universidad de león

DEVELOPMENT OF TRADITIONAL PROCESSED DAIRY PRODUCTS WHICH PROMOTE CARDIOVASCULAR HEALTH THROUGH THE INCLUSION OF CANOLA SEED AND MARJORAM IN EWE´S DIET

Desarrollo de productos lácteos tradicionales con propiedades cardio-saludables a través de la incorporación de colza y mejorana en la dieta de las ovejas.

DOCTORAL THESIS
MANEL BEN KHEDIM

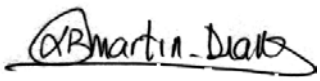
INFORME DEL DIRECTOR DE LA TESIS

La Dra. Carmen Asensio Vegas como la Dra. Ana Belén Martín Diana y el Dr. Ángel Ruíz Mantecón, Directores de la Tesis Doctoral titulada “Desarrollo de productos lácteos tradicionales con propiedades cardio-saludables, a través de la incorporación de colza y mejorana en la dieta de las ovejas” realizada por Manel Ben Khedim en el programa de doctorado Medicina, Sanidad y Producción Animal y Ciencia de Los Alimentos, informa favorablemente el depósito de la misma, dado que reúne las condiciones necesarias para su defensa.

León a 15 de Septiembre de 2014

Fdo. Carmen Asensio Vegas

Fdo. Ana Belén Martín Diana



Fdo. Ángel Ruíz Mantecón

Financiación

Esta tesis se ha llevado a cabo gracias a la financiación del Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), a través de la beca de Formación de Investigadores de tipo Predoctoral (Resolución de convocatoria de 4 de febrero de 2010, BOE 6 de febrero 2010, beca nº 21), y el Instituto Tecnológico Agrario de Castilla y León (ITACyL) a través del proyecto nº 2010/1287 (*Producción Ovina Láctea adaptada a altos estándares de Calidad*), cofinanciado con fondos FEADER.

To my mother

To the memory of my beloved dad

La connaissance est une navigation
dans un océan d'incertitudes
à travers des archipels de certitudes

El conocimiento es una navegación
en un océano de incertidumbres
a través de los archipiélagos de las certidumbres

(Les sept savoirs nécessaires
à l'éducation du future
***Edgar Morin**)*

Acknowledgements

First, I would like to express my sincere gratitude to my thesis directors, Dr. Carmen Asensio Vegas, Dr. Ana Belén Martín Diana and Dr. Angel Ruíz Mantecón for the continuous support during my Ph.D study and research. Your guidance helped me in all the time and particularly during the writing of this thesis. Thank you for your ongoing support and endurance.

I would also like to convey thanks to the "Instituto Tecnológico Agrario de Castilla y León" (ITACyL), en particular to the "Área De Investigación Ganadera" and the "Área De Innovación y Optimización de Procesos", for providing the financial means and laboratory facilities.

I would also like to express my sincere thanks to Luis Rodríguez Ruíz, the responsible of the ETL for his supports and advises

I am grateful to Daniel Rico Bargues for his guidance and assistance in the study of the bioactive properties in milk and dairy products. To Raúl Bodas Rodríguez for his help in the study of the animal performances and also for his encouragement.

My sincere thanks also goes to Dr. Javier Fontecha (Department of Bioactivity and Food Analysis, Institute of Investigation of Food Science (CIAL), Madrid) and to his group of investigation, Luis, Pilar and Marivi, for all what I felt and learned during my stay in your laboratory.

I am deeply grateful to Dr. Nigel Brunton, for his great help and his expert supervision during my stay in the University College of Dublin (Ireland), and also for his goodness and sympathy when my father died. I will never forget those moments. Thank you so much. To the Dr. Dilip Rai, for his supervision during my stay in the agriculture and food development authority in Ireland (Teagasc).

At personal level, I would like to extend my gratitude to the ETL and ITACyL staff and to all my friends, in Spain and Ireland, I truly appreciate your support throughout these years of Ph.D thesis.

To Rim my special friend and sister, miss you so much.

To my special friend Thomas, thank you for your friendship and support during my moments of weakness. Also, a special thanks goes to David for his valuable help and advises. Nines, Elisa, Stefania, Monica and Fran, thanks for everything, help, discussions and sweet moments. To Irene, Maria-Jesus and Marie-Paz for your friendship and kindness.

