of starter cultures for cheese making in order to improve the safety and quality of cheeses (Lipinska, 1973; Maisnier-Patin *et al*., 1992; Delves-Broughton *et al*., 1996 and Ryan *et al*., 1996). These strains should then be combined with other strains, which are bacteriocin-resistant (Wouters *et al.*, 2002).

Enterococcus species that produce bacteriocin are widespread in nature (Brock *et al*., 1963; Giraffa, 1995) and have been isolated from a large number of sources, such as dairy products, vegetables, silage, fermented sausages, fish, and even from the mammalian gastrointestinal tract (Laukova *et al*., 1993; Ben Embarek *et al*., 1994; Kato *et al*., 1994; Franz *et al*., 1996; Cintas *et al*., 1997 and Ennahar *et al*., 1998).

They are thought to play a role in the development of flavour characteristics during ripening due to their proteolytic and lipolytic activities as well as their ability to metabolize citrate and lactose (Sarantinopoulos *et al*., 2001). In fact, their

utilization in certain starter cultures for cheese production (Coppola *et al*., 1998; Parente *et al*., 1989; Litopoulou-Tzanetaki *et al*., 1993; Centeno *et al*., 1996; Centeno *et al*., 1999 Sarantinopoulos *et al*., 2002a,b) suggests their important role in the development of cheese characteristics

Several enterococcal bacteriocins (enterocins) have been characterized at the molecular level and it was verified that all enterocins share a number of common characteristics such as stability to heat and stability over a wide range of pH values, and activity towards *L. monocytogenes* (Giraffa, 1995) *Staphylococcus aureus* and *Clostridium botulinum* (Stiles, 1996), thus they could be exploited as commercial starter culture, if they can be considered as safe (Psoni *et al*., 2006)

There are some reports that *Enterococcus faecalis* and *E. faecium* strains are able to produce bacteriocins against *Listeria* when grown in milk and cheeses (Parente & Hill, 1992; Villani *et al*., 1993; Giraffa *et al*., 1994; Torri Tarelli *et al*., 1994; Garcia *et al*., 1997; Giraffa & Carminati, 1997;; Ennahar *et al*., 1998). These strains are of great technological importance in cheese manufacture, since *L. monocytogenes* can survive the manufacture and ripening conditions of cheeses, such as Camembert (Sulzer & Busse, 1991), Taleggio (Giraffa *et al*., 1994), Mozzarella (Stecchini *et al*., 1995) and Manchego & Chihuahua from Mexico (Solano-López and Hernández-Sánchez, 2000). This can be confirmed by the fact that in Greek Feta cheese, the presence of *Listeria* spp. has never been reported, which could be attributed to the Feta cheese microbiota, where *Enterococcus*, has been frequently reported in the ripened product by Tzanetakis & Litopoulou-Tzanetaki (1992). There are no studies in cheese from algarvian goat breed.

Enterococcus spp., that are considered either a contaminant from the environment or a natural starter culture, depending on their behaviour, can contribute to the development of the organoleptic properties of the ripened cheese (Ordonez *et al*., 1978; Parente *et al*., 1989; Litopoulou-Tzanetaki *et al*., 1993). They can easily grow in the cheeses environment (high salt content and low pH), and many authors believe that they contribute , in addition to the production of bacteriocins, to proteolytic and lipolytic activities, which may play a significant role in cheese maturation process (Wessels *et al*., 1990; Litopoulou-Tzanetaki & Tzanetakis, 1992; Freitas *et al*., 1995; Centeno *et al*., 1996).

The addition of *Enterococcus* as adjunct cultures has been suggested as a mean of controlling numbers of adventitious *Lactobacillus* by, at least initially, competing with them in the cheese and thus controlling cheese flavour development (Martley & Crow, 1993).

The non starter lactic acid bacteria (NSLAB) are bacteria that have the capacity to grow under highly selective conditions prevailing during cheese ripening. These conditions include: lactose depletion in the first hours of cheese manufacture (by the fermentation done by the starter bacteria), temperature below 15 ºC, pH between 4.9 and 5.3, moisture content of 50 %, salt concentration around 4–6 % and low levels of oxygen (Martley & Crow, 1993). This makes the ripening cheese conditions a hostile environment for most microorganism's growth. Nevertheless, the adventitious *Lactobacillus* manage to grow, although at a low rate, but the generally long ripening period allows them enough time to reach considerably high levels of cfu/g in cheese (Martley & Crow, 1993).

Since NSLAB dominate the microbiota of many ripened cheeses, they are believed to contribute to the maturation of cheese. It is already known that strains can affect the development of each other, thus, the development of a secondary microbiota depends also on the properties and composition of the starter, if used (Martley & Crow, 1993). This group of bacteria also possesses several metabolic properties which contribute to the biochemical events of proteolysis and lipolysis, which are of exceptional importance in cheese making (Perez *et al*., 2003).

The presence of *Lactobacillus* may have some negative effects on the quality of the cheese and the presence of *Leuconostoc* species appears to affect the development of adventitious NSLAB (Wouters *et al*., 2002).

LAB carry out the initial acidification of the milk, improving its jellification when the coagulant agent is added. Its ability to produce acid in a rapid way is probably the most important property of starter bacteria (Cogan *et a*l., 1997). They can also contribute, indirectly via acid production, to the expulsion of the whey, the solubilisation of the micelar calcium and, consequently, to the texture of the cheese (Herreros *et al*., 2003). Furthermore, a low moisture content and a relatively low pH in cheese reduces the risk of microbial contamination by pathogens and spoilage microorganisms (Perez *et al*., 2003)

Some LAB can degrade the products derived from the coagulant action on the casein (peptides of different molecular mass), contributing to the proteolysis of cheese. Some strains have proteinases associated with the cell wall, similar to endo- and exopeptidases, which preferentially hydrolyse the casein. After that, the free peptides of the casein are hydrolysed to smaller peptides and free amino acids by the peptidases from the interior of the cells (Herreros *et al*., 2003).

The LAB proteolytic system plays an important role in the development of textural and organoleptic characteristics of the mature cheese (Fox, 1989), since undesirable bitter tasting peptides produced from casein during ripening are degraded by LAB proteinases and peptidases. The formed peptides of lower molecular mass or amino acids contribute directly to cheese flavour and serve as substrates for other flavour-generating reactions (Olson, 1990).

Information on the contribution of LAB to lipolysis, during the ripening period of the cheese is scarce. Some species of LAB are able to hydrolyse milk fat or, at least, some triglycerides (Gobbetti *et al*, 1996), resulting in the formation of free short chain fatty acids (El-Soda *et al*., 1986). This means that lipases of starters and NSLAB (which are generally homo- and heterofermentative species of *Lactobacillus*) can influence the development of cheese flavour to a greater or lesser extent (Herreros *et al*., 2003).

1.3. Aims and Objectives

1.3.1 Overall Aims

Cheese made from the algarvian goat breed is closely associated with the territory of production and its tradition is unique through the historical and cultural environment. Thus, considering there is no published information concerning this product, its characterization is very important.

Cheese sensorial characteristics depend on several factors such as the chemical properties of milk, cheese making practices, goat feeding as well as the composition and dynamics of the native microbial communities. The chemical characterization of the milk and cheese is also important in order to foresee the possible variation in cheese quality during the lactation period.

Spore forming microorganisms can survive in dry cardoon and since its addition is made after boiling the milk (when it is the case), the microbiological characterization of this vegetable coagulant is important in order to determine its contribution to the cheese contamination and to guarantee the cheese safety.

The biodiversity of LAB involved in cheese production is considered a fundamental factor for the maintenance of the characteristic features and quality of traditional cheeses. In order to minimise safety problems, it is advisable to use heat treated milk in cheese production, which implies the destruction of LAB. This suggests that starter cultures should be added to treated milk prior to the cheese manufacture and therefore, there is a great need for new starter cultures to use in the production of traditional cheeses. So, it is important to isolate and characterise wild strains of LAB associated with raw milk and artisanal cheese (across the ripening period) from the algarvian goat breed. The strains should possess good technological properties such as salt tolerance, proteolytic activity, and acid and bacteriocin production, in order to select them as starter cultures to improve the characteristics of cheeses made from heat treated (boiled) milk.

1.3.2. Specific Objectives

This work aims to:

- characterize the gross chemical composition of the algarvian goat breed milk and fresh cheese during all the lactation period;
- characterize the microbiota of dry cardoon thistle flower used as cheese coagulant;
- characterize the microbiota of raw milk and cheese immediately after processing from algarvian goat breed during the lactation period;
- characterize the microbiota of the cheese made from milk of algarvian goat breed during cheese ripening;
- isolate the indigenous lactic acid bacteria from raw milk and from cheese during ripening;
- characterize the isolates and identify them to genus level;
- study the technological characteristics of the identified isolates, namely their bacteriocin production capacity, acidifying capacity, proteolytic capacity and lipolytic

capacity. This information will inform and guide the selection of those with good technological capabilities for cheese manufacture using heat treated (boiled) milk.

Chapter 2. Material and Methods

2.1. Gross Chemical Composition of Milk and Fresh Cheese

2.1.1. Goat Milk

Sampling

Bulk milk (from two days with collection at the beginning and at the end of the day) was directly collected from cheese vats before and after boiling Milk samples were collected twice a month from October to July, from CEAP (Agriculture Experimental Centre of Algarve) and always from the same heard.

pH determination

The pH was measured with a Crison 2001 pH-meter (Crison Instruments S.A., Barcelona, Spain). All determinations were performed in triplicate.

Conductivity

The conductivity was measured with a Crison 2001 conductivity-meter (Crison Instruments S.A., Barcelona, Spain). All determinations were performed in triplicate.

Sodium chloride content

The sodium chloride content was measured with a Crison 2001 conductivity-meter (Crison Instruments S.A., Barcelona, Spain). All determinations were performed in triplicate.

Total solids

Total solids (TS) content was determined in the milk according to the AOAC 990.19 (2005). The milk sample was weighed (3±0.1 g, 38±1 ºC) in a pre-weighed dry (105±1 ºC, ≥ 2 h) porcelain dish. The exact weight was registered. The porcelain dish was placed in a sand bath to pre-dry and after that was transferred to an oven (105±1 ºC), for 3 h. It was cooled to ambient temperature and weighed to constant weight. Two dry porcelain dishes were used as blanks. All determinations were performed in triplicate.

$$\% \text{ total solids} = \frac{(W_2 - W) - B}{W_1 - W} \times 100$$

W – dish weight

W_1 – dish weight + sample

W_2 – dish weight + dry sample

B – average of blank weight

Ash content

The ash content was determined by AOAC, 945.46 (2005). 5 g of sample was Weighed into a dish and allowed to dry in a sand bath and was then ignited to remove all the fat and was placed into a furnace at 550 ºC until it was free of carbon. The samples were then cooled in a desiccator and Weighed to constant weight. The ash percentage was calculated. All determinations were performed in triplicate.

Fat content

The fat content was determined by the Gerber method – NP 469 (1983). All determinations were performed in triplicate.

Total nitrogen

Total nitrogen content was determined by the Kjeldahl method - AOAC 991.20 (2005).. All the analyses were performed in triplicate.

$$\% \text{ N} = \frac{1.4007 \times (V_s - V_b) \times M}{W}$$

V_s e V_b ml of HCl used in the sample and blank titration, respectively.

M – molarity of the HCl solution

W – sample weight

Protein

The nitrogen content value, determined using the Kjeldahl method, was multiplied by the factor 6.38, resulting of the ratio 100/15.65 (milk proteins have 15.65 % of nitrogen), to determine the crude protein content (AOAC 991.23, 2005).

Non-protein nitrogen fraction

The non-protein nitrogen fraction was determined by the Kjeldahl method - AOAC 991.21 (2005). All the analyses were performed in triplicate.

$$\% \text{ N} = \frac{1.4007 \times (V_s - V_b) \times M}{(W_f \times W_m) / [W_t - (W_m \times 0.065)]}$$

Vs e Vb - HCl volume (ml) used in sample titration and blank, respectively.

M – molarity of HCl solution

Wf – weight (g) of 20 ml of filtrate

Wm – milk weight (g)

Wt – milk weight (g) + 40 ml of TCA, 15 %.

Protein nitrogen fraction

The protein nitrogen fraction was calculated by AOAC 991.23 (2005), by the difference between total nitrogen and non protein nitrogen.

2.1.2. Fresh Cheese

Cheese manufacture

Cheeses were manufactured by CEAP (Agriculture Experimental Centre of Algarve) according to traditional procedure for fresh cheese (without starter addition). The milk was boiled up (by law, it must be heat treated for production of fresh cheese) and then cooled to around 30 ºC. The clotting of boiled milk was done by the addition of *Cynara cardunculus* aqueous extracts (15 % w/v) and 12 g/l of NaCl was added. The milk clotting temperature was between 29 and 32 ºC.

Sampling

Cheeses were sampled, immediately after its production, twice a month, during the lactation (cheese production period from October to July)

pH determination

The pH of the cheese samples was measured using a Crison 52-32 electrode for solid samples (Crison, Barcelona, Spain). All the analyses were performed in triplicate.

Total solids

The total solids content was determined in the cheeses by drying the samples (5 ± 0.1 g), in an oven to constant weight at 105 ºC (NP 3544, 1987). All the analyses were performed in triplicate.

$$\% \text{ total solids} = \frac{(W_2 - W) - B}{W_1 - W} \times 100$$

W – dish weight

W_1 – dish weight + sample

W_2 – dish weight + dry sample

B – average of blank weight

Ash content

The ash content was determined by AOAC official method 935.42 (2005). 3 to 5 g of cheese were weighed in a porcelain dish and placed in a sand bath for 1 h. After

that, it was ignited to burn away all the fat and then ignited again in a furnace at 550 ºC. Finally, it was cooled in a desiccator and weighed to constant weight. All the analyses were performed in triplicate.

Fat content

The fat content was determined by the Gerber method, according to Pearsons's handbook (Egan *et al*., 1990). The cheese was homogenised and transferred to a plastic bag. The analysis started with the addition of 10 ml of sulphuric acid and a layer of 6 mm of warm water to a Gerber butyrometer. After that 1 g of cheese sample and 1 ml of amílic alcohol were added. Finally, the butyrometer was filled with warm water. The butyrometer was capped and put in a thermal bath at 65±2 ºC, for 3-10 minutes, mixing until complete dissolution of casein had occurred. It was centrifuged in a Gerber centrifuge, for 5 minutes at 1000-1200 rpm and 65±2 ºC and readings were taken. All the analyses were performed in triplicate.

Total nitrogen

The total nitrogen was calculated using the Kjeldahl method for cheese - AOAC 2001.14 (2005). The cheese was homogenised at a temperature below 20 ºC and put in a plastic bag. All the air in the bag was removed, by pressing the exterior part, in order to homogenize the cheese inside. 1 g of cheese was weighed on a filter paper; the filter was bent and put in a Kjeldahl tube. All the analyses were performed in triplicate.

Protein

The value of nitrogen content, determined using the Kjeldahl method, was multiplied by a factor (6.38) to determine the crude protein content (AOAC 991.23, 2005).

Non-proteic nitrogen fraction

Non-proteic nitrogen fraction was determined by Kjeldahl method - AOAC 991.21 (2005). Sample preparation was prepared as previously described, except for 1 g of cheese that was dissolved in 50 ml of TCA at 12 % and filtrated in a Watman nº 1 filter paper. A sample of 20 ± 0.2 ml of filtrate was taken and analysed as described above. All the analyses were performed in triplicate.

Protein nitrogen fraction

The protein nitrogen fraction was calculated by AOAC 991.23 (2005), as the difference between total nitrogen and non protein nitrogen.

2.2. Microbiology of Cardoon

2.2.1. Collection of Cardoon Flowers

Four types of *Cynara cardunculus* thistle flowers with different phenotypic characteristics and seeds coming from different regions of Portugal and Spain were collected from a single paddock in Tavira (Portugal) during March 2006 and March 2007, after being cultivated for study proposes. The cultivation occurred without any treatments. The seeds were sown in October and the plant was developed until thistle flowers were collected in March. Only water was added to the culture.

Fresh flowers were collected and transported to the laboratory in open plastic containers. Then, they were dried in a monolayer on a surface covered by paper in a dry temperature controlled room, with temperatures between 22 and 25 ºC. After drying, they are stored in the laboratory at around 21 ºC, under dark and dry conditions. As needed, the bracts were separated from the flowers and the extracts were prepared for milk coagulation.

Seven groups were prepared. One resulting from the mixture of the four types (Complete mixture), four individual types (Arouca, Altos without spines, Spain, Tondela) and one (Tondela only flower) constituted only by the violet part of the thistle, where the coagulant activity is present (Faro, 1991). Another one was brought from a local collector (Producer cardoon) in a local market, which is normally the way to obtain the vegetable coagulant used in cheese production.

2.2.2. Water Activity (a_w) Determination

Water activity was determined using a Novasina water activity meter (Novatron, Switzerland) at 24.7 ºC. All the analyses were performed in triplicate.

2.2.3. Sample Preparation for Microbiological Analysis

From each group, seven samples were taken, which were prepared for microbiological analysis, according to NP1829 (1982). They were diluted in sterile Ringer solution (Oxoid) (1/10 v/v) and aliquots (1 ml) were plated in triplicate on each culture medium.

2.2.4. Microbiological Analysis

Total heterotrophic bacteria were enumerated on Plate Count Agar (PCA; Biokar, UK) and incubated at 30±1 ºC for 72±3 h (NP1995 from 1982); faecal coliforms on Violet Red Bile Agar (VRBL) and incubated at 44±0.5 ºC for 24±2 h (NP3788 from 1990) and moulds and yeasts on Rose Bengal Chloramfenicol Agar (Biokar) and incubated at 25±1 ºC for 120±2 h (NP3277-1 from1987). All determinations were done in triplicate and expressed as log colony-forming units per gram of sample (log cfu/g).

2.2.5. Identification of Moulds

Moulds cultivated in Rose Bengal Chloramphenicol agar medium were identified according to cultural and morphological characteristics, based on their vegetative and reproductive structures.

A small piece of tape was applied on the colony and deposited on a slide. A small drop of water was then deposited on top of the tape and microscopic examinations were carried out (Aïssi *et al*., 2009).

2.2.6. Preparation of the Vegetable Coagulant

Dried thistle flowers from *Cynara cardunculus* from all types studied were ground for 1 minute in a domestic grinder (Moulinex); the powder (1.5 g) was diluted in distilled water (10 ml) and rested at room temperature for 5 hours. The homogenate was filtered through Whatman paper n. 1 to give the vegetable coagulant preparation, to be used for the cheese preparation (adapted from Chen *et al*., 2003). The pH values were around 5.5 ± 0.1.

2.2.7. Statistical Treatment

The obtained results for the different determinations of the microbiology of cardoon were subjected to a statistical analysis performed at 95 % level of significance, using SPSS (v.16.0; SPSS, inc.), which was followed by a Dunn test (Dunn, 1964).

2.3. Microbiology Evaluation of Raw Milk

2.3.1. Sample Collection and Preparation

Raw goat milk was collected from the bulk tank of a farm in the Algarve, during a lactation period (from May to July), and transported to the laboratory under refrigerated conditions. Samples were taken and prepared for microbiological analysis, according to NP1829 (1982). Samples were diluted in sterile Ringer solution (Oxoid) (1/10 v/v). Aliquots (1 ml) were plated in triplicate on each enumeration medium.

2.3.2. Microbiological Analysis

Total heterotrophic bacteria, faecal coliforms, yeasts and moulds were enumerated according to NP1995 (1982), NP3788 (1990) and NP3277-1 (1987), respectively, as described before (section 2.2.4). *Lactobacillus* on Rogosa agar (Biokar) incubated at 36±1 ºC for 72 h (instructions for use, Biokar)

All determinations were done in triplicate and expressed as log colony-forming units per ml of sample (log cfu/ml).

2.3.3. Statistical Treatment

The average value and standard deviation from all the microorganisms' enumerations were calculated by Excel 2003.

2.4. Microbiological Quality of Cheese immediately after processing

2.4.1. Cheese Manufacture Procedure and Sampling

Cheeses were prepared at the food processing laboratory at Instituto Superior de Engenharia, of the Algarve University using raw milk from algarvian goat breed to which no starter culture was added. Cheese samples for microbiological analyses were prepared with raw milk because the objective was to study the native microbiota and later follow their dynamic during ripening.

The raw milk was coagulated with an aqueous extract of *C. cardunculus* (Producer cardoon type), prepared as described in 2.2.6, using 0.25 g of cardoon/l of milk, which means 1.67 ml of suspension/l of milk. Eight experimental batches were prepared during the lactation period (from May to July) and each consisted of six cheeses. The clotting temperature for milk was 30±1 ºC. Salt for domestic use was added to the milk in a concentration of 12 g/l milk, immediately before the coagulant agent addition. Curd was cut into 6-8 mm grains and the whey was eliminated. Cheeses, of 8 cm diameter, 3 cm height, and 200 g weight, were hand pressed.

Since these cheeses were prepared with raw milk they can be consumed only after a ripening period of two months. They were analysed in the fresh state in order to study the influence of the lactation period in the microbiota of cheese.

From each batch, two cheeses were analysed.

2.4.2. Sample Preparation

Samples for microbiological analysis were prepared according to NP1829 (1982). Twenty five grams of cheese, representing both the internal and the external part, was homogenized with 225 ml of Ringer solution (Oxoid), in a Stomacher Lab-Blender 400 (Seward Medical, London, U.K.) for 1 minute.

Dilutions of cheese were prepared in sterile Ringer solution (Oxoid) (1/10 v/v) and aliquots (1 ml) were plated on each culture medium.

2.4.3. Microbiological Analysis

Total heterotrophic microorganisms, faecal coliforms, yeasts and moulds were enumerated according to NP1995 (1982), NP3788 (1990) and NP3277-1 (1987), respectively.

All determinations were made in triplicate and expressed as log colony-forming units per gram of sample (log cfu/g).

2.4.4. Statistical Treatment

The average value and standard deviation from all the microorganisms' enumerations were calculated by Excel 2003.

2.5. Microbiology of Cheese During Ripening

2.5.1. Cheese Manufacture Procedure and Sampling

Three batches of algarvian goat's milk cheese were made by CEAP (Agriculture experimental Centre of Algarve), using the procedure described above, but with larger quantities. The cheeses were ripened for 60 days, at 15 ºC and 70 % relative humidity in a ripening chamber. This is the period that this kind of cheese is submitted to ripening conditions in the traditional process.

Every week, including immediately after production, one cheese from each batch was taken and transported to the laboratory under refrigeration. Sampling and microbiological analyses were performed within the following 5 hours.

2.5.2. Sample Preparation

Samples were prepared as described for cheese immediately after processing (section 2.4.2.).

2.5.3. Microbiological Analysis

Total heterotrophic microorganisms, total and faecal coliforms, yeasts and moulds were enumerated according to NP1995 (1982), NP3788 (1990) and NP3277-1 (1987), respectively, as described before (section 2.2.4). Total coliforms were incubated at 44±0.5 ºC for 24±2 h Presumptive *Lactobacillus* on Rogosa agar (Biokar) incubated

at 36±1 ºC for 72 h (instructions for use, Biokar) and presumptive *Lactococcus* on M17 agar (Biokar) incubated at 30±1 ºC for 48 h (instructions for use, Biokar).

All determinations were made in triplicate and expressed as colony-forming units per g (cfu/g) of cheese.

Isolates of lactic acid bacteria were kept at -80 ºC for further studies.

2.5.4. Statistical Treatment

The average value and standard deviation from all the microorganisms' enumerations were calculated by Excel 2003.