I would like to thank Shamol, who as a good friend was always willing to help and give his best suggestions. I learned so much from you and it was a pleasure to know you as a person.

To Laura, Noel, Jafar, Khadija and Angelo, it was a pleasure to meet you boys and hope that destiny will make us meet again.

Before and after all the thankfulness, should be raised to Allah creator of everything and the most merciful and grateful, then to my wonderful parents and brothers. Mum, source of inspiration and strength, thank you for believing in me and in my dreams. Dad, I hope that you are in a better place now.

Last but not least, to my husband Zied, for his love and his ability to adapt and stand my tough moments. For us, life start now and I look forward with hope and excitement.

LIST OF ABREVIATION

AAPH	2,2 E-Azo-bis(2-amidinopropane) dihydrochloride
ABTS	2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)
ADF	acid detergent fiber
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
CLA	conjugated linoleic acid
DM	dry matter
DPPH	2,2-Diphenyl-1-picrylhydrazyl
ET	electron transfer
ESI-MS	electrospray ionisation mass spectrometry
FCR	Folin-Ciocalteu reagent
FL	fluorescein
FRAP	ferric reducing antioxidant property
GA	gallic acid
GAE	gallic acid equivalent
HAT	hydrogen atom transfer
HPLC	high performance liquid chromatography
LCFA	long chain fatty acids
LCT	long chain triglycerides
MCFA	medium chain fatty acids
MCT	medium chain triglycerides
MUFA	mono-unsaturated fatty acids
N	nitrogen
NDF	neutral detergent fiber
OM	organic matter
ORAC	oxygen radical absorbance capacity
PUFA	polyunsaturated fatty acids
RACI	relative antioxidant capacity index
ROS	reactive oxygen species
RA	rumenic acid
SCFA	short chain fatty acids
SCT	short chain triglycerides
SP	sphingomyelin

SSAO	the semicarbazide-sensitive amine oxidase
TEAC	trolox equivalent antioxidant capacity
TP	total phenolic contentç
TRAP	total peroxyl radical-trapping antioxidant parameter
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
TAG	triglycerides
TMR	total mixed ration
UFA	unsaturated fatty acids
VA	vaccenic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidyl-inositol
PLs	phospholipids
PS	phosphatidylserine

TABLE OF CONTENTS

ÌNDICE

LIST OF TABLES

LIST OF FIGURES

LIST OF EQUATIONS

Chapter 1: Introduction

1.1. Functional foods

Among all the innovations introduced in the food industry, functional food was recognize as one of the most interesting areas to satisfy consumer expectations (Bigliardi and Galati, 2013; Bholah and Neergheen-Bhujun, 2013). Functional food plays an outstanding role by their increasing demand derived from the increasing cost of healthcare, the steady increase in life expectancy, and the desire of older people for an improved quality of life (Nagashima *et al*., 2013; Szakály *et al*., 2012). Leatherhead Food International values the functional foods market at 24.2 billion US$ in 2010; the development of the market has been monitored since 2003 and it has been estimated that it increased by almost 1.5 times during the last 10 years. In Spain, the growth rate of the functional foods market was above 14% and the value of the commercialized products was more than 3.500 millions of euro during the last years (Juárez. 2010). Moreover, the functional foods market has been growth significantly faster than the headline rate of growth of the global food and beverage market of around 4% per year. However, the growth rates in other part of the world have dropped over the last couple of years, especially compared with the last decade due to economic reasons caused by the global downturn (Leatherhead Food International. 2012).

1.1.1. Definition and classification of functional foods

The term "functional food" was created in the late 1980's in Japan as a result of a study on the relationships between nutrition, sensory satisfaction, fortification and modulation of physiological systems in order to define those food products fortified with special constituents that possess advantageous physiological effects (Bigliardi and Galati, 2013). Japan was the first nation that has legally defined the term of functional food and the Japanese functional food as consequence is one of the most advanced in the world. To date a number of national authorities, academic bodies and industry have proposed many definitions for functional foods, but up to now this concept still not clear and completely defined. However, the lack of agreement on terminology doesn't seem to be a direct obstacle to the development of this market, which means that consumers are more attracted by a health message rather than the use of a particular legal term.

Many definitions have been proposed by Bigliardi and Galati (2013), in which the main key concepts were underlined; health benefits, nutritional functions and technological

process used. However, the main concept, mostly reported and making the difference between all the definitions, is the fact that functional foods must provide an additional physiological benefit, beyond their nutritional properties, that may prevent disease or promote health and wellbeing (Rodriguez *et al*., 2012; Falguera *et al*., 2012; Bhat and Bhat, 2011; Siró *et al*., 2008).

Moreover, functionality may be an inherent quality of the food raw material or a feature introduced by innovative new processing technologies or by the addition of health promoting food ingredients or probiotic micro-organisms to the food matrix (Briström and Nordström, 2002).

There are two major classifications of functional foods as reported by Bigliardi and Galati (2013); from a product point of view or based on the aim of the functional food. From a product point of view, according to Sloan (2000) and Spence (2006), functional foods include fortified foods (such as juice fortified with vitamin C), enriched foods (such as probiotic), altered products (such as fiber as fat released) and enhanced commodities (such as eggs with increased omega-3 content). And, based on the aim of functional food, functional foods can be classified as foods that improve the conditions of the body (such as prebiotics and probiotics) and foods that may reduce risks to develop diseases (cholesterol-lowering products).

Dairy functional food

In the worldwide functional food market, dairy products are key products and account for nearly 43% of the market, which is almost entirely made up of fermented dairy products (Özer and Kirmaci, 2010). Furthermore, functional beverages are considered the most important fraction in this group (Rodriguez *et al*., 2012).

Functional food acceptance varies across food categories and consumers will reject functional food products if they feel uncertain about the safety of the product (Falguera *et al*., 2012). Consumer acceptance is mainly related to taste, pleasure, security and especially to the degree of familiarity with the functional product (Annunziata and Vecchio, 2011), which may explain the interest that consumers experience respect of fermented milk such as yogurt.

According to Saxelin *et al*. (2003) dairy products can be divided into three groups; basic milk products that includes classical dairy products (such as yogurt, cheese, butter, ice cream, etc), added-value products (such as low-lactose or lactose-free products,

enriched milk with Ca, vitamins, etc) and functional dairy foods which demonstrate health benefits beyond their basic nutritional value (health claims).

1.1.2. Legislation of functional food: Directive 1924/2006/EC

European legislation does not consider functional foods as a specific food category (Coppens *et al.*, 2006), but as part of the food category regulated by the same legislation.

In UE, rather than regulating the product group *per se*, legislative efforts are directed towards restricting the use of nutrition and health claims and tried to reach consensus on specific concepts of functional foods by using the science base that supports specific nutrients that positively affect physiological functions. The main reason of this action has been to protect consumer and promote the innovation of the industry. The Directive 1924/2006/EC complements the general principles in Directive 2000/13/EC, which previously prohibits the use of information that would mislead the purchaser, and lay down specific provisions concerning the use of nutrition and health claims concerning foods to be delivered as such to the consumer.

'Health claim' means any declaration that states, suggests or implies that there is a relationship between a food category, a food or one of its constituents and health (1924/2006/EC). Health claims are permitted if they are in accordance with the lists of authorised claims provided in Articles 13/5 and 14 (Figure 1.1). Claims under those articles are based on developed scientific evidence and for which authorization is required by EFSA on a case-by-case basis.