2.6. Isolation and Phenotypic Characterization of Lactic Acid Bacteria (LAB)

2.6.1. Samples from Raw Milk Obtained During the Lactation Period Between May and July

Isolation

In this study, Lactic Acid Bacteria (LAB) were isolated from raw milk, obtained from the dairy of CEAP, using Rogosa agar (RA), incubated at 36±1 ºC for 72±2 h. Thirty colonies per plate were randomly taken. These selected colonies were purified by subculture in MRS (Man Rogosa Sharp) broth and stored at - 80 ºC in MRS plus glycerol (40 % (v/v), for further characterization.

Phenotypic Characterization

A total of 180 isolates were examined for colony and cell appearance, Gram stain, catalase activity, CO_2 production, tolerance to sodium chloride (4 and 6.5 %), growth at pH 4.4 and 9.6 and two time-temperatures scales (10 days at 6±0.5 ºC, 2 days at 30±1 ºC and 7 days at 45±1 ºC) on MRS agar agar plate or M17 agar plate, for rod and coccus shape, respectively.

From plates of RA incubated aerobically, catalase activity was tested in bacteria under 20 % (v/v) H_2O_2, on a glass slide.

The carbohydrate metabolism was monitored by CO_2 gas production from glucose, detected by inverted Durham tubes in MRS broth. Cultures were examined daily for 1-5 days.

Identification to Genus Level

Identification of the isolates was performed according to methods and criteria described in the Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994) and Axelsson (1998).

A summary of the differentiation of the LAB genera with classical phenotypic tests is shown in Table 2.1.

Table. 2.1. Differential characteristics of LAB (Adapted from Axelsson, 1998).

	Rods				Cocci				
	Carnob	*Lactob.*	*Aeroco.*	*Enteroc.*	*Lactoc Vagoc.*	*Leuc. Oenoc.*	*Pedioc.*	*Streptoc.*	*Weissella*[a]
CO_2 from glucose	-	±	-	-	-	+	-	-	+
Growth at 10 ºC	+	±	+	+	+	+	±	-	+
Growth at 45 ºC	-	±	-	+	-	-	±	±	-
Growth at 6.5 % NaCl	nd	±	+	+	-	±	±	-	±
Growth at pH 4.4	nd	±	-	+	±	±	+	-	±
Growth at pH 9.6	-	-	+	+	-	-	-	-	-

nd not determined, **+** positive, **-** negative, **±** response varies between species [a]*Weissella* strains may also be rod-shaped.

Observing the cell morphology using microscopic observation, LAB could be divided into rods (*Lactobacillus* and *Carnobacterium*) and cocci (all other genera). *Weissella* can show both cocci and rods shapes (Collins *et al*., 1993).

CO_2 production from D-glucose in MRS broth with a Durham tube to collect gas was used to distinguish between the fermentation groups: *Leuconostoc*, *Oenococci*, *Weissella*, and a subgroup of *Lactobacillus* are heterofermentative (produce CO_2); all other LAB are homofermentative (Axelsson, 1998).

Cell growth at various temperatures gave information for the classification as *Enterococcus* - if the cells grew at both 10 ºC and 45 ºC - or as *Lactococcus* and *Vagococcus* if growth occurred at 10 ºC, but not a 45 ºC. *Streptococcus* generally did not grow at 10 ºC. Growth at 45±1 ºC was dependent on the species (Axelsson, 1998).

Tolerance to sodium chloride (6.5 % NaCl) was used to distinguish between *Enterococcus* (positive), and *Lactococcus/Vagococcus*, and *Streptococcus* (negative), although variable reactions can be found among *Pediococcus*, *Leuconostoc*, *Oenococcus* and *Lactobacillus* (Holt *et al*., 1994).

Growth at different pH levels helped to distinguish between *Aerococcus* and *Enterococcus*, which both grow at pH 9.6, compared to *Carnobacteria*, and

Vagococcus which grow at relatively high pH, but not at the tested pH of 9.6 (Axelsson, 1998).

Carnobacteria can be distinguished from *Lactobacillus* by their ability to grow at pH 9.0 and inability to grow on acetate media, which is selective for *Lactobacillus*.

2.6.2. Samples of Cheese Collected During Ripening

Isolation

From each studied stage (samples were taken once a week during two months of ripening), ten colonies were picked up from the first count's plate given separated colonies on Rogosa agar (presumptive *Lactobacillus*) and M17 agar (presumptive *Lactococcus*), as described by Macedo *et al*. (1995). Strain were purified by two consecutives subcultures in MRS and isolated by plating on MRS. The purified strains were frozen at -80 ºC in MRS containing a final glycerol concentration of 50 % (v/v) for further characterization.

Phenotypic characterization

A total of 180 isolates were examined and characterized as described earlier for colonies isolated from cheese immediately after processing during the lactation period.

Identification to genus level

The identification to genus level was performed as described before for colonies isolated from cheese immediately after processing during the lactation period.

2.7. Technological Properties of Isolated LAB

To evaluate the technological characteristics of the isolated strains of LAB from milk and cheese, bacteriocin production, acidification capacity, proteolysis and lipolysis were studied.

Morphological and biochemical characterisation allowed for the selection of isolates that could represent possible starter cultures in the future. These isolates were selected to represent the widest range of properties possible in the combination.

2.7.1. Bacteriocin Production

The presence of bacteriocins was tested against *Weissella paramesenteroides* DSM 5623 strain, which is extremely sensitive to bacteriocin and other antimicrobial compounds. The strains to be tested were sub-cultivated twice in MRS broth at 30 ºC, for 24 h. Two Eppendorff tubes with 1 ml of the culture broth of the selected strain were centrifuged for 15 minutes at 10 000 g. One of these tubes was heated at 100 ºC for 15 min, cooled in ice, and filter-sterilized (syringe filter Acaroids 0.2 mm, Pall-Life Sciences, USA). The other tube was only submitted to filter sterilization. This cell free supernatant (from the two tubes) was then used in bioactivity assays.

The antibacterial activity of cell free supernatant was evaluated and compared by an agar diffusion assay that consisted of a modification of the procedure described by Mayr-Harting *et al*. (1972). Briefly, MRS agar plates were prepared, and an overlay of MRS soft agar (0.75 % agar) was inoculated with a concentration of 10^7 cfu/ml of the indicator bacterium (*Weissella paramesenteroides* DSM 5623). A volume of 20 μL of each cell free supernatant was applied on blank paper disks (Antimicrobial susceptibility test disks, Oxoid, England) and incubated at 30 ºC, for 24 hours. Nisin (50 mg/100 ml water) was used as standard to verify the susceptibility of *Weissella*. The diameter of the clear zone around the discs was measured and recorded. All the analyses were performed in triplicate.

2.7.2. Acidifying Capacity

The acidification capacity was determined by pH measurements in heat-treated (15 min at 80 ºC) reconstituted skim milk powder (Oxoid) (10 % w/v) after incubation of a 1 % v/v inoculum for 0, 3, 6 and 24 h at 30 ºC. Isolates were sub-cultured twice in MRS broth, for 24 h at 30 ºC (1 % v/v inoculum) before growing them in the heat-treated milk. All of the assays were carried out in duplicate.

The cultures were considered as slow, medium or fast-acidifying when a pH variation of 0.4 units was achieved after 6 hours, between 3 and 6 hours or less than 3 hours respectively (Ayad *et al*., 2006).

2.7.3. Proteolysis

The proteolysis of the LAB strains was determined by the o-phthaldialdehyde (OPA) method, described by Church *et al*. (1983). Strains were sub-cultured twice in MRS broth for 24 h at 30 ºC (1 % v/v inoculum). 10 ml of heat-treated (15 min at 80 ºC) reconstituted skim milk powder (Oxoid) was inoculated with 2 % of the above mentionated culture and incubated at 30 ºC overnight. A sample of 5 ml of this culture was added to 500 µL of water, 10 ml of Trichloroacetic Acid (TCA) (0.75 N), mixed and rested for 10 minutes at ambient temperature. This suspension was filtrated with a Whatman paper n. 1, and a sample of 50 µL of this filtrate was added directly to 1.0 ml of o-phtaldialdehyde (OPA) reagent in a quartz *cuvette*. The solution was mix briefly by inversion of the *cuvette* and rested for 2 minutes at ambient temperature. The absorbance at 340 nm was measured in an Ultrospec 1100 pro spectrophotometer (Amersham Pharmacia Biotech). All the assays were carried out in triplicate.

The OPA solution was prepared by adding 25 ml of 100 mM sodium tetraborate to 2.5 ml of 20 % (w/w) Sodium Dodecyl Sulphate (SDS) and 40 mg OPA (dissolved in 1 ml of methanol) and 10 µl of β-mercaptoethanol, and diluted to 50 ml with water. This reagent was prepared daily.

Results were expressed as the increase in absorbance at 340 nm against the uninoculated milk.

2.7.4. Lipolysis

Lipolytic activity was determined following the method described by Sarantinopoulos *et al*. (2001). Strains were sub-cultured twice in MRS broth at 30 ºC, for 24 h and speaded in tributyrin agar (Biokar). The observation of a clear zone around the colonies indicated proteolytic activity. The radius of this halo (mm) was used as arbitrary units of the lipolytic activity. All the assays were carried out in duplicate.

Chapter 3. Results

3.1. Gross Chemical Composition of Milk and Fresh Cheese

Average, minimum and maximum values obtained from results of different milk parameters from October to July are presented in table 3.1.

Table 3.1. Gross composition of milk from algarvian goat breed.

	Raw milk			Boiled milk		
	Average	Minimum	Maximum	Average	Minimum	Maximum
pH	6.67 ± 0.2	5.80	6.86	6.60 ± 0.07	6.45	6.75
Conductivity (mS/cm)	5.12 ± 0.28	4.48	5.61	5.19 ± 0.26	4.55	5.71
NaCl (g/L)	3.01 ± 0.17	2.52	3.29	3.10 ± 0.26	2.72	4.04
Total solids (%)	13.39 ± 1.01	10.09	15.43	13.40 ± 0.89	9.10	14.95
Ash (%)	0.79 ± 0.03	0.73	0.88	0.79 ± 0.02	0.73	0.83
Fat (%)	4.33 ± 0.77	3.10	5.90	4.27 ± 0.50	3.10	5.20
Total nitrogen (%)	0.52 ± 0.08	0.44	0.65	0.45 ± 0.18	0.07	0.83
Non protein nitrogen (%)	0.05 ± 0.02	0.03	0.10	0.07 ± 0.09	0.02	0.30
Protein nitrogen (%)	0.48 ± 0.05	0.36	0.61	0.44 ± 0.08	0.33	0.52
Protein (%)	3.06 ± 0.56	2.28	3.89	2.76 ± 0.55	2.01	3.38

These results confirm that gross composition is similar for raw milk and boiled milk.

Average, minimum and maximum values obtained from results of different cheese parameters are presented in table 3.2.

Table 3.2. Gross composition of fresh cheese from algarvian goat breed, immediately after production.

	Average	Minimum	Maximum
pH	6.58 ± 0.096	6.27	6.73
Total solids (%)	36.27 ± 2.08	31.72	40.77
Ash (%)	2.56 ± 0.15	2.37	3.43
Fat (%)	1.44 ± 0.24	0.90	1.90
Total nitrogen (%)	1.78 ± 0.32	1.23	2.21
Non protein nitrogen (%)	0.18 ± 0.15	0.04	0.47
Protein nitrogen (%)	1.56 ± 0.32	1.19	1.97
Protein (%)	9.94 ± 2.05	7.56	12.56

From milk to cheese the values of almost all items of the gross chemical composition increased three times, except for pH that is practically constant and fat that presented an opposite behaviour, decreasing three times.

Changes occurring in the milk and cheese pH during the lactation (production) period can be observed in Figure 3.1.

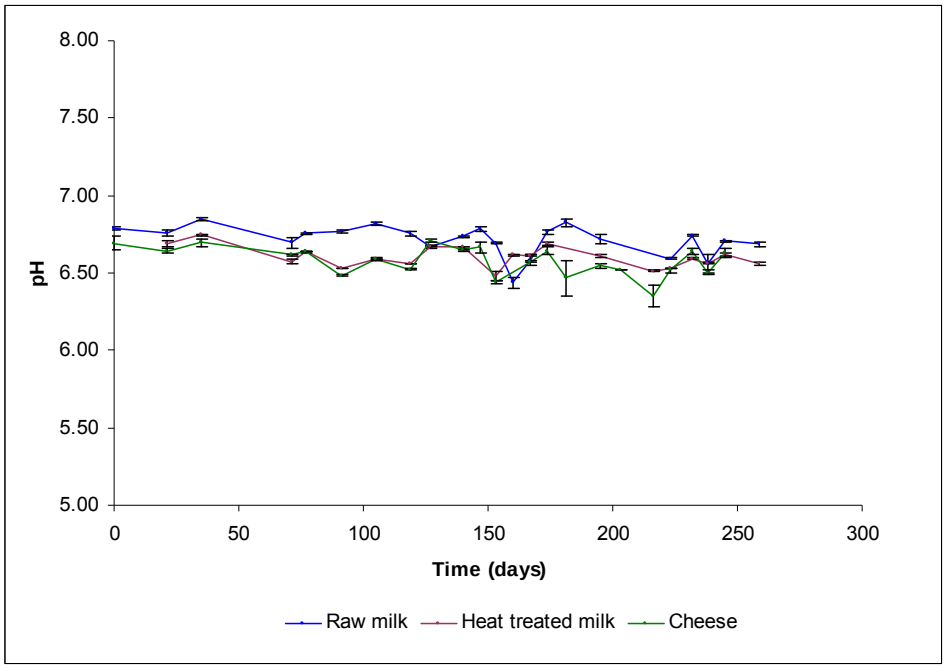

Figure 3.1. pH monitoring of milk and fresh cheese during the lactation period of the algarvian goat breed. Values are means (n=3) with standard deviations (vertical bars) for each data point.

pH values are practically constant during all the lactation period, being slightly higher in raw milk that in heat treated (boiled) milk and cheese. But this slight difference is not important as can be noted by the average values from tables 3.1 and 3.2.

Figure 3.2 shows the variation of conductivity during the lactation period.

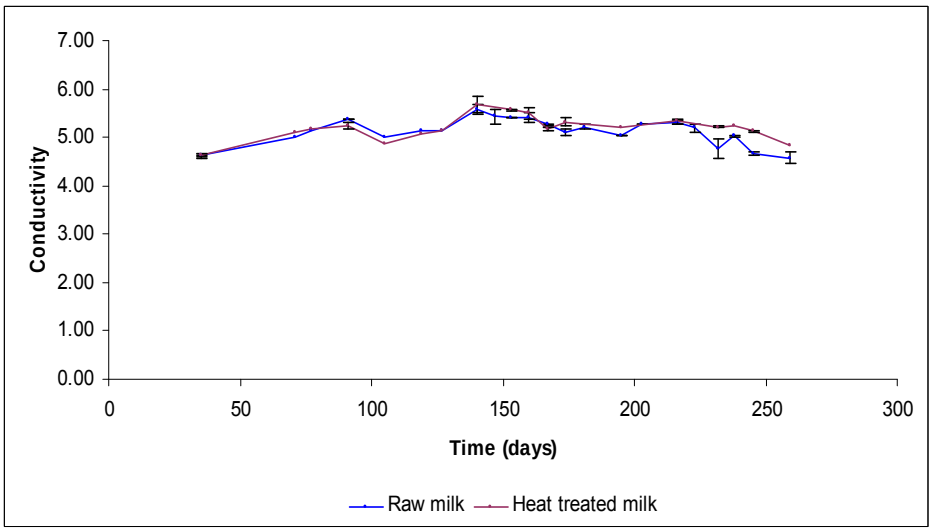

Figure 3.2. Conductivity (mS/cm) monitoring of milk during the lactation period of the algarvian goat breed. Values are means (n=3) with standard deviations (vertical bars) for each data point.

Conductivity didn't present any variation during the lactation period, which is expected, since the chemical composition didn't present any variation too.

Figures 3.3 shows the variation of ash content during the lactation period.

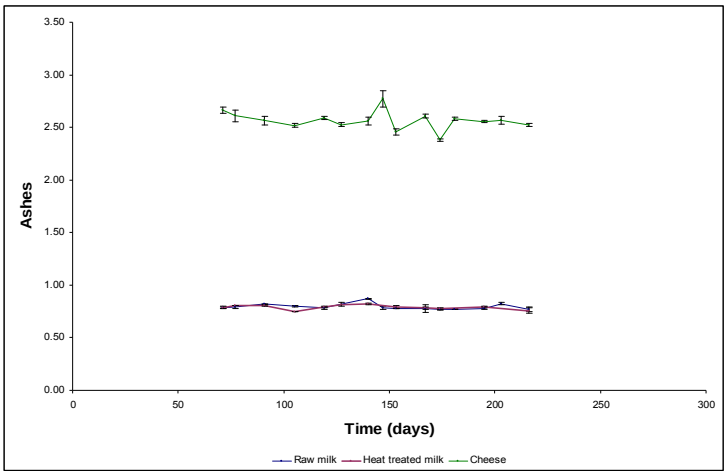

Figure 3.3. Ash content (%) monitoring of milk and fresh cheese during the lactation period of the algarvian goat breed. Values are means (n=3) with standard deviations (vertical bars) for each data point.

Ash content didn't present variations during the lactation period, but was significantly superior in cheese that in milk, which is the result of the water loss during the cheese production process.

Sodium chloride variation during the lactation period is represented in Figure 3.4, the total solids in Figure 3.5 and fat content in Figure 3.6.

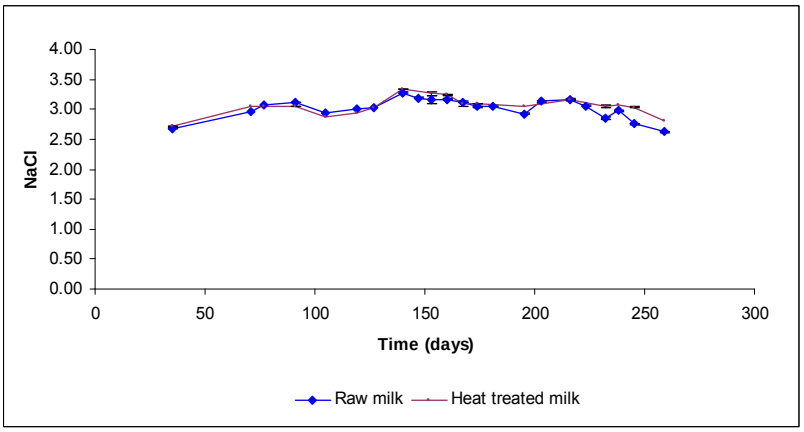

Figure 3.4. NaCl content (g/l) monitoring of milk and fresh cheese during the lactation period of the algarvian goat breed. Values are means (n=3) with standard deviations (vertical bars) for each data point.

Sodium chloride content was approximately the same during all the lactation period.

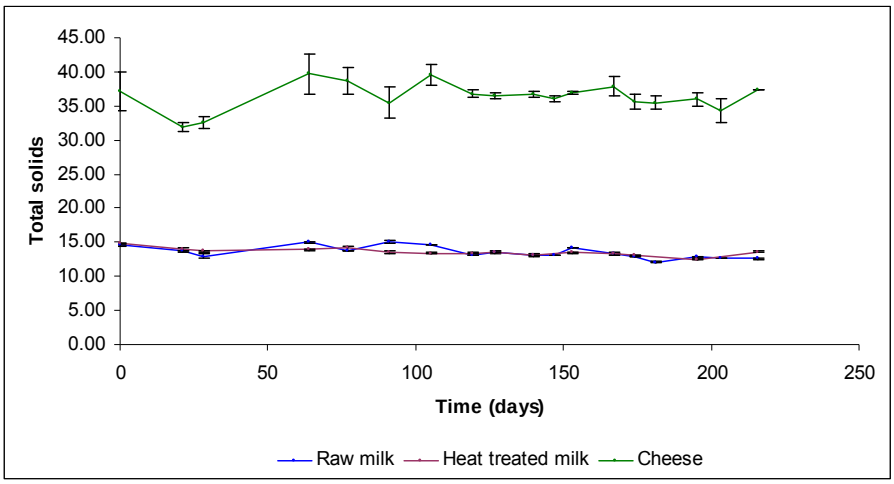

Figure 3.5. Total solids content (%) monitoring of milk and fresh cheese during the lactation period of the algarvian goat breed. Values are means (n=3) with standard deviations (vertical bars) for each data point.

Total solids content didn't present variations during the lactation period, but were much higher in cheese than in milk, which could be explained by the water loss during the production process of the cheese as in the case of ash.

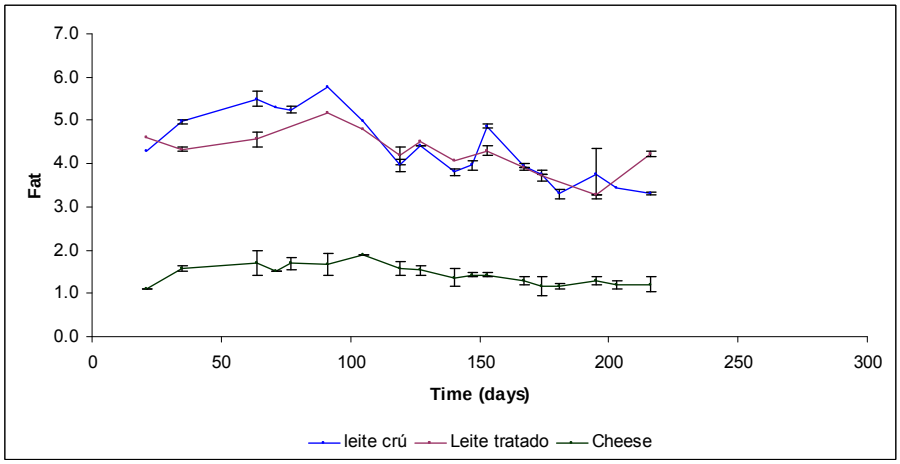

Figure 3.6. Fat content (%) monitoring of milk and fresh cheese during the lactation period of the algarvian goat breed. Values are means (n=3) with standard deviations (vertical bars) for each data point.

Fat content was higher at the beginning of the lactation period, but start to decrease slowly after two months, maintaining this trend until the end of the lactation period.

Figure 3.7 represents the total nitrogen content during the lactation period of the algarvian goat breed.

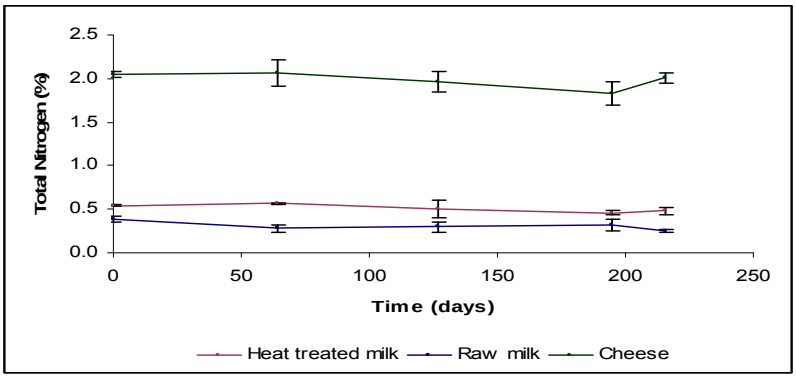

Figure 3.7. Total Nitrogen content (%) monitoring of milk and fresh cheese during the lactation period of the algarvian goat breed. Values are means (n=3) with standard deviations (vertical bars) for each data point.

Total nitrogen presented constant results during all the lactation period, but higher in cheese than in milk. Most of the nitrogen is part of protein molecules and cheese results from the protein precipitation, thus it naturally present a high protein concentration.

Figure 3.8 and 3.9 represent the non proteic nitrogen and the proteic nitrogen content, respectively, during the lactation period of the algarvian goat breed.