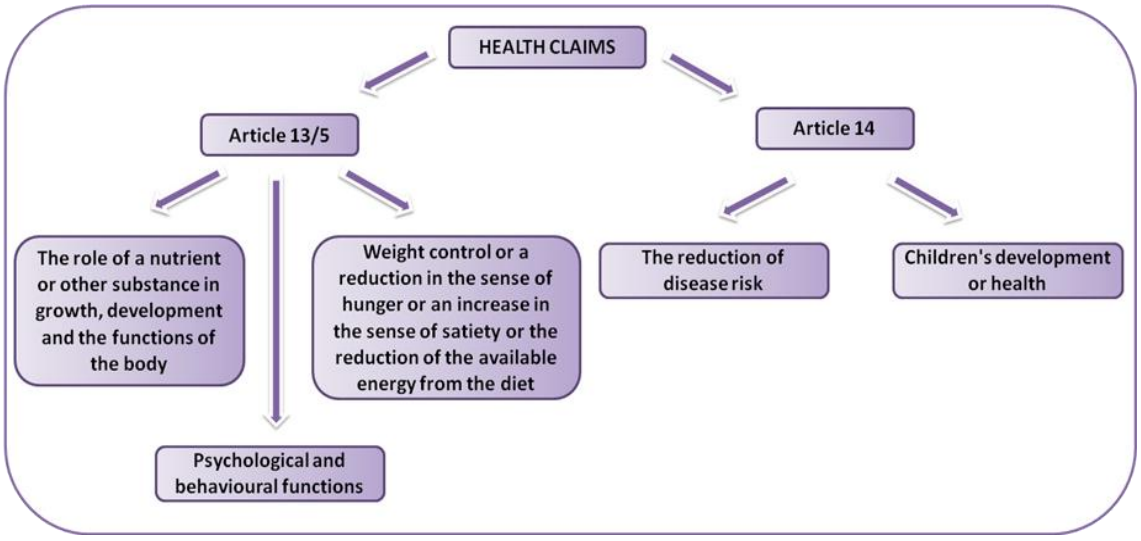


Figure 1. 1. Health claims for functional foods (1924/2006/EC)

Based on scientific information, EFSA has evaluated important number of declaration during the last years. As example EFSA refused that of the relation between the consumption of EPA and DHA and children's brain development. However, declaration on the consumption of sterols from plants and the reduction of the cholesterol LDL or the relation between calcium and vitamin D and the children's growth have been authorized.

1.2. Antioxidants

During the last decade, the use of antioxidants in food industry increased significantly (Franz. 2011). The worldwide market for antioxidants had a total volume of around 0.88 million tonnes in 2007 with Asia accounting for 47% of consumption, followed by Europe at 24%, North America at 22% and the rest of the world at 6% (Additives for Polymers, 2008). And, the volume growth rate is predicted to increase 3.9% per year and the consumption is expected to increase to 1.25 million tonnes in 2016.

On the other hand, antioxidants are very important since they were reported to contribute efficiently in preserving food from oxidative deterioration. Many foods are very sensitive for oxygen, which is responsible for the deterioration of many products either directly or indirectly (Souza Cruz *et al*., 2012). Among dietary lipids, fat rich in poly-unsaturated fatty acids (PUFAs) is the most susceptible to oxidation (Boroski *et al*., 2012) which affects food characteristics by producing modifications on their physico-chemical, nutritional and sensory properties (Boroski *et al*., 2012; Petit, 2009). Moreover, free radical-initiated autoxidation of PUFAs has been implicated in numerous human diseases, including atherosclerosis and cancer (Yin and Porter, 2005).

Additionally, dietary fat can be significant contributors to the free radicals load in animals (Andrews *et al*., 2006) which can decrease performances and compromise immune response (Dibner *et al*., 1996).

1.2.1. Definition and mechanism of action of antioxidants

Halliwell and Gutteridge (2007) defined antioxidant as "any substance that delays, prevents or removes oxidative damage to a target molecule". Antioxidants have been as well defined by Alamed *et al*., (2009) as a synthetic or natural compound that has the ability to slow lipid oxidation. Moreover, antioxidants are also known as free radical scavengers because they can prevent damages due to the reactive oxygen species (ROS) generation (Niki. 2011; Gordon. 2012).

From a chemical point of view, oxidants or free radicals are molecules capable to oxidize target compounds. Free radicals are defined as a collectively term that includes reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Jain *et al*., 2013).

The reactive oxygen species (ROS) are naturally generated in small amounts in the metabolic reactions and can be overproduced under various environmental and stress conditions (Sies. 1997; Scandalios. 2005; Wang *et al*., 2012). Consequently antioxidants can initiate lipid peroxidation in foods, which leads to their deterioration (Miller et *al*., 1993) and they can also induce oxidative stress in the body which is implicated in the etiology of many chronic and degenerative diseases (Prior and Wu, 2013).