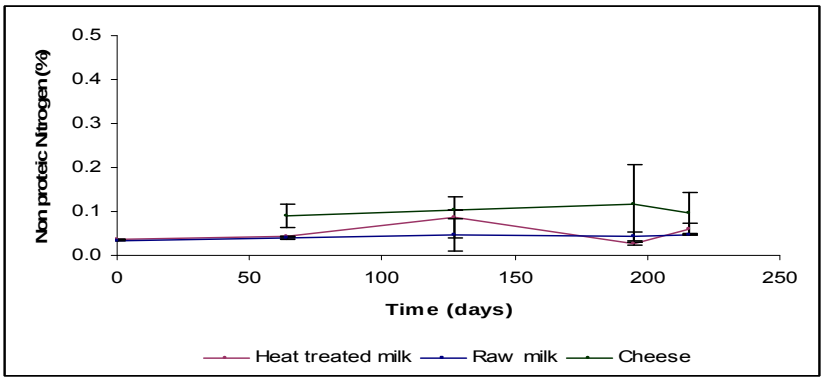

Figure 3.8. Non proteic Nitrogen content (%) monitoring of milk and fresh cheese during the lactation period of the algarvian goat breed. Values are means (n=3) with standard deviations (vertical bars) for each data point.

Non proteic nitrogen was practically constant during all the lactation period and didn't present much variation from milk to cheese. The major part of the nitrogen content increasing from milk to cheese was due to the precipitation of proteins and not to the entrapped of non protein nitrogen.

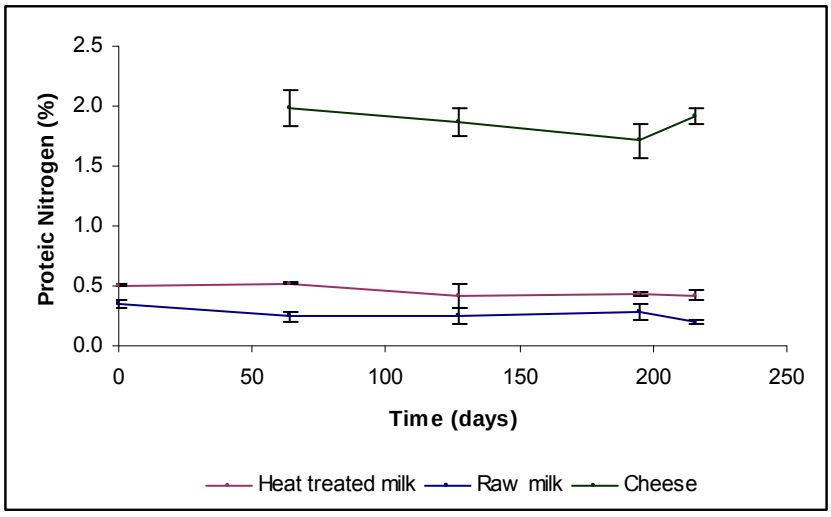

Figure 3.9. Proteic Nitrogen content (%) monitoring of milk and fresh cheese during the lactation period of the algarvian goat breed. Values are means (n=3) with standard deviations (vertical bars) for each data point.

Figure 3.10 represents the protein content, during the lactation period of the algarvian goat breed.

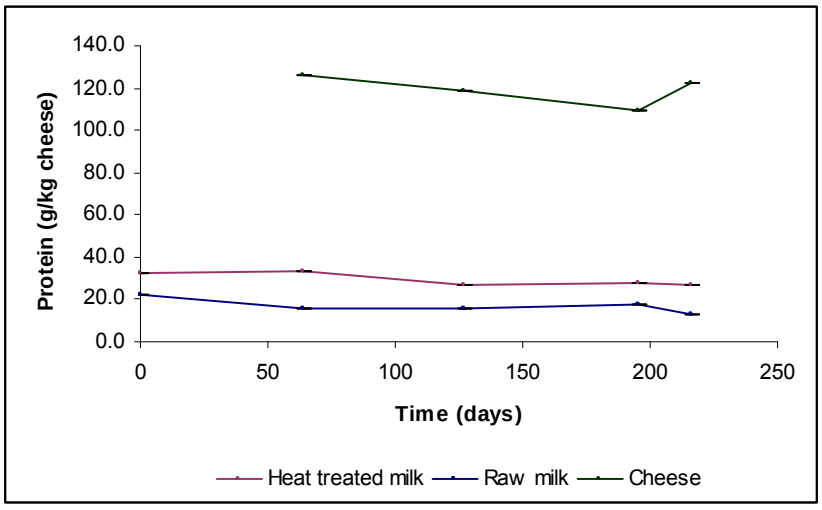

Figure 3.10. Protein content (g/kg of cheese) monitoring of milk and fresh cheese during the lactation period of the algarvian goat breed. Values are means (n=3) with standard deviations (vertical bars) for each data point.

Proteic nitrogen content and protein content are directly related and presented similar behaviour to total nitrogen, which indicates that most of the nitrogen is an integral part of proteins as referred before.

3.2. Microbiology of Cardoon

Figure 3.11 shows mean values obtained for the total heterotrophic microbiota, in the seven studied cardoon types. 21 assays were performed for each type of cardoon.

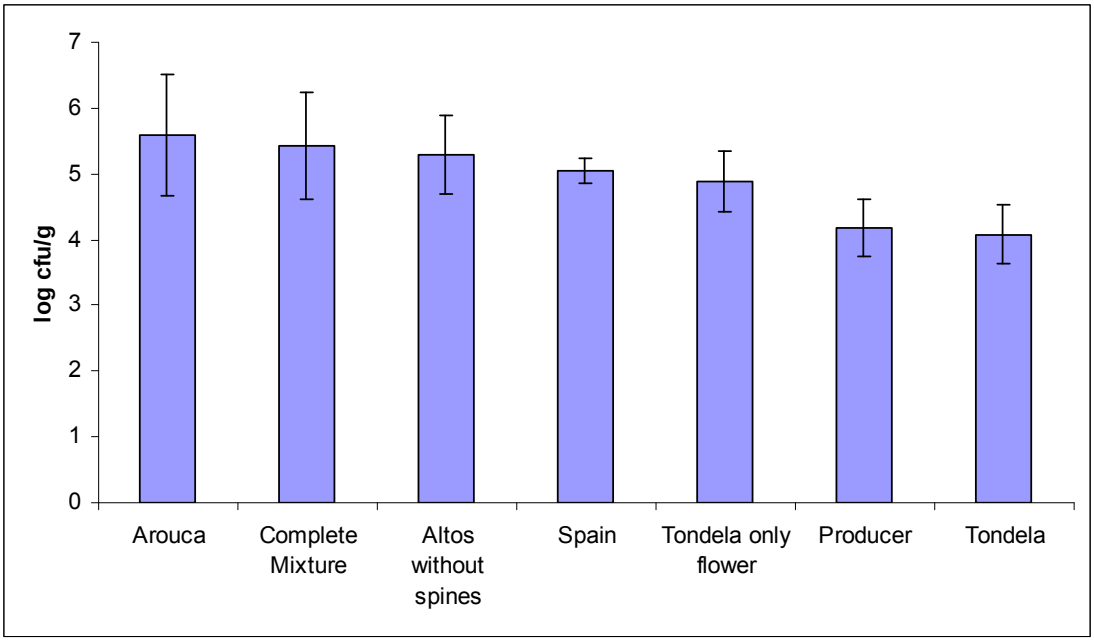

Figure 3.11. Total heterotrophic microbiota in different types of cardoon. Values are means (n=21) with standard deviations (vertical bars) for each data point.

"Arouca" and "complete mixture" showed the higher values for heterotrophic microbiota, being "Producer cardoon" and "Tondela" those who shown lower counts. Considering only this results, the last two types introduce lower contamination in the cheese, which could be important, since cardoon is added after boiling the milk, when it is done.

Figure 3.12, shows the average values of yeasts and moulds in different types of the studied cardoon samples. The same 21 samples referred above were used for this study too.

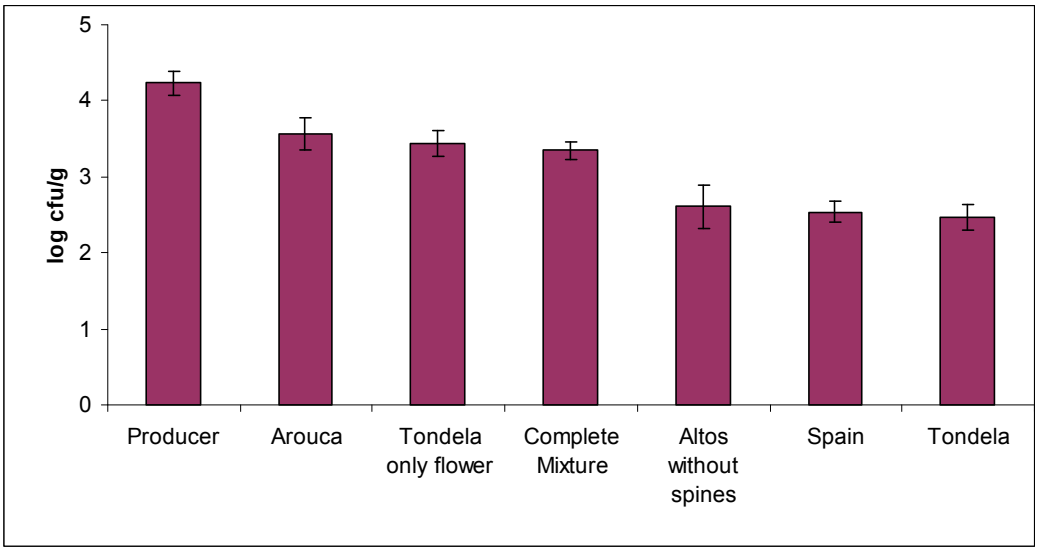

Figure 3.12. Yeasts and moulds in different types of cardoon (21 assays for each type of cardoon). Values are means (n=21) with standard deviations (vertical bars) for each data point.

"Producer cardoon" samples presented the highest counts for yeasts and moulds. "Altos without spines", "Spain" and "Tondela" presented the lower values, thus considering only these results the last three will be responsible for less contamination when used in cheese production. Considering total heterotrophic microbiota and yeasts and moulds counts, "Tondela" was the cardoon type that presented lower level of contamination.

Non-parametric Kruskal-Wallis tests were performed to determine if there was any significant difference between different cardoon samples. This statistical analysis was performed at 95 % level of significance using SPSS (v.16.0; SPSS).

Morphological characterization of the moulds present in the samples was carried out and it was verified that the 90 % of the moulds were from the genus *Aspergillus*. Also genera like *Mucor*, *Rhizopus*, *Phoma* and *Penicilium* were identified, but in very low numbers.

3.3. Microbiological Evaluation of Raw Milk

Figure 3.13 shows the microbiota present in raw milk during a lactation period, from May to July.

The yellow line (5.7 log cfu/ml) represents the legal limit (Regulation (EC) nº 1662/2006, 6th November) for total heterotrophic microorganisms in milk to be used for products without any thermal treatment.

The grey line (6.18 log cfu/ml) represents the legal limit (Regulation (EC) nº 1662/2006, 6th November) for total heterotrophic microorganisms in milk to be used for products that involve thermal treatment. Once the milk is submitted to a thermal treatment, it can legally have more microorganisms present, since most of them will be destroyed during the thermal treatment.

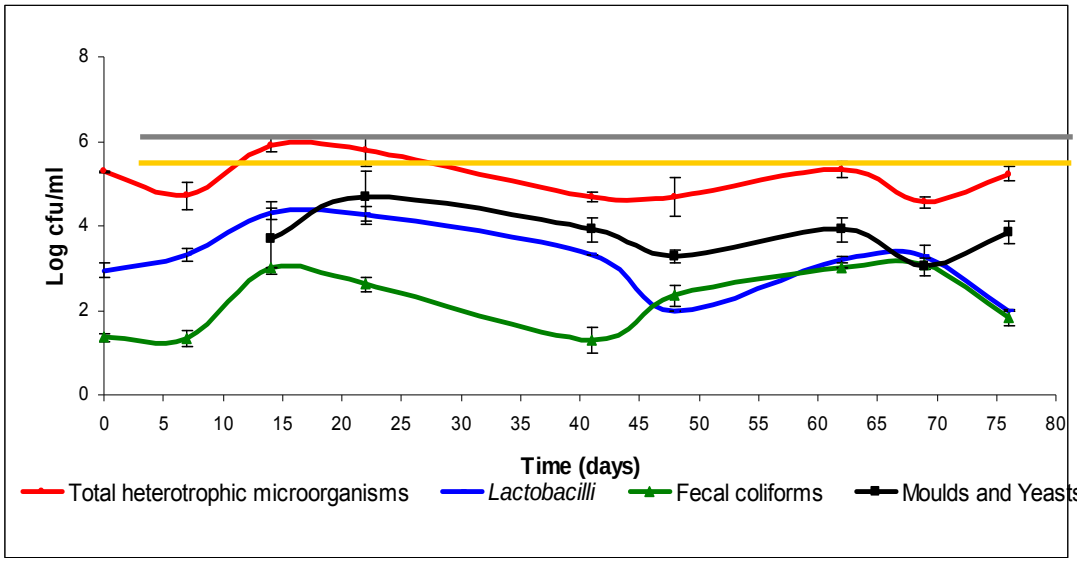

Figure 3.13. Raw milk microbiota observed during the goat lactation period (from May to July). Values are means (n=3) with standard deviations (vertical bars) for each data point.

Samples taken during the lactation period presented no variation in microbiota.

The studied milk could be used in cheese production ever since it was heat treated, since total heterotrophic microorganisms presented values lower than the legal limit for milk to be thermal treated before production (grey line). Total heterotrophic microorganisms are higher than the maximum permitted values for use without thermal treatment (yellow line) thus it could not be used for ripened algarvian goat breed cheese production since currently raw milk is used for it.

3.4. Microbiological Quality of Cheese immediately after processing

Figure 3.14 shows the studied microbiota of cheese immediately after processing, made from raw milk during a lactation period (from May to July).

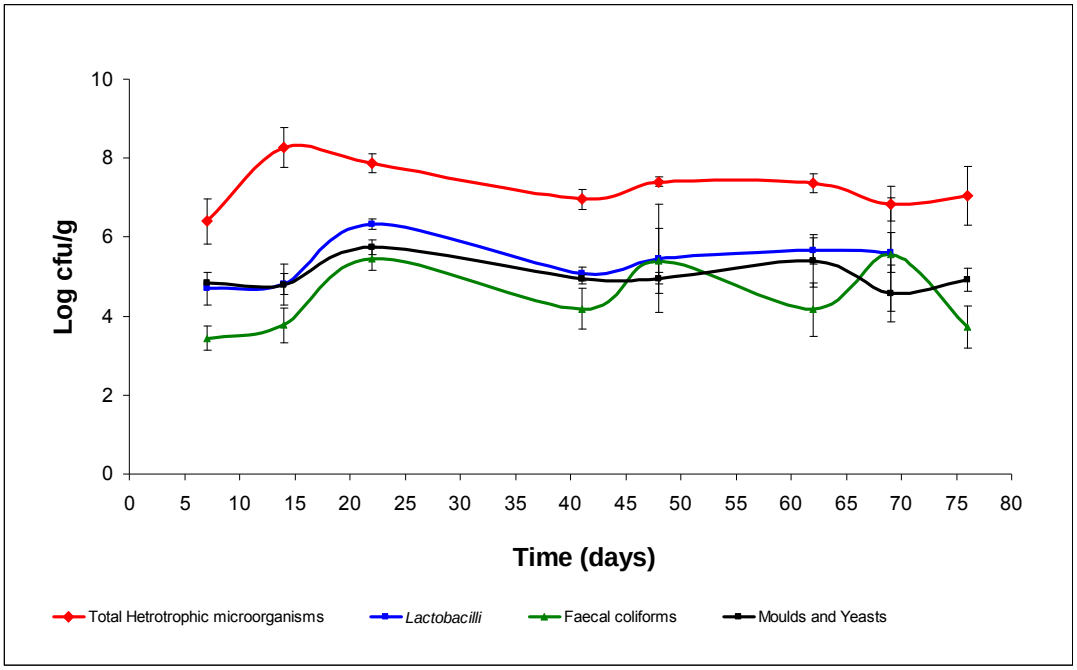

Figure 3.14. Cheese microbiota observed during the goat lactation period (from May to July). Values are means (n=3) with standard deviations (vertical bars) for each data point.

From milk to cheese there were increases of around 2 logs in all microorganisms' counts. These increases could be due to the elapsed time between cheese production and cheese analysis (more or less twenty four hours) which could permit the development of microorganisms in a very rich medium as cheese is. Another factor which could perhaps account for these increases of the microbial counts is the addition of the aqueous cardoon extract.

Table 3.3 shows the maximum and minimum values of aerobic heterotrophic microbiota expressed in cfu/g present in the cardoon samples for all the batches studied.

Table 3.3. Minimum and maximum values of total heterotrophic microbiota in cardoon, used for each batch of cheese production.

Day	7	14	22	41	48	62	69	76
Maximum	2.93E+05	6.67E+05	3.33E+05	1.33E+05	3.33E+05	3.33E+05	3.33E+05	1.33E+05
Minimum	6.67E+03	6.67E+03	3.33E+04	1.33E+03	1.33E+03	1.33E+03	1.33E+03	1.33E+03

3.5. Microbiology of Cheese During Ripening

The counts of different microbial groups in the cheese during ripening are given in figure 3.15.

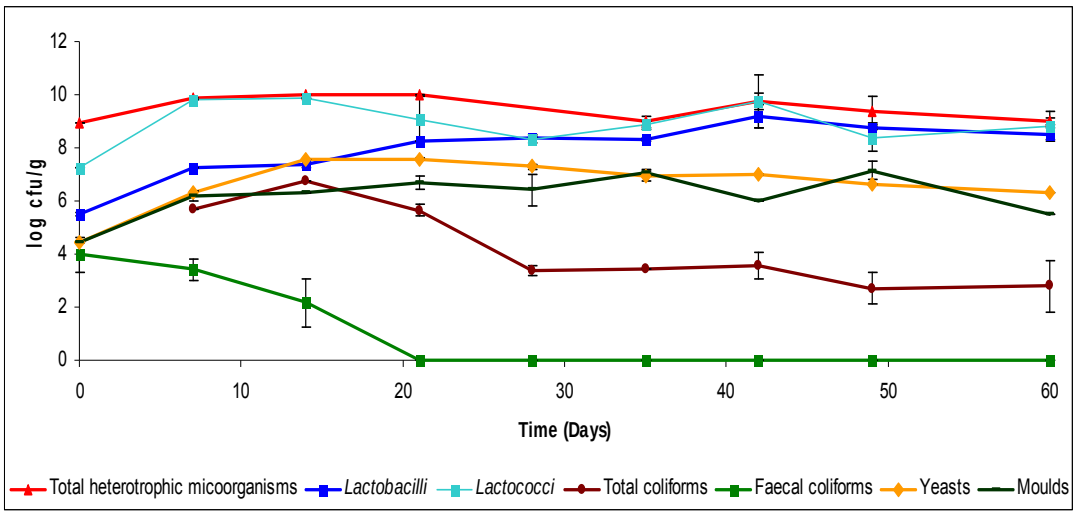

Figure 3.15. Microbiological variation of algarvian goat cheese during ripening. Values are means (n=3) with standard deviations (vertical bars) for each data point.

Faecal coliforms disappeared twenty one days after the beginning of the ripening period, which is good for a safety consumption of cheese.

Lactococci are the predominant microorganisms until the middle of the ripening period, but after that lactobacilli reached the same level and remain both with similar values until the end of the ripening process. The increase of the lactic acid bacteria counts was compensated by the decrease in coliforms numbers. This dynamic change permits that total heterotrophic microorganisms remains at a constant level all over the ripening period.

Table 3.4 shows the pH variation in the studied cheese during ripening.

Table 3.4. pH variation of algarvian goat cheese during ripening.

Days of ripening	0	7	14	21	28	35	42	49	60
pH	6.44±0.05	5.14±0.07	5.11±0.14	5.02±0.09	5.03±0.17	5.02±0.20	4.94±0.10	5.01±0.08	5.02±0.07

There was a decrease in the pH values in the first 42 days of the ripening period, which coincide with the increase of lactic acid bacteria counts. This decrease in pH values was expected since the referred bacteria are producers of lactic acid.

3.6. Isolation and Phenotypic Characterization of LAB

3.6.1. Samples from Raw Milk Obtained During the Lactation Period Between May and July

The 180 isolates, recognized as lactic acid bacteria, were assigned to genus level, by morphology and physiological tests and classified as stated before (Table 2.1.). The results of this identification are showed in Figure 3.16.

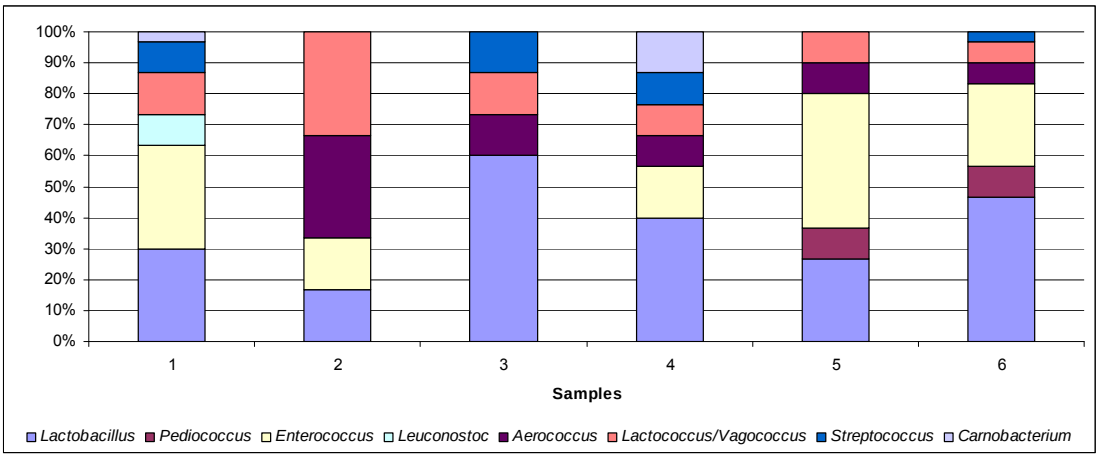

Figure 3.16. Genus of LAB isolated from milk samples during the lactation period (May to July).

The genus isolated more frequently was *Lactobacillus*, followed by *Enterococcus*, except for the 3rd sample where *Enterococcus* was not found.

From the 180 isolates, 34 representing a selection of the widest range of properties used for identification (cell shape, CO_2 production from glucose, growth at 10 and 45 ºC, growth at 6.5 % NaCl and growth at pH 4.4 and 9.6) were choose to characterize for technological properties and the results will be presented further. The selected isolates were 20 *Lactobacillus*, 4 *Lactococcus*, 2 *Streptococcus*, 2 *Enterococcus*, 2 *Pediococcus*, 1 *Carnobacterium*, 1 *Leuconostoc* and 2 *Aerococcus* (Tab. 3.5).

Table 3.5. Selected isolates from milk representing the widest range of properties used for identification. (+) positive result for growth or presence. (–) negative result for growth or presence.