The mechanisms of defense against free radicals can be divided into several mechanisms; quenching oxidants, repairing/ removing oxidative damage or encapsulating non-repairable damage. Indeed, antioxidants may exert their effect by different mechanisms. The first line of defense is by interfering in the initial reaction that generate ROS through the scavenging for free oxygen molecules required to begin the production of ROS or by chelating oxidative elements that speed up oxidative processes, such as iron (Sgorlon *et al*., 2006). The second line of defense is by scavenging the active radicals in order to break the chain propagation reactions (Lobo *et al*., 2010). And the third streak of defense is the repair and de novo antioxidants (Lykkesfeldt and Svendsen, 2007).

1.2.2. Classification of antioxidants

Several classifications have been proposed; they attempted to take into account the nature (enzymatic or non-enzymatic), the chemical-physical properties (hydrophilic or lipophilic), the structure (flavonoids, polyphenols, etc.) and the mechanism (preventive, chain-breaking, etc.) (Vertuani *et al*., 2004). However, the most widely used is that based on the origin of the antioxidant (natural or synthetic).

Synthetic antioxidants

In the last decades, synthetics antioxidants have replaced natural antioxidants for a number of reasons, including low cost, high purity and constant activity (Decker *et al*., 2010). And nowadays, even if in less proportion, they still exist for the same reasons (Table 1.1).

Table 1. 1. US and European antioxidants market by type, 2002±2006

Year (% Sales value)	2002	2006
Synthetics		
BHA (butylated hydroxyanisole)	23.4	23.2
TBHQ (tertbutylhydroquinone)	11.2	11.1
BHT (butylated hydroxytoluene)	3.0	2.9
Propyl gallate	2.4	2.4
Total synthetics	40.0	39.6
Naturals		
Tocopherols	5.5	5.8
Ascorbates and erythorbates	42.6	42.2
Herb extracts	11.9	12.4
Total naturals	60.0	60.4
Grand total	100.0	100.0

Source: Decker *et al*. (2010)

A variety of very effective synthetic antioxidants, such as BHT, butylated hydroxyanisole (BHA), tert-butylhydroxyquinone (TBHQ) and propyl gallate (PG) have been used widely in different food products. BHA, BHT, PG and TBHQ are all hydroxyl substituted phenols, and as such, act as radical scavengers by donating hydrogen atoms, thereby forming more stable phenoxy radicals that do not contribute to the lipid oxidation mechanism (Berdahl, *et al*., 2010).

Natural antioxidants

Natural antioxidants have been used in foods in the past for preservation associated with their antioxidant activity and nowadays there is a renewed interest in them, rather than in synthetic agents (Kindleysides *et al*., 2012).

Natural antioxidants include dietary antioxidants, which are defined as any substance present in food that significantly decreases the adverse effect of reactive species (Jain *et al*., 2103). And, the use of the term "dietary oxidative stress" by Levander *et al*. (1995) confirms the excessive role that has been given to nutrition and to dietary antioxidants from scientists (Veskoukis *et al*., 2012). The addition of natural antioxidants increases the shelf life and reduces the environmental impact associated with nutritional losses in food products by inhibiting and delaying oxidation (Tsuda *et al*., 1994). Moreover, dietary antioxidants can protect human body from free radicals and retard the progress of many chronic diseases (Mathew *et al*., 2010). Many natural antioxidants have been described but vitamins and polyphenols can be considered the main important groups (Reboul *et al*., 2007).

1.2.3. Antioxidant as natural anti-inflammatory

Inflammation is a process caused by many reasons; chronic inflammation is one of the main ways to develop degenerative diseases. Indeed, inflammation plays a key role in the physiology of arthritis, diabetes, heart disease, irritable bowel syndrome, Alzheimer's disease, Parkinson's disease, and many other illnesses (Nair *et al*. 2010). Moreover, clinical trials reveal that inflammation is a potential therapeutic target in cancer treatment (Coward *et al*., 2011, Fernandez *et al*., 2013).

Many antioxidants have natural anti-inflammatory actions by interfering in the different metabolic pathways of the inflammatory process. Inflammation involves a portfolio of cellular and molecular components collectively referred to as inflammation mediators (Issa *et al*., 2006).