Isolate	Cell shape	CO_2	Growth at 45 ºC	Growth at 10 ºC	Growth at 6.5 % NaCl	Growth at pH 4.4	Growth at pH 9.6	Genera
17	Cocci	-	-	+	+	-	+	*Aerococcus*
60	Cocci	-	-	+	+	-	+	*Aerococcus*
29	Rods	-	-	+	-	-	-	*Carnobacteria*
65	Cocci	-	+	+	+	+	+	*Enterococcus*
86	Cocci	-	+	+	+	+	+	*Enterococcus*
20	Rods	+	+	+	+	-	-	*Lactobacillus*
21	Rods	-	-	-	+	+	-	*Lactobacillus*
22	Rods	-	-	-	+	+	-	*Lactobacillus*
59	Rods	-	-	+	+	-	-	*Lactobacillus*
61	Rods	-	-	-	+	-	-	*Lactobacillus*
79	Rods	-	+	-	-	+	-	*LActobacillus*
80	Rods	+	-	-	-	+	-	*Lactobacillus*
85	Rods	+	-	+	+	-	-	*Lactobacillus*
101	Rods	-	+	+	-	-	-	*Lactobacillus*
102	Rods	-	-	+	+	+	-	*Lactobacillus*
103	Rods	-	-	-	-	+	-	*Lactobacillus*
105	Rods	-	-	+	+	-	-	*Lactobacillus*
120	Rods	-	-	+	+	+	-	*Lactobacillus*
125	Rods	-	+	+	+	+	-	*Lactobacillus*
143	Rods	-	-	-	-	+	-	*Lactobacillus*
145	Rods	+	+	+	-	-	-	*LActobacillus*
146	Rods	-	+	-	+	-	-	*Lactobacillus*
147	Rods	-	+	+	-	-	-	*Lactobacillus*
281	Rods	+	-	+	-	+	-	*Lactobacillus*
298	Rods	+	-	+	-	+	-	*Lactobacillus*
13	Cocci	-	-	+	-	+	-	*Lactococcus*
34	Cocci	-	-	+	-	+	-	*Lactococcus*
69	Cocci	-	-	+	-	-	-	*Lactococcus*
139	Cocci	-	-	+	-	-	-	*Lactococcus*
131	Cocci	+	-	+	+	+	-	*Leuconostoc*
89	Cocci	-	+	+	-	+	-	*Pediococcus*
185	Cocci	-	+	-	-	+	-	*Pediococcus*
33	Cocci	-	+	-	-	-	-	*Streptococcus*
110	Cocci	-	+	-	-	-	-	*Streptococcus*

3.6.2. Samples of Cheese Collected During Ripening

Figures 3.17 and 3.18 shows the results from the identification at the genus level of the 180 isolates, recognized as lactic acid bacteria, selected from the Rogosa agar and M17 selective growth media, respectively.

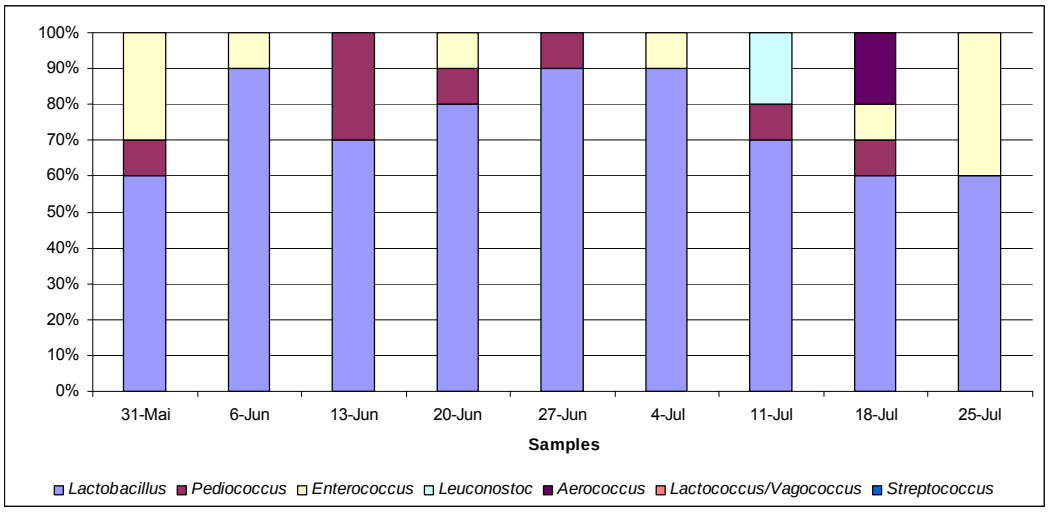

Figure 3.17. LAB isolated during the cheese ripening period, from Rogosa agar.

The genus isolated more frequently was *Lactobacillus.* In much less quantity *Pediococcus* and *Enterococcus* were found.

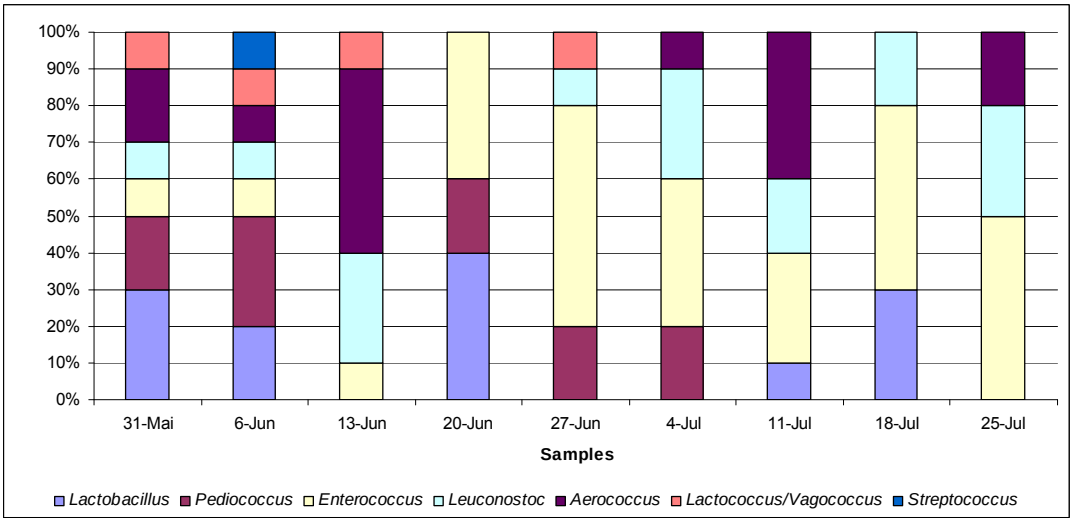

Figure 3.18. LAB isolated during cheese ripening period, from M17 agar.

The genus isolated most frequently was *Enterococcus*, this was the only genus that was present in all the samples.

From the 180 isolates, 22 representing a selection of the widest range of properties used for identification (cell shape, CO_2 production from glucose, growth at 10 and 45 ºC, growth at 6.5 % NaCl and growth at pH 4.4 and 9.6) were chosen to characterize for technological properties and the results will be presented further. The

selected isolates were 10 *Lactobacillus*, 2 *Lactococcus*, 1 *Streptococcus*, 1 *Enterococcus*, 5 *Pediococcus*, 2 *Leuconostoc* and 1 *Aerococcus* (Tab. 3.6).

Table 3.6. Selected isolates from cheese representing the widest range of properties used for identification. (+) positive result for growth or presence (–) negative result for growth or presence.

Isolate	Cell shape	CO_2	Growth at 45 ºC	Growth at 10 ºC	Growth at 6.5 % NaCl	Growth at pH 4.4	Growth at pH 9.6	Genera
Q16	Cocci	-	-	+	+	-	+	*Aerococcus*
Q114	Cocci	-	+	+	+	+	+	*Enterococcus*
Q1	Rods	-	+	+	+	+	-	*Lactobacillus*
Q8	Rods	-	-	+	+	-	-	*Lactobacillus*
Q20	Rods	-	-	+	+	+	-	*Lactobacillus*
Q43	Rods	-	+	+	-	+	-	*Lactobacillus*
Q45	Rods	-	+	+	+	-	-	*Lactobacillus*
Q49	Rods	-	+	+	-	-	-	*Lactobacillus*
Q50	Rods	-	+	+	-	+	-	*Lactobacillus*
Q76	Rods	-	+	+	+	-	-	*Lactobacillus*
Q100	Rods	-	+	+	+	+	-	*Lactobacillus*
Q105	Rods	-	-	+	-	-	-	*Lactobacillus*
Q17	Cocci	-	-	+	-	-	-	*Lactococcus*
Q32	Cocci	-	-	+	-	+	-	*Lactococcus*
Q110	Cocci	+	-	+	-	-	-	*Leuconostoc*
Q132	Cocci	+	-	+	+	-	-	*Leuconostoc*
Q10	Cocci	-	+	+	+	+	-	*Pediococcus*
Q18	Cocci	-	+	+	-	+	-	*Pediococcus*
Q31	Cocci	-	+	-	+	+	-	*Pediococcus*
Q96	Cocci	-	+	+	-	+	-	*Pediococcus*
Q163	Cocci	-	+	+	+	+	-	*Pediococcus*
Q28	Cocci	-	+	-	-	-	-	*Streptococcus*

3.7. Technological Properties of LAB

3.7.1. Bacteriocin Production

Bacteriocin production was detected in Petri plates by a clear zone around the impregnated disc (Figure 3.19).

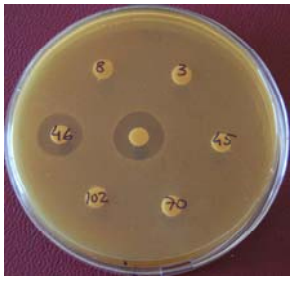

Figure 3.19. Bacteriocin production assay by diffusion method.

Overall, 14 (41.2 %) of the 34 tested strains, isolated from milk (Table 3.7) produced bacteriocins, whereas only 5 (22.7 %) of the 22 isolated from cheese (Table 3.8) produced bacteriocins. The bacteriocins produced by the selected strains were heat-stable.

Table 3.7. Bacteriocin production by strains isolated from milk.

Isolate (Reference n./Genus)	Bacteriocin production (halo diameter in mm)
17 - *Aerococcus*	-
29 - *Carnobacteria*	-
65 - *Enterococcus*	-
20 - *Lactobacillus*	-
21 - *Lactobacillus*	-
59 - *Lactobacillus*	-
61- *Lactobacillus*	-
79 - *Lactobacillus*	-
80 - *Lactobacillus*	-
102 - *Lactobacillus*	-
103 - *Lactobacillus*	-
145 - *Lactobacillus*	-
146 - *Lactobacillus*	-
147 - *Lactobacillus*	-
298 - *Lactobacillus*	-
13 - *Lactococcus*	-
139 - *Lactococcus*	-
131- *Leuconostoc*	-
89 - *Pediococcus*	-
33 - *Streptococcus*	-
60 - *Aerococcus*	13
86 - *Enterococcus*	13
22 - *Lactobacillus*	15
85 - *Lactobacillus*	15
101 - *Lactobacillus*	12
105 - *Lactobacillus*	13
120 - *Lactobacillus*	13
125 - *Lactobacillus*	12
143 - *Lactobacillus*	14
281 - *Lactobacillus*	13
34 - *Lactococcus*	3
69 - *Lactococcus*	13
185 - *Pediococcus*	12
110 - *Streptococcus*	11

Table 3.8. Bacteriocin production by strains isolated from cheese.

Isolate (Reference n./Genus)	Bacteriocin production (halo diameter in mm)
Q16 - *Aerococcus*	-
Q114 - *Enterococcus*	-
Q8 - *Lactobacillus*	-

Isolate (Reference n./Genus)	Bacteriocin production (halo diameter in mm)
Q20 - *Lactobacillus*	-
Q45 - *Lactobacillus*	-
Q49 - *Lactobacillus*	-
Q50 - *Lactobacillus*	-
Q100 - *Lactobacillus*	-
Q105 - *Lactobacillus*	-
Q17 - *Lactococcus*	-
Q32 - *Lactococcus*	-
Q110 - *Leuconostoc*	-
Q132 - *Leuconostoc*	-
Q163 - *Pediococcus*	-
Q18 - *Pediococcus*	-
Q31 - *Pediococcus*	-
Q28 - *Streptococcus*	-
Q1 - *Lactobacillus*	12
Q43 - *Lactobacillus*	15
Q76 - *Lactobacillus*	14
Q10 - *Pediococcus*	14
Q96 - *Pediococcus*	13

3.7.2. Acidifying Capacity

The results for the acidifying capacity of isolates from milk and cheese are presented in Figure 3.20 and Figure 3.21, respectively.

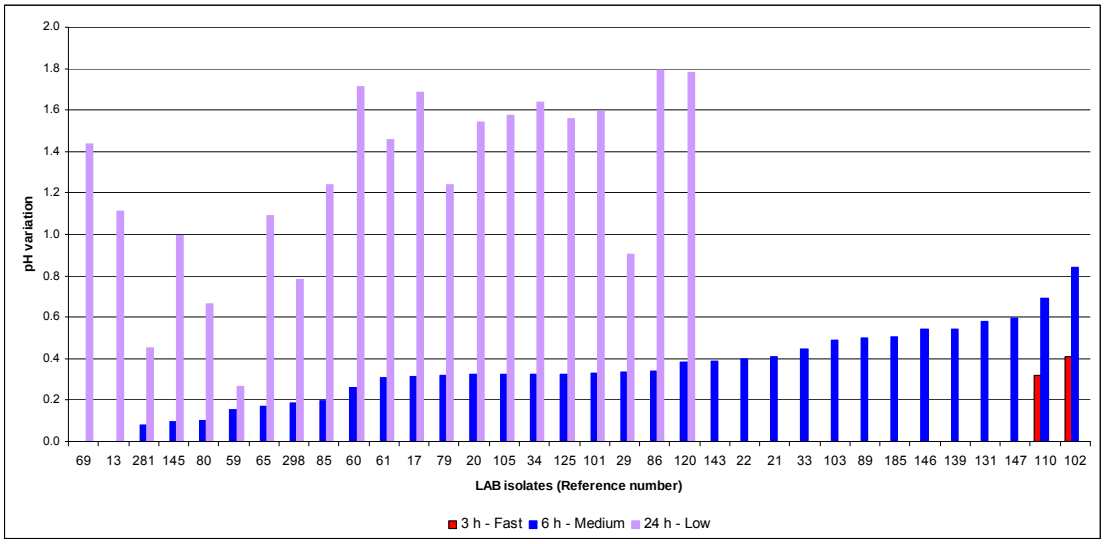

Figure 3.20. Acidification capacity of milk isolates (results after 3 h, 6 h and 24 h incubation).

Only one isolate (referred as 102) (2.9 %) showed fast acidifying capacity. Thirteen isolates (38.2 %) showed medium acidification capacity and the rest (58.9 %) showed slow acidifying capacity.

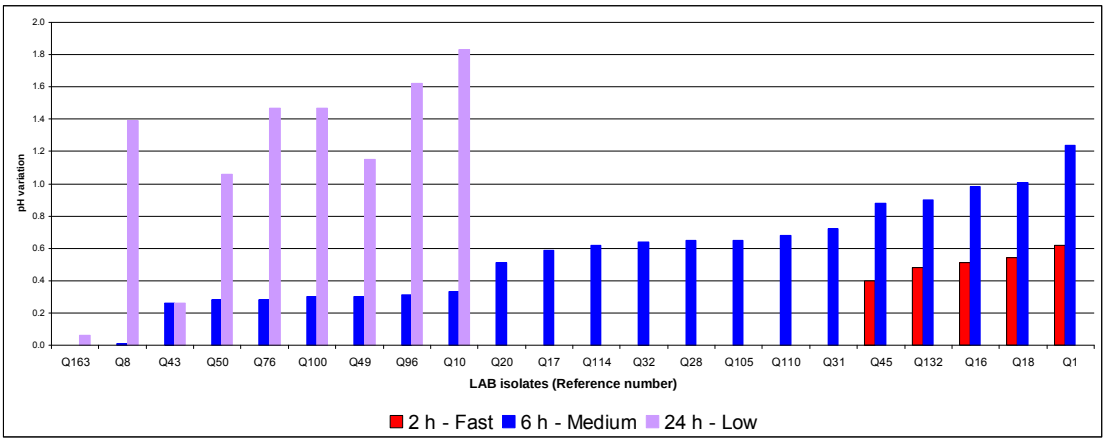

Figure 3.21. Acidifying capacity of LAB isolated from cheese (results after 3 h, 6 h and 24 h incubation).

Five isolates (22.7 %) showed fast acidification capacity. Eight isolates (36.4 %) showed medium acidification capacity and the rest (40.9 %) showed slow acidification capacity.

3.7.3. Proteolysis

Another important technological characteristic of LAB is proteolysis. The results for average optical density (OD) obtained by the o-phthaldialdehyde (OPA) method, described by Church *et al*. (1983) are presented in tables 3.9 and 3.10.

Table 3.9. Proteolytic activity of the milk isolates.

Isolate	Genera	OD Average	OD SD
60	*Aerococcus*	0.000	0.00
103	*Lactobacillus*	0.000	0.00
102	*Lactobacillus*	0.000	0.00
79	*Lactobacillus*	0.000	0.00
105	*Lactobacillus*	0.000	0.00
120	*Lactobacillus*	0.000	0.00
145	*Lactobacillus*	0.000	0.00
69	*Lactococcus*	0.000	0.00
22	*Lactobacillus*	0.01	0.01
281	*Lactobacillus*	0.01	0.01
65	*Enterococcus*	0.05	0.01
101	*Lactobacillus*	0.01	0.02
80	*Lactobacillus*	0.04	0.03
298	*Lactobacillus*	0.04	0.01
20	*Lactobacillus*	0.05	0.02
61	*Lactobacillus*	0.05	0.02
131	*Leuconostoc*	0.02	0.02
185	*Pediococcus*	0.08	0.08
33	*Streptococcus*	0.02	0.03

Isolate	Genera	OD Average	OD SD
17	*Aerococcus*	0.41	0.06
29	*Carnobacteria*	0.32	0.03
86	*Enterococcus*	0.19	0.05
85	*Lactobacillus*	0.10	0.04
125	*Lactobacillus*	0.15	0.02
146	*Lactobacillus*	0.21	0.09
21	*Lactobacillus*	0.32	0.05
59	*Lactobacillus*	0.32	0.02
147	*Lactobacillus*	0.39	0.00
143	*Lactobacillus*	0.39	0.05
139	*Lactococcus*	0.15	0.04
34	*Lactococcus*	0.17	0.01
13	*Lactococcus*	0.19	0.02
89	*Pediococcus*	0.22	0.05
110	*Streptococcus*	0.16	0.02

From the 34 isolated strains from milk, 8 (23.5 %) did not present any increase in absorbance when assayed with the OPA reagent, meaning that they did not cause any proteolysis. Two (5.9 %) yielded an increase in absorbance <0.01 at 340 nm, nine (26.5 %) presented an increase between 0.01 and 0.1 and fifteen (44.1 %) superior to 0.1.

Table 3.10. Proteolytic activity of the cheese isolates.

Isolate	*Genera*	OD Average	OD SD
Q10	*Pediococcus*	0.00	0.00
Q114	*Enterococcus*	0.01	0.02
Q8	*Lactobacillus*	0.01	0.01
Q20	*Lactobacillus*	0.03	0.03
Q110	*Leuconostoc*	0.09	0.04
Q96	*Pediococcus*	0.01	0.01
Q31	*Pediococcus*	0.03	0.03
Q28	*Streptococcus*	0.08	0.06
Q16	*Aerococcus*	0.41	0.06
Q1	*Lactobacillus*	0.15	0.02
Q100	*Lactobacillus*	0.29	0.09
Q45	*Lactobacillus*	0.32	0.03
Q76	*Lactobacillus*	0.32	0.01
Q43	*Lactobacillus*	0.38	0.01
Q49	*Lactobacillus*	0.39	0.00
Q50	*Lactobacillus*	0.47	0.02
Q105	*Lactobacillus*	0.56	0.04
Q17	*Lactococcus*	0.13	0.00
Q32	*Lactococcus*	0.19	0.02
Q132	*Leuconostoc*	0.43	0.03
Q163	*Pediococcus*	0.17	0.01

Isolate	*Genera*	OD Average	OD SD
Q18	*Pediococcus*	0.22	0.05

From the 22 isolated strains from cheese, one (4.5 %) did not present any increase in absorbance at 340 nm when assayed with the OPA reagent, seven (31.8 %) presented an increase between 0.01 and 0.1 and fourteen (63.6 %) more than 0.1.

From the 20 *Lactobacillus* isolated from milk, 7 (35 %) presented high proteolytic activity (> 0.1), 5 (25 %) presented medium activity (between 0.01-0.1) and other 2 (10 %) presented low activity (<0.01). Six isolates (30 %) didn't show any absorbance.

From the 10 *Lactobacillus* isolated from cheese, 8 (80 %) presented high proteolytic activity (> 0.1) and 2 (20 %) presented medium activity (between 0.01-0.1) .

3.7.4. Lipolytic Activity

The lipolytic capacity of the isolated strains that are considered representative of the total studied isolates (after morphological and biochemical tests) was evaluated. In isolates from cheese, only one isolate showed lipolytic activity. This isolate was referred as Q163 and identified as *Pediococcus* (Figure 3.22).

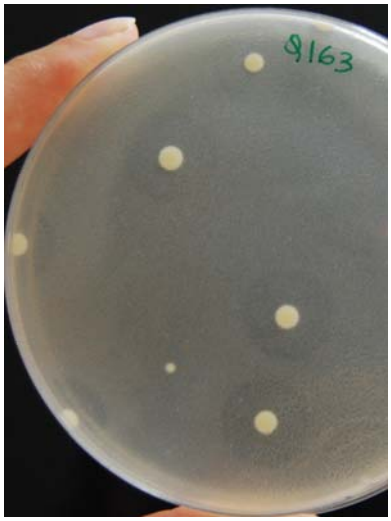

Figure 3.22. Lipolytic activity testing, in tributyrin agar (isolate Q163).

Chapter 4. Final discussion

4.1. Gross Chemical Composition of Milk and Fresh Cheese

There were no significant differences, in gross composition, between the raw and heated treated milk. If milk monitoring during all the lactation (production) period was taken into account, it could be observed that milk pH (Figure 3.1), conductivity (Figure 3.2) and ash (Figure 3.3) values presented no significant variation. pH varied from 5.8, 6.45 to 6.86 and 6.75; conductivity changed from 4.48 mS/cm, 4.55 mS/cm to 5.61 mS/cm and 5.71 mS/cm and ash varied from 0.73 % for both raw and treated milk to 0.88 % and 0.83 % for raw and treated milk, respectively. Sodium chloride (Figure 3.4) was maintained practically constant during all the lactation period (from 2.52 g/l, 2.72 g/l to 3.29 g/l and 4.03 g/l for raw and treated milk, respectively), but slightly superior in treated milk. This result could be due to the loss of water during the thermal treatment. The total solids (Figure 3.5) varied from 10.09 %, 9.10 % to 15.43 % and 14.95 % for raw and treated milk, respectively, but didn't show any tendency for variation during the lactation period.

In raw milk, fat content (Figure 3.6.) presented an increase from 4.3, in October (day 20) to 5.8, in February (day 90) and than a decrease to 3.3 at the beginning of July (day 220). This could be explained by the fact that the goats were taking feed supplements when they are feeding their kids. After the middle of January (day 65) they get food outside in the pasture. These findings are in accordance with Raynal-Ljutovac *et al*. (2005). Treated milk presented a similar variation, as was expected. The slightly lower levels registered in this milk could be due to the loss of some fat during the thermal treatment, since part of it stayed adhered to the thermal treatment tank walls.

Breed is a factor for fat content variation. Variation also exists between countries, average contents being more than 5.0 % for southern Europe and about 3.0–3.5 % for northern Europe (Kalantzopoulos *et al*., 2002). Bernacka (2005) stresses that goat's milk obtained in the different seasons' feeding conditions do not show clear differences in terms of the gross chemical composition.