1.2.4. Methods of analysis of the antioxidant and anti-inflammatory activity

During the oxidation process in food systems, where lipids, proteins and carbohydrates are the target molecules, different reactive oxygen species and different mechanisms are involved. Therefore, the evaluation through different antioxidant assays with different chemistry is very important. Measurements of antioxidant capacity have been developed to assess total antioxidants in fruits and vegetables and other dietary components and to provide another biomarker of oxidative stress in the form of plasma and tissue antioxidant capacity (Prior *et al*., 2003; Prior and We, 2013). These antioxidant capacity assays can be classified into two groups based on their principle mechanisms of reaction with free radicals: assays based on hydrogen atom transfer (HAT) reactions and assays based on electron transfer (ET) (Huang *et al*., 2005).

Although anti-inflammatory activity is not a direct measurement of the antioxidant activity, it can give information on the oxidative process. Numerous methods were used to measure the anti-inflammatory capacity such as the measurement of the inhibition of the pro-inflammatory cytokines (for example, tumor necrosis factor-α (TNF-α)) or the pro-inflammatory mediators (such as the cyclooxygenase 2 (COX2) and the inducible nitric oxide synthase (iNOS)). However, the measurement of the semicarbazide-sensitive amine oxidase (SSAO) inhibitory activity is of special interest because as enzymatic based method it could be easily correlated with the antioxidant assays.

1.2.4.1. Electron transfer (ET) based assays

The ET-based assays involve a redox reaction with the oxidant as an indicator of the reaction endpoint. These methods involve two components in the reaction, antioxidants and oxidant. And method is based on the following electron transfer reaction (Huang *et al*., 2005).

Equation 1.1

$$[\text{Probe (oxidant)} + \text{e (from antioxidant)} \rightarrow \text{reduced probe} + \text{oxidized antioxidant}]$$

The probe itself is an oxidant that abstracts an electron from the antioxidant, produce colour changes of the probe. The degree of the colour change is proportional to the antioxidant concentration.

Different methods are indicators of the antioxidant activity; the electron transfer ET-based assays include: 1) the total phenols assay by Folin-Ciocalteu reagent (FCR), 2) the diphenyl-1-picrylhydrazyl (DPPH) assay, 3) Trolox equivalence antioxidant capacity (TEAC), 4) ferric ion reducing antioxidant power (FRAP), and 5) microsomal lipid peroxidation assay. The assays described forwards are the main used for the determination of antioxidant capacity on food samples.

Total phenols assay by Folin-Ciocalteu reagent

Total polyphenol content (TP) is a spectrophotometrical method based on the use of Folin–Ciocalteu's reagent (FCR) in order to determine the amount of phenolic compounds present. The FCR measures the reducing capacity; the method is based on the reduction of phosphomolybdic-phosphotungstic acid. After the reaction, an increase absorbance is observed and measured spectrophotometrically between 550 nm and 750 nm. The total phenolic content is expressed in mg of gallic acid per gram of dry weight extract (mg GAE/g DWE).

Folin–Ciocalteu's reagent (FCR) was initially used for the analysis of proteins taking advantage of the reagent's activity toward protein tyrosine (containing a phenol group) residue. After that, it was extended by Singelton *et al*. (1999) to the analysis of total phenols mainly in wine. Although the Folin–Ciocalteu's reagent is not specific for polyphenols, the total phenolic assay was reported to be a good indicator to evaluate phenols and estimate antioxidant activity (Escarpa and González, 2001; Singleton *et al*., 1999). There is a very significant linear correlation between the "total phenolic profiles"

and "the antioxidant activity" because FCR is considered as an ET-based antioxidant capacity assay (Huang *et al*., 2005).

DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging capacity assay

DPPH assay is an easy and accurate method with regards to measuring the antioxidant capacity of several matrices such as beverages, fruits, vegetables and extracts (Huang *et al*., 2005; Sanchez-Moreno. 2002). However, the DPPH is long-lived nitrogen radical, which bears no similarity to the highly reactive and transient peroxyl radicals involved in lipid peroxidation. Therefore, many antioxidants that react quickly with peroxyl radicals may react slowly or may even be inert to DPPH. And, DPPH is not present in *vivo* but is quite stable, unlike radicals present in living organisms (Pérez-Jiménez *et al*., 2008).