No published values for goat milk from Algarve could be found in the literature, the work described here represents the first study in this area, but values reported for other goat breeds outside of the Algarve regarding pH (Reynal-Ljutovac *et al*., 2004,

Zumbo *et al*., 2004), total solids (Reynal-Ljutovac *et al*., 2004), ash (Zumbo *et al*., 2004), protein (Reynal-Ljutovac *et al*., 2004, Zumbo *et al*., 2004), fat (Zumbo *et al*., 2004), total nitrogen content (Estepar *et al*., 1999) and non protein nitrogen content (Estepar *et al*., 1999), are similar to that observed in the present work. Masle & Morgan (2001) published work showing that goat milk composition depends on the lactation stage, which explain the different acidification propensities of goat milk that they found during the lactation periods. A high percentage of proteins and minerals favour the activity of the lactic acid bacteria, thus the milk composition has a direct impact on its acidifying properties.

In the present work goat milk presented a non-protein nitrogen value of 0.05 %; however Bozanic (2002) found much more (6%). Nevertheless, in both cases care should be taken in the milk storage since, when refrigerated (4 ºC) for a longer period than 72 h, milk can be submitted to protein degradation, through the solubility of the nitrogen material, lowering the technological capacities and cheese yield (Raynal-Ljutovac *et al.,* 2005)

Results by Erreifej (2006) confirm that the chemical composition of cheese does not vary significantly during lactation period; this is also in accordance with the work by Fresno (1996) on Spanish goat's milk cheese (Armada variety). Some other results from Erreifej (2006) suggest that ash content varied significantly during the whole lactation period, which are in agreement with similar values reported by Guo *et al*. (1998) and Haenlein (1998). However, it is not the case in the present work.

pH values from milk and cheese were approximately similar during all the lactation period. In fact, pH values of fresh cheese immediately after production varied from 6.27 to 6.73. After the coagulation, the pH of fresh cheese rose to the initial values of the milk pH due to changes in the micellar structure. This is in accordance with Fox (1987), who obtained pH values varying from 6.2 to 6.5 for some cheeses immediately after production and with Estepar *et al*. (1999) who obtained values from 6.2 to 7.0.

From milk to cheese, the fat content decreased significantly, since only a fraction of it stays entrapped in the cheese protein mesh and the relative concentration of the protein increased, which is to be expected as the cheese is mostly the result of casein coagulation.

As expected, the ash content and total solids presented higher values in cheese that in milk (3.7 times higher for ash and 3 times higher for total solids). This difference could be due to the loss of fat and liquids during the coagulation process. The fat variation presented the same trend as that observed in milk, but its quantity was 3 times less, since a significant amount is lost in the whey. Estepar *et al*. (1999) found that total solids doubled from milk to cheese.

The fat content decreases around 3 % from milk to cheese. This is probably due to the fact that part of it doesn't stay entrapped in the protein structure and was lost with the whey.

The high moister content obtained in the fresh cheese studied in this work (63.7 %) could be due to the very slow whey drainage caused by the very slow acidification like in PenJamellera cheese studied by Estepar *et al*. (1999).

Total protein content determination give the result of 3.36 %, which is in accordance with Prata *et al*. (1998) that presented values of 3.27 % and Grappin *et al*. (1981) that presented 3.08 %. Values for true protein were 3.09 % in this work and 2.97 % for Prata *et al*. (1998) and 2.81 % for Grappin *et al*. (1981). Non proteic nitrogen found in this work was 0.05 %, which is quite different from Prata *et al*. (1998) with 0.30 % and Grappin *et al*. (1981) with 0.27 %.

Results on nitrogen content showed that 91.9 % of the total nitrogen in raw milk corresponds to true protein and 1.37 % to the non-proteic fraction. Studies from Prata *et al*. (1998) obtained 90.83 % and 9.17 %, respectively for the same parameters in raw milk of goats other then algarvian breed. The same authors presented a correlation between true protein and total protein of $y = -0.33437 + 1.0195x$ with an $r^2 = 0.9921$ which are in accordance with this work were the correlation obtained was $y = -0.3937 + 1.0367x$, with an $r^2 = 0.9968$.

This work showed that milk from the algarvian goat breed could be boiled without undergoing any quality loss. It also showed that the milk gross chemical composition is practically constant during all the lactation period which contributes to maintain a regular quality in cheeses throughout all the production period. These results are important because they prove that boiled milk could be used in cheese production, which permit the increase of safety concerned with microbiological population, without loss of properties. The use of native starters which have been

isolated in this work may make it possible to maintain the typical characteristics of the studied cheeses. Milk that will undergo heat treatment could present a higher microbial load which permits the utilization of more milk that is produced in good, but not excellent hygienic conditions which will help the small producers in being able to use more of their milk. This increment in the production could contribute to the increasing interest of the producers in working better every day and to improve the quality of the cheese. The uniform quality obtained during all the production period is a very important factor for every product because consumers expect to buy a consistent product, with the same quality and the same characteristics. With this conditions the purchases increase, the income of producers increase too and consequently the social development of the region occur.

4.2. Microbiology of Cardoon

Cynara cardunculus extracts contain aspartic proteases (chemosin) that possess specificity for cleaving casein in particular sites (Faro *et al*., 1993; Mecedo *et al*., 1993) which confer to the cheese manufactured with cardoon flower extract very good sensory properties (taste and texture) when compared with cheeses made with rennet preparations (Chen *et al*., 2003). Esteves *et al*. (2002) verified that at low temperature (algarvian goat cheese undergoes coagulation at 30 ºC), the coagulum was formed very fast and the proteolysis was not excessive, which has a favourable effect on texture and flavour of the final products. In addition to this being of vegetable origin which constitutes a valorisation for the utilization of this kind of cheese in lacto-vegetarian and ecological markets (Gómez *et al*., 2001) and can also be certified by Kosher rules (Prados *et al*., 2007).

In spite of the fact that no published information for cardoon from Algarve could be found in the literature, it is known that during cheese manufacture the cardoon is added after boiling the milk. So it is important to study the microbiota of the dried cardoon in order to try to increase the safety of the cheese.

In this work the cardoon was studied only in dry conditions, since it is used for cheese production only after drying procedures. During the drying process all the vegetative cells of microorganisms should not survive due to the low water activity, eventually only sporulated forms remaining.

The high standard deviation values (Figure 3.11) suggested a great heterogeneity in the different samples. Cardoon samples composed of dry stigmas, stylets, bracts and remaining leaves, that are different structures with varied geometries, could explain the different microbial loadings.

Values obtained varied from a minimum of log 3.12 to a maximum of log 7.25 cfu/g, which is in accordance with Gomez *et al*. (2001) who registered values of log 5.13±0.63 cfu/g.

"Tondela only flower" was the cardoon type in which the samples were more handled, since the stigmas were separated from the rest of the flower before the drying process. When it is compared with "Tondela", which is the same type of cardoon, but without undergoing any process of stigmas separation, the latter presented indeed less microbial counts.

The "Producer cardoon" was bought in a local market and all the other samples were prepared and stored under known and controlled hygienic conditions. As can be observed, the differences are not significant, which indicates that the cardoon brought in the market was treated with care, maintaining an adequate quality as far as microorganism numbers are concerned, which is of a major importance, since it is this product which is normally used in cheese production.

The microorganisms detected in cardoon, did not appear to have a spoilage action since samples kept in the laboratory (dry and dark conditions) for three years, did not present any sign of degradation. This can be explained by the very low water activity of cardoon, with a value of 0.54, observed in the studied samples. This value is in accordance with Martins *et al*. (1996), who reports water activity of 0.6. This situation creates difficulties for the microorganism's growth, although, they could remain in a dormant stage or as spores. This fact could represent a problem when the cardoon is added to the milk since its addition is made after boiling the milk (when heat treatment is done).

Relatively to yeast and moulds, the maximum value (log 4.23 cfu/g) was registered from the "Producer cardoon". Since it presented one of the lowest values (log 4.17 cfu/g) for the total heterotrophic microorganisms, it indicates that the dominant microbiota in this type of cardoon were yeasts and moulds. The lower value

observed was for “Tondela” samples, which is expected, since it presented the lowest values for the total heterotrophic microorganisms too.

Figure 3.12, shows that the average values of yeasts and moulds varied from log 2.53 to log 4.27 cfu/g, which is in accordance with Gomez *et al*. (2001), who reports values around 4.07 cfu/g.

In almost all studied cardoons, the concentrations of moulds and yeasts were approximately two logs lower than those of total heterotrophic microorganisms, except for “Producer cardoon”, where both concentrations are similar. The high value found in “Producer cardoon” is, perhaps, due to the storage conditions. Usually it is stored under dry conditions and protected from draught to prevent later contamination. Low water activity may have been adequate to prevent bacterial growth, but not sufficient to prevent the growth of fungi and yeasts, which are generally able to tolerate dryer conditions. It should also be noted that the “Producer cardoon” was one year older than the other samples thus it had more time in contact with air and consequently more opportunity to be contaminated by moulds.

Non-parametric Kruskal-Wallis test showed that there were significant differences between samples. As a result of this, a Dunn test (Dunn, 1964) was performed. This test showed that, in terms of total heterotrophic microbiota, samples of “Arouca”, “complete mixture”, “Altos without spines”, “Spain” and “Tondela only flower” form a group while “Producer cardoon” and “Tondela” forms another group. The first mentioned group was the one which presented higher microbial counts.

As far as yeasts and moulds are concerned, tested samples could be arranged in three groups, one composed by “Arouca”, “Tondela only flower” and “Complete mixture”, another group constituted by “Altos without spines”, “Spain” and “Tondela”, and finally, a third one with only an element – “Producer cardoon”.

In this work, most of the identified moulds from the cardoon samples are from the genus *Aspergillus*, which is common in soil and all kind of decaying material and reported as being as one of the genera most responsible for spoilage during storage of a wide range of products (Filtenberg *et al*., 1996). Also genera like *Penicilium, Mucor*, *Rhizopus* and *Phoma* were identified, but in very low numbers. Both *Aspergillus* and *Penicilium* are microaerophilic which permit their growth in the

interior part of cheese (Montagna *et al*., 2004), constituting an important quality problem.

Although for some types of cheeses moulds and yeasts can contribute to improve organoleptic qualities, it was not possible to find published results about algarvian goat cheese that indicates this. On the contrary, visible growth of fungi in this type of cheese represents a quality problem, affecting the appearance of the cheese and making it inappropriate for sale. It is also reported by authors like Hayaloglu & Kirbag (2007); Jodral *et al*. (1993); Barrios *et al.* (1998) and Wouters *et al.* (2002) that some moulds can produce toxic secondary metabolites (mycotoxins), and other may be able to produce undesirable flavours. *Penicilium verrucosum*, one of the species most commonly isolated from cheeses is an example of a species that is considered toxigenic (Scott, 1981; Bullerman, 1979).

In the presence of these findings it could be suggested therefore that the second group of cardoon types stated above ("Altos without spines", "Spain" and "Tondela") was the one that presented better mycological properties for the production of this kind of cheese. However the cardoon that is used in cheese making is a wild plant that is collected in non cultivated regions, so it is impossible to recommend the utilization of a particular type. What is important is that the collectors should have taken much care during the collection, transport and drying operations. They should choose days with dry weather conditions for the collection, for instance. Care should be taking too during transport (fulfilling all the hygienic conditions with dedicated transport for instance); drying process and storage conditions (care should be taken with relative humidity of the storage place, ambient contamination, presence of plagues and animals). Similar care should be taken by the cheese makers during the storage of the cardoon prior to the utilization in cheese manufacture.

Prados *et al*. (2007) suggested the utilization of a powder coagulant from the crude aqueous extract which has been patented by Fernández-Salguero *et al*. (2003) and tested in 'Los Pedroches' cheese (ewes milk) with satisfactory results. This powdered coagulant is free from viable microorganisms, is soluble in milk, shelf stable in the absence of air without the need for preservatives and easy to handled (Prados *et al*., 2007). It should be interesting to study its utilization in the algarvian goat breed milk in order to verify if the traditional characteristics will be the same. In

case of maintenance of these characteristics the use of this type of vegetable coagulant will be suggested in order to increase the safety of the algarvian goat breed cheese.

The knowledge of the microbiological properties of cardoon makes it possible to be aware of the problems that it can cause to the cheese when added after to the milk heat treatment and to advice the cardoon collectors and the cheese makers of some hygienic rules that could improve cheese safety.

4.3. Microbiological quality of Raw Milk

No significant differences were observed in the studied microbiota from the raw milk across the studied period (from May to July), which means that the microbiological quality of the milk is stable and consistent during all the lactation period. No published values for goat milk from algarvian goat breed could be found in the literature as this is the first study in this area, but other goat breeds were studied by other authors, like Ereifej (2006), and no significant differences were found in their studies, which is in keeping with the current work.

Values between 4.67 and 5.61 log cfu/ml for heterotrophic microorganisms and from 3.49 to 5.9 log cfu/ml, for moulds and yeasts were reported by Ereifej (2006). Other workers reported 5.54±2.44 log cfu/ml for heterotrophic organisms, 5.82±1.7 log cfu/ml for yeasts and moulds and 3.62±1.16 log cfu/ml for faecal coliforms (Zárate *et al*., 1997). These results are broadly in accordance with the results obtained in the present work with average values of 5.13±0.5 log cfu/ml for heterotrophic microorganisms, 3.82±0.69 for yeasts and moulds and 2.12±0.77 for faecal coliforms. No comparable results for *Lactobacillus* in milk were found.

The lower number of faecal coliforms obtained in this work could indicate better hygienic conditions than those reported by Zárate *et al*. (1997), namely good practices of hygiene in the cattle-shed and in milking process as well as in the milk transport from the farm to the manufacture place.

Moulds present in milk were not identified in this work, but other workers like Torkar & Vengust (2008) reported the isolation of strains from the genera *Geotrichum*, *Aspergillus*, *Mucor*, *Fusarium* and *Penicillium*, will the first one being the most important in quantity. Also Jodral *et al*. (1993) reported *Geotrichum* as the genus most frequently isolated in the raw milk followed by *Fusarium* and *Aspergillus*.

If the milk was to be used after thermal treatment, as in the production of fresh cheese, moulds and yeasts do not survive it (Jodral *et al*., 1993; Nelson, 1981) and consequently their presence in raw milk is not a problem.

If it was to be used raw as in case of the ripened cheeses, the presence of yeasts and moulds could be a problem that will be reflected in cheese, with both economic and sensory losses (Torkar & Vengust, 2008).

Total heterotrophic microorganisms are currently subject to legislation. By Regulation (EC) nº 1662/2006, 6th November, the legal limit for total heterotrophic microorganisms in milk to be used for products without any thermal treatment is 5.7 log cfu of heterotrophic microorganisms per ml of milk and 6.18 log cfu/ml for total heterotrophic microorganisms in milk to be used for products that involve thermal treatment. In the present study, only two samples were above the limit for the utilization of raw milk. Thus, if this milk was to be used after a thermal treatment all the samples were under the legal limit. This fact indicate that more milk, obtained during the lactation period, could be used for cheese production, if the heat treated milk was used and indigenous starters were added in order to maintain the regional cheese characteristics. These findings indicate that the heat treatment of milk is important to the safety of the cheese and contribute to reinforce the importance of the study of native microbiota in order to produce the above referred starters.

4.4. Microbiological Quality of Cheese immediately after processing

There were no significant differences in the studied microbiota of cheese immediately after processing (made from raw milk) across the studied period (from May to July), which means that this product had a consistent microbiological quality.

The slight differences observed between the studied microbiological parameters of cheese from different batches may be attributed to the natural variation of microbiological results or may be to the contribution of the processing environment, handling and raw milk contamination.

No published values for cheese from algarvian goat breed could be found in the literature, as the work described here is the first study in this area, but other authors, like Olarte *et al*. (1999), who studied cheese (curd) made from goats of other breeds, reported results from 3.8 to 8 log cfu/g for heterotrophic microbiota; from 2 to 5 log

cfu/g for yeasts and moulds and up to 5 log cfu/g for faecal coliforms. As it can be seen in Figure 3.14, heterotrophic microorganisms varied from 6.4 to 8.3 log cfu/g, yeasts and moulds varied from 4.6 to 5.7 log cfu/g and faecal coliforms varied from 3.4 to 5.5 log cfu/g, which means that all the results in the present work, are in accordance with those reported by other authors.

All studied microorganisms increased approximately two orders of magnitude from milk to cheese. This increment could be due to the entrapment of the microorganisms in the curd after drainage of the whey and their multiplication (Tatini *et al*., 1971) in the elapsed time from the milk clotting to the beginning of the analysis (approximately 24 hours), as the milk provides a good support for microorganisms development, due to its high nutrient content. Also Fernandez del Pozo *et al*. (1988); Poullet *et al*. (1991) reported similar results in goats' and ewes' cheeses.

It is important to remember that, in Portugal, it is forbidden by law (Reg (CE) n. 1662/2006 from European Commission, 6th November) to sell fresh cheese made from raw milk. Thus these cheeses must pass through a ripening process. During this period the concentration of faecal coliforms and moulds should decrease as it was showed in the results chapter of this work (section 3.5) and will be discussed further, which are in accordance with was described by Caridi *et al*. (2003). However we must think that some of these microorganisms could be toxin producers and that even if they disappeared during ripening due to the hostile environment created at that time, toxins eventually produced stays in cheese, which can cause a safety problem. A solution for this problem is the thermal treatment of the milk before cheese manufacture, thus preventing toxin production.

Considering the maximum values of microbial numbers registered from the cardoon samples and the amount of cardoon used for cheese production, it can be assumed that this addition had no immediate significant importance. However, it is possible that some of the species present in the cardoon may have grown quickly to high levels in the milk, adding to, and contributing new species to the cheese. This includes yeasts and moulds. These microorganisms could have important positive or negative effects on the cheese quality.

Moulds isolated from cheese immediately after processing were not identified in this work, but Chapman & Sharpe (1990) identified *Penicillium* as the most prevalent

mould in samples of cottage cheese (similar to cheese immediately after processing) while *Mucor*, *Geotrichum* and *Cladosporium* were detected at lower percentages.

The microbiota monitorization of the cheese immediately after processing during the lactation period was very important because it indicated that there are no significant differences from the microbiological point of view during this period. This fact contribute to the guarantee that constant quality of the cheese during the production period could be expected, but it was important also to isolate some microorganisms that, due to their technological properties, could be used as component of a native starter to use in cheese production using boiled milk.

4.5. Microbiology of Cheese During Ripening

During the ripening period, total viable microorganisms showed almost no differences, presenting values of 9.02±0.13 log cfu/g after 60 d of ripening. No published values for ripening of cheese made from algarvian goat breed could be found in the literature, but other authors studied cheeses made from milk from other regions. Thus these results are in agreement with studies of Centeno *et al.* (1994), Centeno *et al.* (1996), De Giori *et al*. (1983), Medina *et al*. (1995) and Quinto *et al*. (1994). According to Souza *et al*. (2003), values of 9 log are normal in cheese produced with raw milk, which is the case of the cheese studied in this work.

The initial increase of 1 log could be due to the lactic acid bacteria growth, but, in this case, it was probably compensated by the reduction of faecal coliform numbers. In fact the overall constant results obtained for total bacterial counts masks a very dynamic situation as evidenced by changes in populations of coliforms and *Lactobacillus*.

Total coliforms reached maximum counts after 14 days (6.77±0.01 log cfu/g) of ripening, decreasing thereafter gradually by about 3 log units until 28 days (3.37±0.21 log cfu/g) of ripening, after which they become approximately constant. Studies by De Giori *et al*. (1983) showed that total coliforms were not detected in Tafi cheese after 30 days of ripening due to the increase of NaCl concentration by water loss (low a_w). The coliform population is influenced also by the temperatures of the maturation room (Nuñez *et al*., 1985). Research studies done by Fernandez del Pozo *et al*. (1988)

with La serena cheese showed that coliforms had a higher decrease rate when ripened at 16.9 ºC than those ripened at 13.6 ºC. In this work the ripening occurred at 15 ºC.

Mean log counts of 3.81 for total coliforms have been reported in La Serena cheese made with vegetable coagulant and ripened at 14 ºC (Medina *et al*., 1991), but Núñez *et al*. (1991) obtained enumerations greater than 5.00 log cfu/g at 60 days of ripening in the variety of the studied cheese.

The coliforms and faecal coliform enumerations were similar to the values reported for goats' milk used in the manufacture of Majorero, Gredos and Valdeon cheeses (Fontecha *et al.,* 1990; Medina *et al.,* 1991; Lopez-Diaz *et al.,* 1995).

Faecal coliforms depart from a maximum of 3.98±0.65 log cfu/g at the production day to a zero value by the 21 days of ripening, indicating a safe food product from this day until the end of the study.

Other factor that can contribute to the decline of these groups during ripening is the inhibition by lactic acid bacteria (Nuñez *et al.,* 1985), which causes a decrease in the pH values due to the increase of lactic acid production.

However, the numbers of microorganisms indicative of the bacteriological quality (coliforms and faecal coliforms) were low, suggesting good hygiene practices during milking and cheese manufacturing.

During cold room ripening, lactic acid bacteria were the most abundant microbial group in milk, wich is in accordance with Tejada & Salguero (2003) and Zàrate *et al*. (1997). In fact, in this work the concentration of lactic acid bacteria enumerated on Rogosa agar (mostly *Lactobacillus*) and on M17 agar (mostly *Lactococcus*) increased during the first week of ripening by 1.70 and 2.55 log cycles, respectively, with respect to their initial numbers in the curd (approximately 5.52 and 7.26 log cfu/g of cheese, respectively). Thereafter, viable *Lactobacillus* tended to increase at a much slower rate from 7 to 42 d of ripening and eventually stabilized or slowly decreased until 8.52±0.25 log cfu/g of cheese at 60 d. Although we could not find other studies with the milk and cheese types studied here, results reported by other authors, in different types of cheeses, are similar. Results include 8.30±0.19 log cfu/g by Vioque *et al*. (2000); 8.76±0.33 log cfu/g by Tejada & Salguero (2003) and 7.58±1.64 log cfu/g by Zàrate *et al*. (1997).

Lactococcus, being predominant during the early stages of ripening, reached a maximum enumeration after 14 d, decreasing only slightly after that. In other studies with raw milk cheeses, by Nuñez (1978), Litopoulou-Tzanetaki & Tzanetakis (1992), Fontán *et al*., (2001), Ordóñez & Burgos (1977), Dervisoglu & Aydemir (2007) and Centeno *et al*., (1996), the predominance of *Lactococcus* during the early stages of ripening has also been reported.

After 28 days, the enumeration of *Lactococcus* and *Lactobacillus* were practically the same and approximately constant during the second month of ripening, which is in accordance with Nufiez (1978) studies.

Lactic acid bacteria, especially *Lactobacillus,* were probably responsible for the fall in the cheeses' pH, presenting minimum values at 42 days (Table 4.5). According to Peterson *et al*., (1990), during the cheese ripening the formation of several metabolites, such as lactate, citrate, glycerol, and aminoacids, among others, which are better utilized by *Lactobacillus*, takes place. According to Steele (1995), lactic acid bacteria influence the ripening process not only by the production of lactic acid, reducing pH, but by a decrease in the oxidation-reduction potential, and by proteolysis and lipolysis.

Yeasts showed maximum concentrations after about 14 days of the ripening period and then presented a slight decreased until the end of the maturation.

Moulds increased until 35 days of ripening, keeping approximately constant numbers after that, except for the last week of this study, when they decreased by two orders of magnitude. Some authors, in studies conducted with cheeses produced from different goat breeds milk, found lower values for yeasts and moulds, respectively 4.28±1.46 and 3.91±1.92 log cfu/g (Zàrate *et al*., 1997), but others like Floréz *et al*. (2006) found 7.04±0.38 and 7.34±0.58 at the end of ripening. This probably means that the cheese studied in this work is more close to that studied by Floréz *et al*. (2006), namely in respect to initial contamination of milk and/or manufacture conditions, including low initial contamination by cardoon addition.

In some cases, yeasts and moulds may increase the quality of ripening by metabolizing lactic acid and liberating alkaline compounds by proteolytic and lipolytic activities (Macedo *et al*., 1995; Alonso-Calleja *et al*., 2002; Gerasi *et al*., 2003). This slight increase of pH encourages growth of bacteria that are sensitive to

acidic environments, helping to initiate the second stage of maturation (Sousa & Malcata, 1996).

However, some other species of moulds and yeasts could cause quality problems and economic losses in the cheese production. Some moulds can produce secondary metabolites which are toxic (mycotoxins) and undesirable flavours (Hayaloglu & Kirbag, 2007). Also the cheese appearance could be affected by the presence of moulds, making it unfit for sale. In this work, non identified species of moulds presented visible growth in the interior of some cheeses during ripening, which definitively represented a quality problem.

As ripening advances, there is an increase in cheese matrix compactation, which reduces the diffusion of the oxygen required for the multiplication of yeasts and moulds (Souza *et al*., 2003), but due to the small size of the cheeses studied in this work, this influence was not noticeable.

In the beginning of the ripening period the moulds identified were from genus *Aspergillus, Penicilium, Chaetomium* and *Phoma*, but after two weeks only *Penicilium* was detected. These results are in agreement with Scott (1981) in which *Aspergillus*, *Penicillium*, *Cladosporium*, *Geotrichum* and *Mucor* were the most important moulds occurring in cheese. Considering the incidence of moulds in a wide variety of cheeses, it was found that *Penicillium* is the most common genus found, especially on refrigerated cheese (Scott 1981). All this genera of moulds were isolated from the cardoon samples too (except for *Chaetomium*) which seems to confirm that the principal source of moulds was the vegetable coagulant.

If we consider the general microbiological point of view the cheese made from algarvian goat breed is similar to other goat cheeses. However each cheese (not cheese type) has a different microbiota depending on the synergetic relations among the microorganisms presents and there are differences that make each cheese so interesting from the organoleptic point of view.

The microbiological assessement through the presence of coliform bacteria showed that the studied cheese presented an adequate safety condition at the end of the ripening period.

This section of the work allowed the isolation of native LAB which then allowed identification to genus level and the study of their technological characteristics. This

knowledge then allows the design of native starters to use with boiled milk in order to obtain a cheese even more safe and with similar organoleptic characteristics to tradicionaly made Algarve cheese.

4.6. Isolation and Phenotypic Characterization of LAB

4.6.1. Samples from Raw Milk Obtained During the Lactation Period Between May and July

Considering the isolates as a whole, *Lactococcus* and *Lactobacillus* formed the prevalent groups, followed by *Enterococcus* and *Aerococcus* (both present at almost all stages of lactation), with the other studied groups being the minority.

No published values for goat milk from Algarve could be found in the literature but comparison with other authors that studied other breeds is possible. The *Leuconostoc* group was isolated only once, which is in accordance with findings stated by Beresford *et al*. (2001). *Leuconostoc* spp. have the ability to metabolise sugars by the phosphoketolase pathway but have a poor ability to grow in milk, perhaps due to the lack of a proteinase system to degrade milk proteins and release growth substrates.

Pediococcus were isolated only at the end of lactation period, perhaps due to contamination by the vegetable food supplied to the herd, since they are primarily of vegetable origin.

Streptococcus showed a poor representation across the sampling period, perhaps due to their need for amino acids which are not present in milk, unless in the presence of some *Lactobacillus*, which could degrade milk proteins and release amino acids through their proteolytic systems, allowing their growth (Wouters *et al*., 2002). Probably, this type of degradation is limited by the short time occurring between milk and cheese manufacture.

Enterococcus were present in most of the samples, which is in accordance with findings reported by Wouters *et al.* (2002) according to whom their presence has generally been a consequence of insufficient sanitary conditions during the milking process. However, several strains show biochemical properties that make them possibly useful as starters for technological application in cheese manufacturing.

Although, care should be taken since some strains of *Enterococcus* have been recognised as potential pathogens, their virulence factors should be taken into account before any selection for starter cultures is considered (Sarantinopoulos *et al*., 2001; Andrighetto *et al*., 2002).

Apparently there is a big variability of genera during the lactation period, which could cause problems of uniformity of the cheese. However it is important to note that colonies for identification were collected randomly from Petri dishes used in the lactic acid bacteria enumeration. Thus there were many more colonies present and if all of them were identified, probably the variability of microorganisms between samples could be much lower.

4.6.2. Samples from Cheese During Ripening

Despite the large number of different bacteria found, *Lactococcus* group (M17 medium) seems to dominate the microbiota in cheeses samples representative of all ripening stages (Figure 3.15), reaching maximum levels of 9.81 log cfu/g at the 7th day and remaining relatively constant throughout ripening. Their growth tendency was similar to that observed for total heterotrophic aerobic microorganisms (PCA medium).

Lactobacillus also reached a high colony density (1.5×10^{9} cfu/g), by the 42nd day, after which they declined slowly during the maturation process. However, their numbers were always lower than those for *Lactococcus* (Figure 3.15).

After identification tests it was verified that *Lactococcus* were present mostly at the early stages of the ripening period, as reported by Centeno *et al*. (1996).

Estepar *et al*. (1989) reported that M17 is a poor selective medium and, thus, counts using this medium might reflect the real relationship between the different lactic acid bacteria in the cheese. This means that other LAB could grow in M17 agar medium and thus enumerating other microorganisms as being *Lactococcus* could occur, giving the impression that it was present much more commonly than was actually the case.

In accordance with the expectations, identification to genus level of bacteria isolated from Rogosa agar medium showed *Lactobacillus* as the most numerous group, followed by *Enterococcus*.

Considering the isolates as a whole, *Lactobacillus* formed the prevalent group (44.4 %), followed by *Enterococcus* (22.8 %) and *Pediococcus* (10.6 %), *Leuconostoc* (10 %) and *Aerococcus* (9.4 %) (both attaining levels of about a quarter of those of the *Lactobacillus*), with *Lactococcus*/*Vagococcus* (2.2 %) and *Streptococcus* (0.6 %), being the minority groups.

This prevalence of *Lactobacillus* has also been found in other goat's milk cheeses studies (Tornadijo *et al*., 1995; Zárate *et al*., 1997), which probably reflects a combined effect of high salt concentration and low pH values, which *Lactobacillus* tolerate better than other LAB genus members (Axelsson, 1998).

Previous studies showed that the majority of non starter lactic acid bacteria (NSLAB) (natural) found in most types of cheeses are made up of mesophilic *Lactobacillus* (Demarigny *et al*., 1996; Williams & Banks, 1997; Bouton *et al*., 1998). Although NSLAB require several amino acids for growth, they possess numerous proteases and peptidases that permit their growth in cheeses without fermentable carbohydrate, by metabolizing peptides, amino acids and sugars released from enzymatic hydrolysis of casein, and products resulting from the degradation of starter bacteria. The NSLAB population is widely variable, but is composed, predominately, of *Lactobacillus plantarum* and *Lactobacillus casei*, both homofermentative and present throughout the ripening period, and *Lactobacillus brevis*, which is heterofermentative and was present especially in the later ripening period, around 60 days, when it seems to replace partially the homofermentative microbiota. To a lesser extent, species of *Pediococcus* seem to appear. (Peterson & Marshall, 1990). Thus *Lactobacillus* and *Pediococcus* are the most important genera of NSLAB.

In general, more desirable flavours were detected in cheeses containing *Lactobacillus casei* and *Lactobacillus plantarum* and more objectionable flavours, typically fermented, were associated with cheeses with *Lactobacillus brevis*. Openness and discoloration are associated with this species too, however only after long ripening periods as in Cheddar cheese (Peterson & Marshall, 1990). The same authors showed that, sometimes, mixed cultures of *Lactobacillus* produced better cheese than when strains are added alone, suggesting complementary effects in the mixed cultures. As described above, *Lactobacillus brevis* can confer undesirable

properties to the cheese, but the ripening of algarvian goat cheese takes around 60 days, which may indicate that *L. Brevis* is unlikely to be a problem in this product.

The distinction between *Lactococcus* and *Enterococcus* can be difficult, however, it is likely that *Enterococcus faecalis* and *Enterococcus faecium*, would be the most common species of *Enterococcus* and *Lactococcus lactis* and *Lactococcus raffinolactis* would be the commonest species of *Lactococcus* found in cheese. But these two groups can be distinguished from each other at the genus level by their growth abilities at 45 °C and in 65 % of NaCl as shown by Cogan *et al.* (1997) with different cheeses from goat milk.

Litopoulou-Tzanetaki & Tzanetakis (1992) showed values for *Enterococcus* of approximately 29 % during ripening of cheese made from raw goat's milk.

The high numbers for *Enterococcus* are in accordance with enumerations found in other cheese varieties, manufactured from goat's milk (Tornadijo *et al*., 1995; Suzzi *et al*., 2000). Several strains of *Enterococcus* have even been isolated as dominant bacteria in different artisanal cheeses (Nuñez, 1978; 1983; Devoyod & Muller, 1969; Fontecha *et al*., 1990; Centeno *et al*., 1996).

The presence of this high numbers of *Enterococcus* could be due to their tolerance to a wide range of environmental conditions (Holt *et al*., 1994). It is important to note that some strains of *Enterococcus* are able to produce bacteriocins against *Listeria* when grown in milk and cheeses (Parente & Hill, 1992; Villani *et al*., 1993; Giraffa *et al*., 1994; Torri Tarelli *et al*., 1994; Garcia *et al*., 1997; Giraffa & Carminati, 1997; Ennahar *et al*., 1998). This group of bacteria also may influence the ripening process due to their lipolytic and proteolytic activities, including their marked ability to ferment lactose and to stimulate acid production by some *Lactococcus* and gas production by *Leuconostoc* (Devoyod & Desmazeaud, 1970; Sarantinopoulos *et al*., 2001). *Leuconostoc* may also have an important role in ripening (Martley & Crow, 1993.

The relationship between *Leuconostoc* isolates and all other isolates did not vary significantly across the ripening period in accordance with Arizcun *et al*. (1997). *Leuconostoc mesenteroides* subsp *mesenteroides* and *Leuconostoc mesenteroides* subsp *dextranieum*, together with *Lactococcus*, can produce diacetyl, acetic acid, and ethanol, which can contribute to the aroma formation in cheese but, on the other hand,

they have sometimes been associated with flavour defects (Peterson & Marshall, 1990). The CO_2 produced by their metabolism may also contribute to the undesirable formation of small eyes (gas bubbles) in the cheese. However, CO_2 also inhibits the growth of some contaminating moulds (Arizcum *et al*., 1997).

After the isolation and morphological and biochemical characterization of the microorganisms present in milk and cheese, the study of their technological properties can make an easier selection of isolates that could represent possible starter cultures in the future. In this work, the isolates to study were selected so as to represent the widest range of properties used for identification (cell shape, CO_2 production from glucose, growth at 10 and 45 ºC, growth at 6.5 % NaCl and growth at pH 4.4 and 9.6). The remaining microorganisms were considering as being replicates of the studied ones.

4.7. Technological Properties of LAB

4.7.1. Bacteriocin Production

In this work, the bacteriocin production was tested against *Weissella paramesenteroides* DSM 5623 strain. Bacteriocin-like antagonism by *Enterococcus* has also been reported against *Listeria* by Giraffa *et al*. (1994). Also an important number of enterococcal strains isolated by Simonetta *et al*. (1997) from Argentinian milk and milk products were found to have an antagonistic activity against *Vibrio cholerae*. These data could suggest that antibacterial activity of *Enterococcus* could be related with particular habitats or areas, and that they may play an important role in preservation and safety of traditional food (Suzzi *et al*., 2000).

Nunez *et al*. (1997) showed that counts of *Listeria monocytogenes* present in Manchego cheese inoculated with *Enterococcus faecalis* (bacteriocin-producing strain) decreased by 6 log in a week, whereas the survival of other microorganisms in cheese made with commercial starter culture was not affected.

Muñoz *et al*. (2004) inoculated milk with an enterococcal strain (enterocin producer) as an adjunct culture in combination with a commercial starter culture for cheese manufacture and verified that growth of the starter was not affected neither the physicochemical properties of the cheese. Simultaneously, enough bacteriocin was

produced in the cheese to ensure inhibition of *Bacillus cereus*. Therefore it was considered important to study the bacteriocin production of isolates obtained from milk and cheese.

From the 20 *Lactobacillus*, isolated from milk, 8 (40 %) presented bacteriocin activity, whereas from the 10 isolated from cheese, only 3 (30 %) presented that characteristic. Just one of the two *Enterococcus* isolates, from milk, showed the capacity to produce bacteriocin. Two *Enterococcus* spp were isolated from cheese, but none produced bacteriocin. The presence of *Enterococcus* in cheese made from algarvian goat breed could be considered an advantage in *Listeria* control since there are reports by Parente & Hill (1992), Villani *et al*. (1993), Giraffa *et al*. (1994), Torri Tarelli *et al*. (1994), Garcia *et al*. (1997), Giraffa & Carminati (1997) and Ennahar *et al*. (1998) that indicate some species of *Enterococcus* as being able to produce bacteriocins against this microorganisms when grown in milk and cheeses.

As in studies conducted by Peterson & Marshall (1990) with Cheddar cheese, the secondary microbiota in the cheese, studied in this work, consisted mostly of *Lactobacillus* (10) and *Pediococcus* (5). These bacteria are collectively referred to as non-starter lactic acid bacteria (NSLAB). However, the composition of the NSLAB in the cheese varied with the age of the cheese and even with the day of manufacture (Williams & Banks, 1997 and Fitzsimons *et al*., 2001).

In this work only two *Pediococcus* were isolated from milk samples, one presented bacteriocin activity and the other one did not. Five isolates from this genus were obtained from cheese but only two (40 %) presented bacteriocin activity. Some of the strains of *Pediococcus* widely used as starter, such as *P. udibctici* and *P. pentosuceus,* produce bacteriocins (Stiles & Holzapfel, 1997). However, since they require a fermentable carbohydrate to grow, they grow poorly in milk, because lactose is not readily utilised by them. As they have only medium capacity to acidify the medium (Figures 3.20 and 3.21), they are of some economic importance as starter cultures (Stiles & Holzapfel, 1997), but are more important as NSLAB cultures. More importantly, Bacteriocins produced by *Pediococcus* (Pediocins) have a large spectrum of activity against Gram-positive bacteria. Pediocins are produced by *Pediococcus ucidiktici* (Bhunia *et al*., 1988; Nieto-Lozano *et al*., 1992; Cintas *et al*., 1995) and *Pediococcus pentosucrous* (Piva & Headon, 1994). Pediocin produced by

Pediococcus spp. has been demonstrated capacity to inactivate *Listeria monocytogenes* (Barnes *et al*., 1989; Berry *et al*., 1990; Ming *et al*., 1997; Zheng *et al*., 1999; Ariyapitipun *et al*., 2000 and Pucci *et al*., 1988). *Pediococcus cerevisiae* has also been reported by Haines & Harmon (1973) to inhibit *Staphylococcus aureus*.

Pediococcus have a positive role in proteolysis and lipolysis of certain cheeses during ripening as the case of Parmigiano Reggiano cheese (Gobbetti *et al*., 2002).

From the four *Lactococcus* spp. isolated from milk in this work, two (50 %) presented bacteriocin activity. From the two isolated from cheese, none presented this kind of activity. This is in line with Klijn *et al.* (1995); Cogan *et al*. (1997) and Estepar *et al*. (1999) and in accordance with the general observation that *Lactococcus* isolated from non-dairy natural environments show a higher incidence of antimicrobial production than strains isolated from a dairy niche, including industrial ones. This phenomenon can be due to the fact that the ability to produce antimicrobial compounds offers these wild type strains the power to resist the competition from other microorganisms, being able to survive in their relatively hostile natural environment (Martley & Crow, 1993).

Studies from Ayad *et al*. (2002) showed that 32 out of 79 isolated strains from Ras cheese (40.5 %) were active against microorganisms. In 17 of these strains (53 %), the antimicrobial peptide nisin was found, whereas the other produced diplococcin (6.3 %), Lactococcusin (9.4 %) or an unidentified bacteriocin-like compound (31.3 %).

Because of their potential to produce interesting flavour compounds (through lipolysis and proteolisys) and in addition with their antimicrobial activity, the bacteriocin-producing lactococcal strains may be useful as starters in cheese making. Although care should be taken in combining them with other strains, which can be bacteriocin-sensitive (Martley & Crow, 1993). In this instance, part of the microbiota will be destroyed.

4.7.2. Acidifying Capacity

A rapid decrease in pH is essential for coagulation and the prevention or reduction of the growth of adventitious microbiota, during the initial steps of cheese

preparation, making it of crucial importance in cheese manufacture (Sarantinopoulos *et al*., 2001).

In this work strains were classified as presenting fast acidification capacity if a pH variation of 0.4 occurred in less then three hours, medium acidification capacity if pH variation of 0.4 occurred in less them six hours and slow acidification capacity if a pH variation of 0.4 occurred in twenty four hours (Ayad *et al.*, 2002).

In milk (figure 3.20), 20 isolates presented slow acidification capacity, 13 presented medium acidification capacity and only one (isolate no. 102) presented rapid acidifying capacity.

In the present work, *Lactobacillus* was the genus more frequently isolated both in milk and cheese, thus it is likely that it is the prevalent genus in all the acidification capacity categories. From the isolates with slow acidification capacity, 16 of 24 (60 %) were *Lactobacillus*, 7 of 13 (53.8 %) of the isolates with medium acidification capacity were *Lactobacillus* and the only isolate which presented fast acidification capacity belonged to the genus *Lactobacillus* also.

Relative to isolates with medium acidification capacity, *Lactobacillus* (7 isolates) were followed by *Streptococcus* (2) and *Pediococcus* (2), both with 15.4 % and *Lactococcus* (1) and *Leuconostoc* (1), both with 7.7 %.

Studies of Herreros *et al*. (2003) showed that *Leuconostoc* had significantly lower acidifying capacity than *Lactococcus lactis* subsp. *lactis*, which could be due to the fact that the capacity of *Leuconostoc* to metabolize lactose was much lower than those of *Lactococcus.*

In the present study, twelve strains of *Lactobacillus*, two of *Enterococcus*, three of *Lactococcus*, two of *Aerococcus* and one of *Carnobacterium* isolated from milk samples showed slow acidification capacity, presenting a pH variation superior to 0.4 pH units only at 24 hours.

Studies of Suzzi *et al*. (2000) showed that *Enterococcus faecalis* isolated from an artisanal Italian goat cheese presented slow acidifying activity, being able to lower the pH after 24 h fermentation in agreement with results from this work.

Results by Perez *et al*. (2003) and Nuñez *et al*. (1981) seems to be opposite to the results of the present work, since the first showed that *Lactococcus* isolated from Tenerife cheese presented the highest acidifying activity in milk and the former

suggested that strains of *Lactococcus lactis* subsp. *lactis*, could be used as starter culture in cheese manufacture due their acidification capacity. However, although studies by Herreros *et al*. (2003) stated that a strain of *Lactococcus lactis* subsp. *lactis* isolated from Armada cheese showed a higher acidifying capacity than *Lactobacillus* strains, with the pH of the culture medium decreasing to values lower than 4.2, they also stated that some strains of *Lactobacillus casei* subsp. *casei,* were developing acidity as rapidly as some *Lactococcus*, after 24 h of incubation. This observation could be due to the fact that *Lactobacillus* spp. were able to metabolize lactose more slowly than *Lactococcus* but the final acid production could be similar to, or even higher than, that of *Lactococcus.*

Results for isolates from cheese (Figure 3.21), showed that there were more isolates with fast acidification capacity (5 isolates), than in milk (only one). Two (40 %) of these isolates belonged to the *Lactobacillus* genus, followed by *Pediococcus*, *Aerococcus* and *Leuconostoc* both representing 20 %. There were 8 isolates with medium acidification capacity, being distributed between several genera with no particular preponderance for any one. *Lactobacillus*, *Lactococcus*, both with 25 %, *Pediococcus, Leuconostoc*, *Enterococcus* and *Streptococcus*, both with 12.5 %. There were 9 isolates with slow acidification capacity, 66.7 % (6) *Lactobacillus*, and 33.3 % (3) *Pediococcus*.

Studies by Huggins & Sandine (1984) showed that none of *Lactococcus lactis* subsp. *lactis* strains could be characterized as fast, which is in accordance with the present study.

Weak production of acid by *Lactococcus* isolated from raw-milk cheeses has been reported by other researchers such as Devoyod & Muller (1969) and Nuñez & Medina (1979).

Studies by Durlu-Ozkaya *et al*. (2001) showed that different *Lactobacillus* strains showed different ability to reduce the pH of milk at the beginning of the process and there were strains that did not change the pH of milk in 6 h, but were able to after 24 h of incubation.

Dorlu-ozkaya *et al*. (2001) also concluded that the acidifying capacity of *Enterococcus* at 30 °C is, generally, low. Nevertheless, the performance of *Enterococcus* was, most of the time, higher than that of *Lactobacillus* within 6 h, but

there was a tendency for the strains to become slow after 5 h, which could explain the medium capacity observed in this work.

Strains of *E. faecium* and *E. faecalis* have been reported to degrade lactose in caprine milk more slowly than *Lactobacillus paracasei*, which is itself a slow acidifier (Freitas *et al*., 1999).

Although *Enterococcus* strains were poor acidifiers in milk, and therefore of little importance as starter organisms, they may be useful as adjunct cultures (Sarantinopoulos *et al*., 2001). These cultures are constituted by microorganisms without good acidification capacity that are added to the starter because they posses other properties which are important for cheese ripening like bacteriocin production, proteolytic or lipolytic activities. They are frequently called non starter lactic acid bacteria (NSLAB).

Studies done by Cogan *et al*. (1997) showed that *Leuconostoc* and *Lactococcus* isolated from different cheeses made from goat milk were poor acid producers. Nevertheless, Perez *et al*. (2003) showed that most *Lactobacillus* and *Leuconostoc* were weak acid producers, but *Lactococcus* and particularly *L. lactis* ssp. *lactis* showed the highest acidifying activity in milk. This means that different species of the same genus have different behaviour relatively to the capacity to produce acid conditions.

To design a starter culture to be use in cheese made with milk from algarvian goat breed strains with fast acidification capacity should be chosen in order to accelerate the coagulation made by the vegetable coagulant. It seems that the isolates referred as 102, Q1 and Q16 presented very good acidification characteristics to be used as starter microorganisms in this cheese.

4.7.3. Proteolysis

Studies from Perez *et al*. (2003) showed that among the genera *Lactococcus*, *Lactobacillus* and *Leuconostoc* isolated from Tenerife cheese, *Lactococcus* presented the highest proteolytic activity with six of the seventeen strains tested producing an increase in absorbance >0.1 at 340 nm. In accordance with these results, in the present study 75 % of *Lactococcus* showed values higher than 0.1 OD. *Lactobacillus* also

presented higher activities, but only 80 % of the tested strains presented values higher then 0.1 OD.

Streptococcus and *Enterococcus* presented similar proteolytic activity (both <0.1), which is in accordance with the findings of Dorlu-Ozkaya *et al*. (2001) who, in particular, showed that the proteolytic activity of *Enterococcus* during growth in milk is comparable, in general, to that of *Streptococcus thermophilus*.

Although in this study identification was just conducted at the genus level, the fact that almost all *Enterococcus* presented proteolytic activity, is in accordance with studies of Mucchetti *et al*. (1982) in which *E. faecalis* isolates were shown to possess high proteolytic activity, and in particular the variety *Enterococcus faecalis* var. *faecalis*. Other *Enterococcus*, like *E. faecium* possessed a weak proteolytic activity. These properties together with the fact that they could play an important role in providing the typical flavour of the cheese makes *Enterococcus* possibly useful for technological applications in food fermentation. The typical flavour is a result of citrate metabolism and lipolytic activity, which are very characteristic in strains of *E. faecalis* var. *liquefaciens* (Schdmit & Lenoir, 1972; Muchetti *et al*., 1982).

Studies conducted by Cogan *et al*. (1997) from Fontina cheese, showed that the most proteolytic organisms were *Streptococcus thermophilus* strains followed by *Enterococcus* isolates from Fiore Sardo and Toma cheeses and thermophilic *Lactobacillus* from Grana, Gioddu, Toma, Scotta innesto and Fontina cheeses.

Although only two isolates of *Leuconostoc* were tested in the present study, the differences in their proteolytic activity were in accordance with Herreros *et al*. (2003) who indicated that there were significant differences between the values of proteolytic activity for *Leuconostoc* strains. Additionally, the proteolytic activity of the genera *Leuconostoc* was inferior to that of the majority of *Lactococcus*, but higher than that of the *Lactobacillus* (Herreros *et al*., 2003).

The data reported here suggest that there was no relationship between the proteolytic and acidifying activities of the strains, as also suggested by Fontina *et al*. (1998) for strains of *Lactobacillus.* The strains with the strongest acidifying abilities (e.g. 102, Q1, Q18) did not exhibit the highest proteolytic activity (Q132, Q105) and there were strains with very low acidifying but high proteolytic activity (e.g. 13) and strains with high acidifying and low proteolytic activity (for example, 102, Q8).

To produce a mix of microorganisms for use as starter culture the isolates that present the highest proteolytic activities should be chosen. Thus the isolates referred as Q132 and Q105 could be good choices. In addition to this Q132 produced CO_2 which can contribute to the formation of little holes in the cheese mass as in the traditional texture. However more studies should be done in order to verify that they are not too much proteolytic being able to hydrolyse proteins before coagulation occurs, which will stop the cheese production.

4.7.4. Lipolytic Activity

An increase in the concentration of free fatty acids during ripening has a great importance for the cheese taste. A determined concentration of free fatty acids is necessary for an optimum taste, particularly when they are in a correct equilibrium with products from proteolytic reactions or other reactions (McSweeney & Sousa, 2000).

It is generally accepted that LAB have low lipolytic activity, nevertheless some authors like Freitas *et al*. (1999) demonstrated that some strains of *Lactobacillus* and *Lactococcus* had lipolytic enzymes, which are able to hydrolyse the milk fat.

This low lipolytic activity of LAB could represent an important advantage when they are used as starter cultures, since a slight breaking of the milk fat during maturation is enough to induce aroma production without giving a rancid flavour in cheese (McSweeney & Sousa, 2000).

Authors like Mucchetti *et al*. (1982), and Cogan *et al*. (1997) suggested that *Enterococcus* play an important role in the development of the typical flavour of the cheese, as a result of their lipolytic activity, but no isolate identified as *Enterococcus*, in this study, presented any signs of this activity. Centeno *et al*. (1996) reported that lipolytic activity was variable between different strains and most *Enterococcus* isolates exhibited low lipolytic activity with few exceptions. Perez *et al*. (2003) studied 130 isolates from Tenerife cheese and none of these showed any lipolytic activity on solid media.

Studies conducted by Menéndez *et al*. (2001) showed that lipase activities were very low, or not detected, for most strains of *Lactococcus* and Herreros *et al*. (2003)

showed that lipase activity was` not detected, or were very low, in *Leuconostoc* strains.

Studies conducted by Herreros *et al*. (2003) shows that no lipase activity was detected for the strains of *L. plantarum*, although some strains of *L. casei* subsp. *casei* exhibited a very low lipase activity. Menéndez *et al*. (2001) detected weak lipase activity in most strains of *Lactobacillus* tested. Certain strains of *Lactobacillus* can contribute to cheese lipolysis when releasing intracellular lipolytic enzymes during autolysis (Khalid & Marth, 1990). These enzymes contribute to an increase in the concentration of free fatty acids in cheese which, in low concentrations, contribute to the flavour of cheese, particularly when they are correctly balanced with the products of proteolysis (McSweeney & Sousa, 2000).

Villani & Coppola (1994) found that LAB showed low lipolytic activity when grown in whole milk and suggested that this could signify substrate specificity. This could be the reason for there being only one isolate showing lipolytic activity in this work.

4.7.5. Possible combination of microorganism's to produce a native starter in the future

Different combinations of microorganisms should be tested in cheese production and ripening at a pilot scale in order to determine which the best are. It will be interesting to test combinations of strains which presented fast acidifying capacity and elevated proteolytic and lipolytic capacities. Table 4.1 present suggestions for combinations of microorganisms to design a native starter.

Table 4.1. Constitution of native starters for testing in cheese production

Batch	Acidifying strain	Proteolytic strain	Lipolytic strain
A	102 - *Lactobacillus*	Q132 - *Leuconostoc*	Q163 - *Pediococcus*
B	102 - *Lactobacillus*	Q105 - *Lactobacillus*	Q163 - *Pediococcus*
C	Q1 - *Lactobacillus*	Q132 - *Leuconostoc*	Q163 - *Pediococcus*
D	Q1 - *Lactobacillus*	Q105 - *Lactobacillus*	Q163 - *Pediococcus*
E	Q16 - *Aerococcus*	Q132 - *Leuconostoc*	Q163 - *Pediococcus*
F	Q16 - *Aerococcus*	Q105 - *Lactobacillus*	Q163 - *Pediococcus*
G	102 - *Lactobacillus*		Q163 - *Pediococcus*
H	Q1 - *Lactobacillus*		Q163 - *Pediococcus*
I	Q16 - *Aerococcus*		Q163 - *Pediococcus*
J	102 - *Lactobacillus*	Q105 - *Lactobacillus*	-

Batch	Acidifying strain	Proteolytic strain	Lipolytic strain
K	Q1 - *Lactobacillus*	Q105 - *Lactobacillus*	-
L	Q16 - *Aerococcus*	Q105 - *Lactobacillus*	-

The microorganisms chosen for tests in cheese production at a pilot scale have different characteristics that are important to be present in a starter for cheese production. The isolates referred as 102, Q1 and Q16 were those that presented the fast acidification capacity and Q1 presented bacteriocin production which can help with the safety of the cheese. Q105 presented the highest proteolytic capacity and Q132 presented CO_2 production (to form holes in cheese) besides high proteolytic capacity. Q163 was the only that presented lipolytic capacity among all the isolates of this study.

The batches G, H and I (without proteolytic strains) allows the study of possible synergetic effects between the microorganisms of the native starter in the medium acidification as observed by Lee *et al*. (1990), Lynch *et al*. (1997) and Hynes *et al*. (2003). Lynch *et al*. (1997) studied the contribution of the different strains of lactobacilli added as adjunct cultures in cheese ripening of cheddar cheese produced in aseptic conditions. These authors found lower pH values (0.2-0.3 units) in the cheeses with adjunct lactobacilli, which could be explain by the lactose residual metabolism (producing lactic acid) presented by lactobacilli. A lower acidification retard the whey drainage (Stanley, 1998), but the absence of a proteolytic strain could negatively influences the alkaline compounds released, stopping the partial neutralization of cheese pH.

The sensory analysis of certain cheeses shows that certain combinations of microorganisms cause defects like absence of typical taste, bitterness and dry texture (Requena *et al.*, 1992 and González-Crespo & Mas, 1993). These authors related de appearing of these defects with the microorganism's higher acidifying capacity. Lee *et al*. (1996) found that certain microbial combinations could cause a higher decrease in pH which could lead to bitterness formation, off flavours and texture problems (higher friability).

The batches J, K and L (without lipolytic strains) are useful to compare the effect of the lipolytic strain in the ripening process from the sensorial point of view, since the presence of certain lipolytic strains can cause rancidity. On the other hand, it

permits testing of the interaction between two strains of lactobacilli (batches J and K). Some authors showed that the presence of more than one strain of lactobacilli could increase the aminoacids concentration in cheddar cheese (Lane & Fox, 1996, Lynch *et al.*, 1997) Tetilla (Menédez *et al.*, 2004) and Sant-Paulin (Hynes *et al.*, 2003), stressing the important contribution from lactobacilli peptidase in the release of aminoacids.

Nevertheless, before cheese production occur in a pilot plant scale; compatibilities of microorganisms should be tested because it is important that none of the strains have an inhibitory effect over other present in the same starter.

After the production of cheeses with the chosen combinations of microorganisms, they should be subject to sensorial evaluation in order to select the combination which gives to the cheese the typical characteristics of algarvian goat breed cheese. Soft 'goat-like' taste, cream-colored and firm mass with very small holes.

Chapter 5. Conclusion

From the present work it can be concluded that:

● Traditional boiling of the milk did not affect the milk gross chemical composition, which permits to use these treatement in cheese production increasing the product safety. This safety product could maintain its traditional characteristics if native starters are added to the milk in the cheese production process.

The milk and cheese gross chemical composition was practically constant during all the lactation period which contributed to maintain a regular quality in cheeses throughout all the production period. This uniformity of cheeses is important for consumer acceptance, since consumers hope to find always the same product with similar characteristics.

These findings can contribute to the utilization of boiled milk with native starters, increasing the safety of the product and the cheese production as more milk can be used since the microbiological criteria for milk to be heat treated are less exigent.

● In consideration of the fact that the cardoon is added to the milk after boiling, its microbiota could cause safety problems.

The microbiota present in the studied cardoon doesn't represent a spoilage problem during its storage, considering the low a_w of this type of material, but isolated sporulated forms could represent a safety problem when the cardoon is used.

From the studied cardoon types "Producer cardoon" and "Tondela" were those which presented lower microbial counts, thus these two types of cardoon seems to be more adequate for cheese making by the traditional process. The lower the contamination of cardoon, the higher is the safety of the cheese. The knowledge of the microbiota of the cardoon allows establishment of control conditions for the collection, drying and transport of this vegetable coagulant.

● There were no significant differences in the studied microbiota of raw milk and cheese immediately after processing (made from raw milk) during the studied period (from May to July), which means that this product did not show important variations in its microbiological quality. This microbiota regularity in addition to the uniformity in gross chemical composition indicates that it is possible to produce cheeses of similar quality during all the production period, which is very important to the consumer.

● During the ripening period there was a decrease of faecal coliforms, which disappeared by the 21st day of ripening, indicating a safe food product from this day until the end of the study.

In the beginning of the ripening period the moulds identified were from genus *Aspergillus, Penicillium, Chaetomium* and *Phoma*, but after two weeks only *Penicilium* was detected. The capacity of *Penicilium* to resist the cheese conditions of ripening, with a decrease of pH and a_w and a possible presence of its mycotoxins, could be a safety problem.

● After the identification of the lactic acid bacteria isolated from raw milk obtained during the lactation period it was found that *Lactococcus* and *Lactobacillus* were the prevalent groups, followed by *Enterococcus* (present in most of the samples) and *Aerococcus* (both present at almost all stages of lactation), with the other studied groups being the minority. During ripening *Lactobacillus* formed the prevalent group, followed by *Enterococcus* and *Pediococcus*, *Leuconostoc* and *Aerococcus* with *Lactococcus*/*Vagococcus* and *Streptococcus*, being the minority groups.

● Bacteriocin activity detection tests showed that 10 *Lactobacillus*, 1 *Enterococcus*, 1 *Pediococcus* and 2 *Lactococcus* isolated from milk presented this activity. From the isolates from cheese only 3 *Lactobacillus* and 2 *Pediococcus* presented bacteriocin activity. All the other isolated produced no bacteriocin.

Bacteriocin production could be an important characteristic, since it helps to destroy some pathogenic microorganisms thus contributing to the safety of cheese. This is a factor to have in account in the selection of strains to be used as starter microorganisms.

● Twenty four isolates from milk presented slow acidification capacity, fourteen presented medium acidification capacity and only one (102 - *Lactobacillus*) presented rapid acidifying capacity. Probably this one is a strong candidate to be part of a possible native starter, since the rapid decrease of pH accelerates the milk coagulation.

Both isolates with medium and slow acidification capacity could be used as part of the native starter also, but acting as non starter lactic acid bacteria (NSLAB) if they possess good proteolytic or lipolytic activities. In every case, compatibility studies

should be done to investigate the possibility of mixing the isolates in the same fermentation process.

In cheese, there were more isolates with fast acidification capacity (6 isolates), than in milk (only one). Half of these isolates belonged to the *Lactobacillus* genus, followed by *Pediococcus*, *Aerococcus* and *Leuconostoc.*

One of the *Pediococcus* isolate that present slow acidification capacity (Q163) presented lipolytic capacity and four of the *Lactobacillus* isolates also with slow acidification capacity (Q43, Q49, Q50, Q76) presented high proteolytic capacity, there are properties that can indicate that they can be used as NSLAB.

Lactobacillus was the genus more frequently isolated both in milk and cheese.

From all the isolates, *Lactobacillus* genus (Q1, Q132, Q67, Q45, 102), *Pediococcus* (Q18) and *Aerococcus* (Q16) showed the fast acidification capacity, which could be an indicator of good potential for their use as starter bacteria.

It is important to note that *Lactobacillus* Q1 produced bacteriocin which could destroy other added strains (disadvantage), but also some pathogenic organisms present in milk (advantage). Studies of compatibility should therefore be made, before choosing this strain as a starter. The other strains above mentioned didn't present any bacteriocin production.

● The isolates which presented more proteolytic activity were 17 (*Aerococcus*), Q132 (*Leuconostoc*), Q50 and Q105 (both *Lactobacillus*). These strains could be used as adjunct cultures due to the importance of proteolysis during cheese ripening.

The data reported here suggest that there was no relationship between the proteolytic and acidifying activities of the strains.

● No positive results for lipolytic activity were found in the isolates from milk and in the isolates from cheese, only one isolate showed lipolytic activity. The isolate referred as Q163 and identified as *Pediococcus*.

● The identification of the microorganisms and the knowledge of their technological properties is very important since it permits the investigation of different combinations of microorganisms that could be used as a native starter.

This starter should be composed of one isolate with fast acidifying capacity, one with high proteolytic activity and one with high lipolytic activity.

Isolates referred as 102 (*Lactobacillus*), Q1 (*Lactobacillus*) and Q16 (*Aerococcus*) were those that presented the fastest acidification capacity and Q1 presented bacteriocin production, which can help with the safety of the cheese. Q105 (*Lactobacillus*) presented the highest proteolytic capacity, Q132 (*Leuconostoc*) presented CO_2 production (to form holes in cheese) and high proteolytic capacity. Q163 (*Pediococcus*) was the only that presented lipolytic capacity among all the isolates of this study. It can be therefore being suggested that different batches made up of different combinations of these microorganisms could be trialled for cheese production. Since only one isolate showed lipolytic activity (Q163) it can be suggested that it is combined with other two isolates, as for example (Q163+Q132+102). Q132 has high proteolytic and CO_2 producer and 102 has a fast acidification capacity. Nevertheless, before cheese production occurs in a pilot plant scale compatibilities of microorganisms should be tested because it is important that none of the strains have an inhibitory effect over others present in the same starter.

After the production of cheeses with the chosen combinations of microorganisms, they should be subject to sensorial evaluation in order to select the combination which gives to the cheese the typical characteristics of algarvian goat breed cheese (soft 'goat-like' taste, cream-colored and firm mass with very small holes).

● Considering the lack of published data about milk and cheese from the algarvian goat breed and the necessity to increase the traditional production in the interior region of Algarve, the results obtained in this work are very important in order to encourage production through the improvement of milk and cheese quality. This improvement will be achieved by the utilization of heat treated milk (more safe) and native starters to maintain the traditional organoleptic characteristics. In this work the native microbiota was characterized and some combinations of microorganisms were suggested in order to produce a native starter in the future.

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Chapter 6. Suggestions for Future Work

● The identification of microorganisms only based on phenotypic characteristics and biochemical analysis is not complete and so, representative strains of the different genera isolated should be subject, in near future to more reliable and sophisticated techniques in order to identify them to species level and thus better estimate the extent of biodiversity present among the isolates from the algarvian goat breed milk and cheese.

● The isolation and characterization of LAB conducted in this work will contribute to the selection of those with good technological capabilities for cheese manufacture using heat treated milk, but further research of their pathogenesis before using them in pilot plant production is needed.

● The technological properties of yeasts, their positive microbial interactions and inhibitory effects against spoilage organisms should ensure successful application of appropriately selected strains as starters. So, yeasts could also be isolated from milk, cheese during ripening and cardoon and tested for use as starter cultures in production of cheese from milk of the algarvian goat breed.

● After bacteria and yeasts with better technological characteristics are chosen and the tests for patogeneicity are done, tests of compatibility should be done also. These tests are important to avoid species with the capacity to destroy each other, through bacteriocin production for instance, when used in the same starter.

● After all the referred tests, batches of cheeses with different combinations of microorganisms could be produced. These cheese batches should be analysed for biochemical, microbiological and organoleptic characteristics in order to choose the best combination to be used as starter.

● To disseminate the results of this work through the ANCRAAL (Associação Nacional de Criadores de Raça Algarvia), which is an association of goat producers from algarvian goat breed.

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Apendix A

A.I. Poster presentations

I. Ratão; M. Costa & L. Dionísio. 2006. Microbial Quality of Goat Milk for Cheese Processing. *CEFood2006*. Sofia. BULGUERIA.

S. Broa, I. Ratão, & L. Dionísio. 2007. Enumeration of Total Microbiota, Yeasts and Moulds in Different Types of Cardoons. *II International Conference of Environmental, Industrial and Applied Microbiology - BioMicroWorld2007*. pag 330. Sevilha. SPAIN.

I. Ratão, R. Matos & L. Dionísio. 2008. Microbiological Monitoring of Cheese Made From Algarvian Goat Milk. *Foodmicro2008*. Aberdeen. SCOTLAND.

I. Ratão & L. Dionísio. 2008. Contribution of cardoon to the microbiological quality of goat cheese, made from pasteurized milk. *Foodmicro2008.* Aberdeen. SCOTLAND.

I. Ratão; R. Matos & L. Dionísio. 2008. Microbiological Monitoring of Cheese Made From Algarvian Goat Milk During Frozen and Ripening Period. P44. *4th symposium of Foodprotection* . Lisboa. PORTUGAL.

I. Ratão; A. Anastácio & L. Dionísio. 2009. Design of control charts to monitor the microbiological quality of raw milk from Algarvian goat breed. *4th IDF Dairy Science and Technology Week*. Pag 99. Rennes. FRANCE.

I. Ratão & L. Dionísio. 2009. Chemical monitoring of milk and cheese from an Algarvian Goat breed. *World Dairy Summit 2009*. Pag 99. Berlim. GERMANY.

I. Ratão, D. Guerreiro & L. Dionísio. 2009. Microbiological Changes in Cheese of Algarvian Goat Breed During Ripening. *BioMicroWorld2009. III International Conference on Environmental, Industrial and Applied Microbiology.* Pag. 319. Lisboa. PORTUGAL.

MICROBIAL QUALITY OF GOAT MILK FOR CHEESE PROCESSING

I. RATÃO(a,b), M. COSTA(a) and L. DIONÍSIO (a)
(a)Natural Resources Engineering Faculty, University of Algarve. Campus de Gambelas, 8000-139 Faro, Portugal
Tel./Fax: + 351 289 800 040/+ 351 289 818 419, E-mail: mecosta@ualg.pt
(b)Superior School of Technology, University of Algarve. Campus da Penha, 8005-139 Faro, Portugal
Tel./Fax: +351 289 800 151/+ 351 289 888 405, E-mail: iratao@ualg.pt

Introduction

It is known that the physical-chemical characteristics of milk vary during the lactation period (Maio, 1989). One of the objectives of this study is to monitor the microbiological characteristics along this period.

Material and methods

Total heterotrophic microbiota, *Lactobacillus*, faecal coliforms, moulds and yeasts were determined in raw goat milk and cheese obtained from May to July of 2005.

Total heterotrophic microorganisms were enumerated in Plate Count Agar (30±1 °C, 48 h); *Lactobacillus* in Rogosa Agar (36±1 °C, 48 h) and moulds and yeasts on Cook Rose Bengal (25±0.5 °C, 5 days).

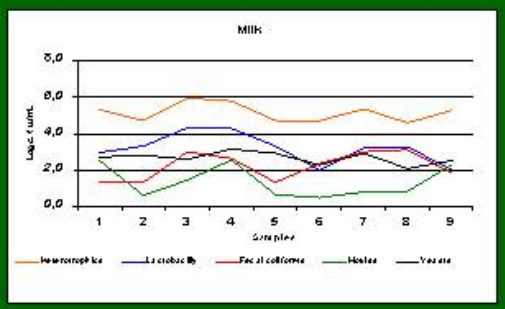
Fecal coliforms had been cultivated in anaerobic conditions in Violet Red Bile Agar (44.5±0.5 °C, 48 h).

The crude extract from thistle flower was prepared as an aqueous extract at 15 % (w/v) and it was kept frozen and used directly for milk coagulation.

Results and discussion

Figures 1 and 2 show that there are no sig[illegible]s in the studied microbiota along the studied period.

Yeasts and moulds concentrations were almost the same in milk and cheese.



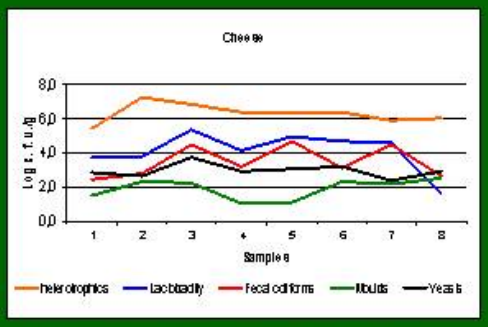


Fig. 2 Cheese microbiota observed during the studied period

Fecal coliforms and *Lactobacillus* also increased approximately one order of magnitude from milk to cheese. This increase could be due to the elapsed time from the milk clotting and the beginning of the analysis (approximately 24 hours), as the milk gives a good support for their development due to its high lactose content.

The variation from 10^5 to 10^7 c.f.u./mL in the total heterotrophic enumeration could be explained by the sum of the individual variation of the species that compose it.

Conclusion and future developments

The studied microbiota did not show significa[illegible] during the sampled period. However, it is intended to do more trials, using boiled milk, thus eliminating original microbiota from raw material and to verify in which extension the use of thistle suspensions without any treatments contributes for the microbiological contamination of the goat cheeses. Physical and chemical parameters from milk and cheese will be analysed to compare with milk and cheese obtained from traditional production methods.

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ENUMERATION OF TOTAL MICROBIOTA, YEASTS AND MOULDS IN DIFFERENT TYPES OF CARDOONS

S. BROA[1], I. RATÃO[1,2] and L. DIONÍSIO[1]

[1]Natural Resources Engineering Faculty, University of Algarve. Campus de Gambelas, 8005-139 Faro, Portugal
Tel./Fax: + 351 289 800 040/+ 351 289 818 419, E-mail: ldionis@ualg.pt

[2]Superior School of Technology, University of Algarve. Campus da Penha, 8005-139 Faro, Portugal
Tel./Fax: +351 289 800 151/+ 351 289 888 405, E-mail: iratao@ualg.pt

Introduction

Cynara cardunculus, common designed by cardoon, is a plant from the *Asteracea* family, a prickly variety of thistle similar to the globe artichoke[1], which aqueous extracts of the flowers have been successful used for ages in the manufacture of goat's milk cheeses in several rural areas of Portugal, like Algarve[2].

Cardoon is considered one of the factors of major importance for cheese quality. Some of them has a Protected Origin Denomination (POD), and the legislation determine the use of this vegetable clotting agent in the cheese making process[3].

The main goal of this work was to understand the contribution of *Cynara cardunculus* as a contamination source during the cheese making process, since it is added after the heat treatment of the milk.

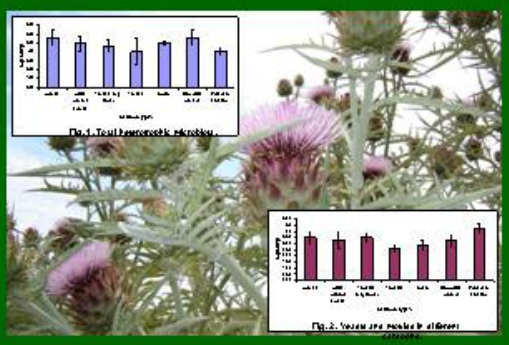
Material and methods

Seven types of cardoon, were analysed. All assays were done in triplicate.

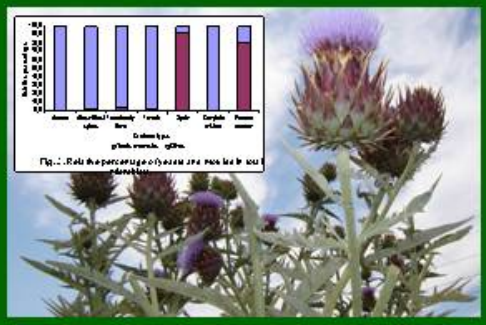
Total heterotrophic microbiota was enumerated in Plate Count Agar medium (Biokar) at 30±1 °C, during 48 h, by incorporation technique. For moulds and yeasts Cook Rose Bengal medium (Biokar) was used at 25±0.5 °C, for 5 days, by spreading technique.

Results and discussion

As it can be observed in figure 1, the total heterotrophic microbiota varied from log 4.01 to log 5.59, in different types of cardoon samples.



Since this kind of material has a very low water activity, approximately 0.6[3], microorganisms face problems to survive, unless they are sporeformers, like moulds, yeasts and others. As it can be seen in figure 2, the number of yeasts and moulds varied from log 2.57 to log 4.24 cfu/g. The relation between moulds and yeasts and other microorganisms could be observed in figure 3.



The total amount of microorganisms found in this kind of material could represent an important source of contamination, when used for cheese production. Studies should be done in order to find the best methods to eliminate or reduce this contamination. In near future suspension of thistle flowers will be submitted to UV radiation, ozone and/or electrolysed water to reduce total microbiota. Studies of enzymatic activity should be conducted to clarify the effects of these treatments in the enzymatic activity and subsequent clotting capacity of cardoon.

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MICROBIOLOGICAL MONITORING OF CHEESE MADE FROM ALGARVIAN GOAT MILK

I. RATÃO[1,2], R. MATOS[1] and L. DIONÍSIO[1]

[1]Natural Resources Engineering Faculty, University of Algarve. Campus de Gambelas, 8005-139 Faro, Portugal
Tel./Fax: + 351 289 800 040/+ 351 289 818 419, E-mail: ldionis@ualg.pt
[2]Superior School of Technology, University of Algarve. Campus da Penha, 8005-139 Faro, Portugal
Tel./Fax: +351 289 800 151/+ 351 289 888 405, E-mail: iratao@ualg.pt

Introduction

Goat milk cheese is one of the traditional products from Algarve (Portugal). This cheese is produced with milk from Algarvian breed goats (Fig. 1) and is coagulated[1] by aqueous extracts from cardoon flowers (*Cynara cardunculus)* (Fig. 2), a plant from the *Asteracea* family, a prickly variety of thistle similar to the globe artichoke[2]

Goat cheeses from Algarve are usually produced under artisanal conditions and is handled at various stages of manufacture, which may permit several contamination during manufacture procedures.

The main goal of this work was to monitor the microbial variation of the milk and the cheese along the maturation process in order to improve the hygienic conditions and procedures for this particular cheese making.

Material and methods

Total heterotrophic microbiota was enumerated in Plate Count Agar medium (30±1 °C, 48 h), by incorporation technique; *Lactobacillus* in Rogosa Agar (36±1 °C, 48 h), by incorporation technique, and moulds and yeasts on Cook Rose Bengal (25±0.5 °C, 5 days), by spread technique. Total and Fecal coliforms had been cultivated in anaerobic conditions in Violet Red Bile Agar (30±1 °C , 48 h and 44.5±0.5 °C, 48 h, respectively), by incorporation technique.

All assays were done in triplicate.

Fig. 1. Algarvian breed goats.

Fig. 2. Cardoon (*Cynara cardunculus*).

Results and discussion

In Figure 3, it can be observed the variation of the total heterotrophic microbiota, Lactobacilli, yeasts and moulds, as well the total and faecal coliforms from milk and cheese along the ripening process.

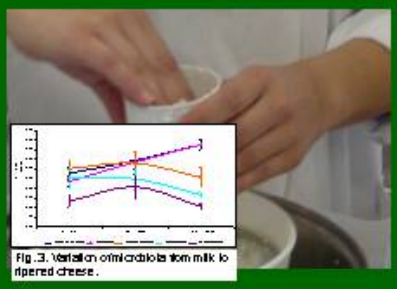

Fig .3. Variation of microbiota from milk to ripened cheese.

Table 1 shows the relationship between the studied microrganisms.

Table 1. Relationship between the studied organisms

As it can be seen, the dominant microbiota in raw milk and fresh cheese are the yeasts and moulds, but after the ripening period, the lactobacilli overcome their number.

Conclusion and future developments

The total amount of the studied microorganisms showed an increase along the ripening process. During this period all the species suffered a significant reduction, except lactobacilli, which became the dominant specie.

The knowledge of the degree of the decaying of each microorganism present in the milk could be a useful tool to predict the quality of the final product and to decide which measures and decisions should be taken about the raw materials.

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Contribution of cardoon to the microbiological quality of goat cheese, made from pasteurized milk

I. RATÃO[1,2] and L. DIONÍSIO[1]
[1]Natural Resources Engineering Faculty, University of Algarve. Campus de Gambelas, 8005-139 Faro, Portugal
Tel./Fax: + 351 289 800 040/+ 351 289 818 419, E-mail: ldionis@ualg.pt
[2]Superior School of Technology, University of Algarve. Campus da Penha, 8005-139 Faro, Portugal
Tel./Fax: +351 289 800 1514/+ 351 289 888 405, E-mail: iratao@ualg.pt

Introduction

Cynara cardunculus, common designed by cardoon (Fig. 1), is a plant from the *Asteracea* family, a prickly variety of thistle similar to the globe artichoke[1]. The aqueous extracts of the flowers have been successful used for ages in the manufacture of goat's milk cheeses in several rural areas of Portugal, like Algarve[2].

Cardoon is considered one of the factors of major importance for cheese quality. Some of them has a POD (Protected Origin Denomination), and the respectively legislation determined the use of this vegetable clotting agent in the cheese making process[3].

The main goal of this work was to study the existent microbiota in the aqueous extract of *Cynara cardunculus* used in cheese making process.

Material and methods

Seven types of cardoon samples were analysed. Total heterotrophic microbiota was enumerated by incorporation technique, in Plate Count Agar medium (Biokar) and incubated at 30±1 °C, during 48 h. For moulds and yeasts Cook Rose Bengal medium (Biokar) was used, and incubated at 25±0.5 °C, during 5 days, by spreading technique. Faecal coliforms were determined using Mfc Agar medium (Biokar), and incubated at 44.5±0.5 °C, during 48 h. All trials were done in triplicate.

Results and discussion

Figure 2, showed the enumeration of the total heterotrophic microbiota which varied from 4.9 to 6.5 log cfu/g; yeasts and moulds varied from 3.4 and 5.1 log and Faecal coliforms from 0 to 2 log.

Since this kind of material has a very low water activity, approximately 0.6[3], most of the microrganisms have problems to survive, unless they are sporeformers, like moulds and yeasts.

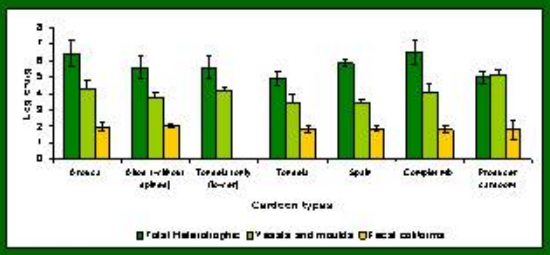

Fig. 2. Microbiota in the different studied cardoons.

However colonies like faecal coliforms were detected, which lead to conclude that sporeformers microorganisms grew in the used medium. These micoorganisms were Gram negative and able to ferment lactose in order to acidify the medium, which is revealed by the blue coloration of the colonies.

Conclusion and future developments

Other microorganisms, beside coliforms can growth in Mfc Agar. There is a need to implement other analytical methods, such as molecular biology, to identify such microorganisms in order to be sure of their real impact in the safety of the final product.

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MICROBIOLOGICAL MONITORING OF CHEESE MADE FROM ALGARVIAN GOAT MILK DURING FROZEN AND RIPENING PERIOD

I. RATÃO[1,2], R. MATOS[1] and L. DIONÍSIO[1]

[1]Natural Resource s Engineering Faculty, University of Algarve. Campus de Gambelas, 8005-139 Faro, Portugal
Tel./Fax: + 351 289 800 040/+ 351 289 818 419, E-mail: ldionis@ualg.pt

[2]Superior School of Technology, University of Algarve. Campus da Penha, 8005-139 Faro, Portugal
Tel./Fax: +351 289 800 1514/+ 351 289 888 405, E-mail: lratao@ualg.pt

Introduction

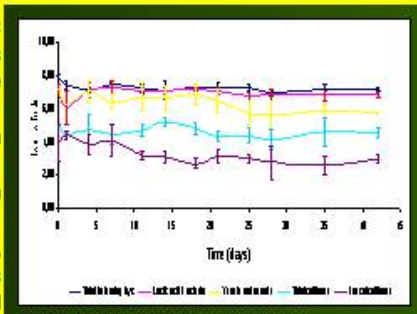
Goat milk cheese is one of the traditional products from Algarve, in Portugal. It is produced with milk from Algarvian breed goats and is coagulated(1) by aqueous extracts from cardoon flowers (*Cynara cardunculus*), a prickly variety of thistle similar to the globe artichoke(2).

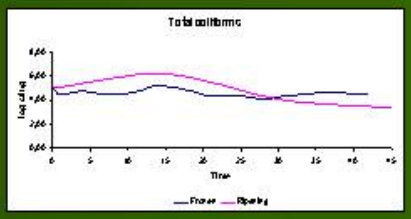
These cheeses are usually produced under artisanal conditions and is handled at various stages of manufacture, which may permit several contamination during manufacture procedures, and could have some influence during storage.

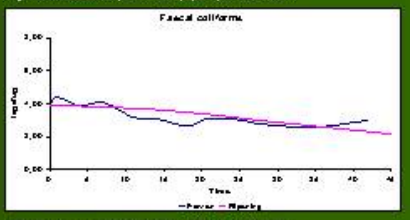
The main goal of this work was to monitor the microbial variation of the cheese along a determined period, both under frozen and maturation conditions, in order to verify which is the better way to maintain or improve microbiological

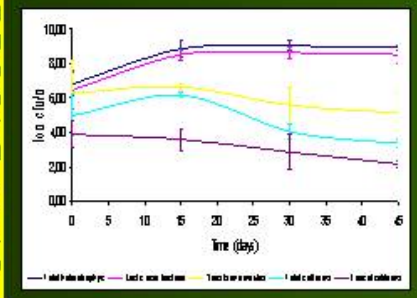
Material and methods

Total heterotrophic microbiota was enumerated in Plate Count Agar (30±1 °C, 48 h), by incorporation technique; Lactic Acid Bacteria (LAB) in Rogosa Agar (36±1 °C, 48 h), by incorporation technique, and moulds and yeasts on Cook Rose Bengal (25±0.5 °C, 5 days), by spread technique. Total and faecal coliforms had been cultivated in anaerobic conditions in Violet Red Bile Agar (30±1 °C , 48 h and 44.5±0.5 °C, 48 h, respectively), by incorporation technique. All assays were done in triplicate.









Results and discussion

The organoleptic characteristics of goat cheese changes along the ripening process due to microbial activity, among other factors.

After forty five days, samples under frozen conditions showed lower concentrations of total microorganisms when compared to the ripened ones (7.5 and 9.0 log cfu/g). Nevertheless, coliforms, which may cause safety problems, were lower for samples submitted to ripening (3.0 and 2.0 log cfu/g). These results should be due to the competition with LAB, which increased during the first period of the ripening process (from 6.5 to 8.5 log cfu/g), becoming the dominant specie.

Conclusion and future developments

In samples under ripening, coliforms showed a decrease of one to two orders of magnitude and a tendency to decrease. However, the ripening period could not be extended due to the excessively hardness acquired by this type of cheese.

These results suggested that the quality of the milk should be controlled before processing.

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DESIGN OF CONTROL CHARTS TO MONITOR THE MICROBIOLOGICAL CONTAMINATION OF RAW GOAT MILK FROM ALGARVIAN GOAT BREED

L RATÃO[1,2], A. ANASTÁCIO[2] and L. DIONÍSIO[1]

[1] Science and Technology Faculty, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal
Tel./Fax: + 351 289 800 940+ 351 289 818 419, E-mail: [illegible]

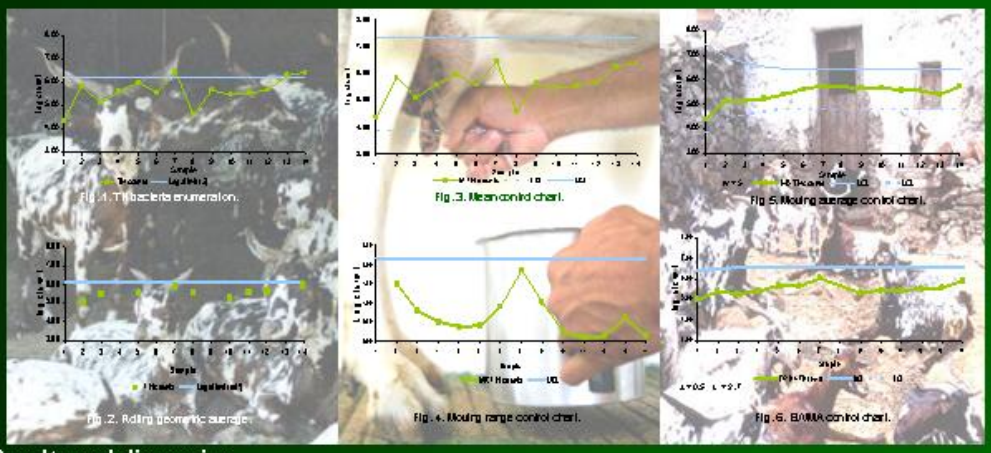
[2] Superior Institute of Engineering, University of Algarve, Campus da Penha, 8005-139 Faro, Portugal
Tel./Fax: +351 289 800 161+ 351 289 800 406, E-mail: [illegible]

Introduction

In Algarve region an artisan goat cheese is produced using milk from autochthonous goat breed, submitted to a thermal treatment. To ensure product hygiene and safety, the number of total heterotrophic (TH) bacteria in raw milk, must not exceed a specific limit. The use of control charts to monitor TH bacteria from samples collected at the bulk tank raw milk, at the farm level, may contribute to prevent unacceptable results.

Material and Methods

Samples of raw goat milk were collected from the bulk tank of a farm in Algarve, between October and June. TH bacteria was enumerated in Plate Count Agar medium (30±1 °C, 72 h), by incorporation technique. All assays were done in triplicate. Before performing statistical analysis, TH bacteria counts and rolling geometric average results were converted into decimal logarithms, since log expression was independent and normally distributed, as reported previously[1]. Four types of control charts for individual data were used: Mean (M) (Fig. 3), Moving Range (MR) (Fig. 4), Moving Average (MA) (Fig. 5) and Exponentially Weighted Moving Average (EWMA) (Fig. 6).



Results and discussion

TH bacterial enumeration (Fig. 1) was between 4.37 and 6.48 log cfu, with an arithmetic average and standard deviation of 5.62 and 0.61 log cfu/ml, respectively. According to the European Regulation (EC) No 853/2004[2], the rolling geometric average of TH counts for goat raw milk used to produce heat-treated products must not exceed 6.18 log cfu/ml, considering periods of 60 days. Number of samples under this limit were 79 for individual samples and 100 % for rolling geometric average samples (Fig 2). Upper control limits of M and MR were 7.36 and 2.14 log cfu/mL (0.66 standard deviation). For MA and EWMA (large shift detection[3]) charts, the limits were 6.44 and 6.57, respectively. These results showed that there are not out of control points.

Conclusion

Since milk hygiene quality assessment involve raw data transformation using geometric moving average, a control chart where smoothing of TH counts is used, like MA chart, is more user-friendly by the farmer.

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CHEMICAL MONITORING OF MILK AND CHEESE FROM ALGARVIAN GOAT BREED

I. RATÃO[1,2], and L. DIONÍSIO[1]

[1]Science and Technology Faculty (CDCTPV), University of Algarve. Campus de Gambelas, 8005-139 Faro, Portugal
Tel./Fax: + 351 289 800 040/+ 351 289 818 419, E-mail: ldionis@usig.pt

[2]Superior Institute of Engineering, University of Algarve. Campus da Penha, 8005-139 Faro, Portugal
Tel./Fax: + 351 289 800 151/+ 351 289 888 405, E-mail: iratao@usig.pt

Introduction

A traditional cheese from Algarve region (Portugal) is produced with milk from an autochthonous breed of goats (Fig 1) and it is coagulated by aqueous extracts from cardoon thistle flowers (*Cynara cardunculus*).

These cheeses are usually produced under artisanal conditions and in small quantities, but are an important income to the rural population of the interior part of Algarve. In order to improve its quality and increase the income of the producers it is important to know their characteristics.

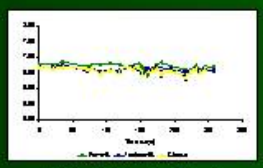
Material and methods

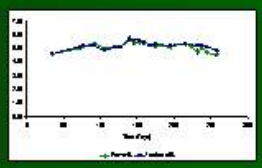
Milk from lactating Algarvian goat breed was collected at Messines (Algarve), along a lactation period (from October to July) and was sampled twice a month. Also samples of cheese are taken in the same days, immediately after its production.

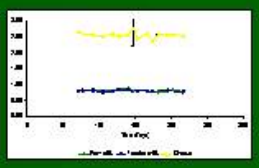
Chemical analyses of milk: pH, conductivity and sodium chloride (NaCl) by potentiometer. Aches were determined by AOAC[1] official method 945.46 for milk, total solids by AOAC 990.19 and fat by Gerber method[2].

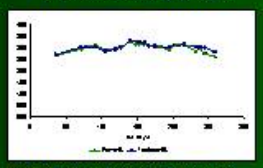
Chemical analyses of Cheese: pH by potentiometer, aches by AOAC official method 935.42 for cheese, total solids and fat, by Pearsons's[3] chemical analysis book method.

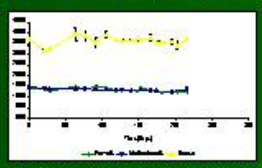
Results and discussion

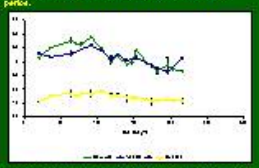












In milk, the pH (Fig.2), conductivity (Fig.3) and aches (Fig. 4) values presented no variation during the studied period. Sodium chloride (Fig. 5) was maintained practically constant during all the lactation period, but slightly superior in treated milk, may be due to the loss of water during the thermal treatment. Total solids (Fig. 6), didn't present any tendency for variation too. In accordance to previous studies[4] with other milk type, the fat quantity (Fig. 7) varied along the lactation period. It rises from 4.3 in October to 5.8 in February and than it starts decreasing to 3.3 at the beginning of July. In the increasing period the goats were taking feeding supplements, because they are feeding their kid and after January they were feeding in the pasture.

In cheese, the pH (Fig. 2) is almost the same of the milk. The values for percentage of aches (Fig. 4) and total solids (Fig.6) presented higher values in cheese (respectively 3.7 and 3 times superior), which could be due to the lost of fat during the coagulation. Two third part of it (Fig. 7) wasn't entrapped in the curd and was lost with the whey.

Conclusion

Almost all the gross components of the milk and cheese suffered no variation during the lactation period, except for fat, that increased until middle period and decreased after that.

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MICROBIOLOGICAL CHANGES IN CHEESE OF ALGARVIAN GOAT BREED DURING RIPENING

I. Ratão[1,2], D. Guerreiro[1] and L. Dionísio[1]
[1]Faculty of Science and Technology, University of Algarve. Campus de Gambelas, 8005-139 Faro, Portugal Tel./Fax: + 351 289 800 040/+ 351 289 818 419, E-mail: ldionis@ualg.pt
[2]Superior Institute of Engineering, University of Algarve. Campus da Penha, 8005-139 Faro, Portugal Tel./Fax: +351 289 800 151/+ 351 289 888 405, E-mail: iratao@ualg.pt

Introduction

A traditional goat cheese from Algarve region (Portugal) is produced under artisanal conditions. During the processing procedures several contaminations may occur, which could influence its quality during ripening. The main goal of this work was to monitor the cheese microbial quality along a ripening period of sixty days.

Material and methods

Samples were prepared for microbiological analysis according to NP1829 (1982)(1). heterotrophic bacteria, coliforms, yeasts and moulds were enumerated according to NP1995 (1982)(2), NP3788 (1990)(3) and NP3277-1 (1987)(4), respectively. Presumptive lactobacilli were incubated on Rogosa agar (RA) (Biokar) at 36±1 ºC, during 72 h(5) and presumptive lactococci were incubated on M17 agar (Biokar) at 30±1 ºC, during 48 h(6). All assays were done in triplicate and expressed as colony-forming units per gram (cfu/g) of cheese.

Results and discussion

During cheese ripening, the heterotrophic microorganisms showed no considerable differences. The initial increase of 1 log should be due to the Lactic Acid Bacteria (LAB) growth, but it was rapidly compensated by the decrease of coliform bacteria.

The enumeration of presumptive lactobacilli reached a maximum after 42 days of ripening and for presumptive lactococci at 14 days. A slightly decrease was observed after that. Nevertheless, the concentrations of both LAB bacteria reached similar levels after 28 days of cheese ripening.

Total coliforms reached maximum concentrations values after 14 days of ripening, decreasing thereafter gradually by about 4 log units until 28 days of ripening. After this period, their concentrations become approximately constant.

Faecal coliforms reached zero value by the 21 days of ripening. Moulds and yeasts reached maximum values at 14 days of ripening and then decreased slightly to the end of the ripening period.

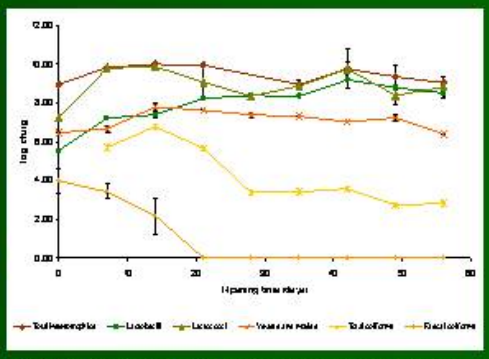

Fig. 1. Microbiological monitorization of Algarvian breed goat cheese during ripening.

Conclusion

These results suggested that the lactic acid produced by the LAB in the beginning of the ripening period contributed to the decrease of the remaining microbiota, including the inhibition of the faecal coliforms, which can contribute to the safety of the ripened cheeses.

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