The synthetic compound 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$, radical form) is one of a few stable and commercially available organic nitrogen radicals and has a UV-vis absorption maximum at 515 nm (Huang *et al*., 2005). DPPH is used as a redox probe for determining the free radical scavenging capacity of plant phenolic compounds (Apak *et al*., 2007). The concentration that causes a decrease in the initial DPPH concentration by 50% is defined as EC_{50}.

Trolox equivalent antioxidant capacity assay (TEAC)

The TEAC assay was firstly reported by Miller *et al*. (1993) and later modified by Re *et al*. (1999). For the oxidant, $ABTS^{\bullet-}$, is generated by persulfate oxidation of 2,2´-azinobis(3- ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{2-}$) (Huang *et al*., 2005). The method used is based on the capacity of a sample to inhibit the ABTS radical ($ABTS^{\bullet+}$) compared with Trolox as a reference antioxidant standard.

TEAC assay has been broadly applied in assaying food samples (Huang *et al*., 2005). However, According to Pérez-Jiménez *et al*. (2008), ABTS is not present in *vivo* and the antioxidants, besides reacting with the radical to yield the original molecules, generate other compounds.

The TEAC value is based on the ability of the antioxidant to scavenge the blue-green coloured $ABTS^{\bullet+}$ radical cation relative to the $ABTS^{\bullet+}$ radical cation scavenging ability of the water-soluble vitamin E analogue, Trolox.

1.2.4.2. Hydrogen atom transfer (HAT) based assays

The HAT-based assays quantify hydrogen atom donating capacity. Most HAT-based assays monitor competitive reaction kinetics, and the quantitation is derived from the kinetic curves. HAT-based methods generally have the following components: (a) an azo radical initiator, normally AAPH; (b) a molecular probe (UV or fluorescence) for monitoring reaction progress; (c) antioxidant; and (d) reaction kinetic parameters collected for antioxidant capacity quantitation (Huang *et al*., 2005). Hydrogen atom transfer (HAT) assays include: 1) oxygen radical absorbance capacity (ORAC), 2) total radical trapping antioxidant parameter (TRAP) assay, 3) crocin bleaching assay, 4) β-Carotene bleaching assay, and 5) inhibition of induced low-density lipoprotein autoxidation.

ORAC assay is the most widely used HAT-based antioxidant capacity assay. ORAC is considered particularly biologically relevant since use the peroxyl radical, which is the most common radical found in biological systems. Moreover, ORAC assay has found even broader application than TEAC and DPPH assays for measuring the antioxidant of botanical and biological samples (Huang *et al*., 2005). However, the fact that the procedure to generate peroxyl radicals is not physiological and that protein may have an interfering effect represent the limitation of this method (Pérez-Jiménez *et al*., 2008).

The ORAC assay used fluorescein (FL) (3´,6´-dihydroxyspiro[isobenzofuran-1[3H], 9´[9H]- xanthen]- 3-one) as a synthetic non protein probe (Ou *et al*., 2002). The fluorescence decay of FL is an indication of damage from its reaction with the peroxyl radical. Antioxidants protects the fluorescent probe from the damaging effect of peroxyl radical generated by radical generator eg., 2,2_-azobis (2-amidinopropane) dihydrochloride (AAPH). ORAC assay measures the extent of protection of the fluorescent probe by the antioxidants against the radicals generated in the reaction.

1.2.4.3. The hypothetical concept of relative antioxidant capacity index

In many cases, it is very difficult to compare results of antioxidant activity obtained from different methodologies (Frankel and meyer, 2000) because the antioxidant activity measured by an individual assay reflects only the chemical reactivity under the specific conditions applied in that assay and it is inappropriate and misleading to generalize the data as indicators of "total antioxidant activity" (Huang *et al*., 2005). Therefore, Sun and Tanumihardjo (2007) proposed a new concept, RACI, from the perspective of statistics by integrating the food antioxidant capacity data determined by

several methods in order to compare the food antioxidant capacity derived from different chemical methods. The best proposed idea is to transform the values of antioxidant into standard scores. In statistics, a standard score (also called z- or normal score) is a dimensionless quantity derived by subtracting the mean from the raw data divided by the standard deviation (Sharma. 1996; Sun and Tanumihardjo, 2007).

1.2.4.4. The assay of the semicarbazide-sensitive amine oxidase (SSAO) inhibitory activity

The oxidative deamination of endogenous and exogenous amines in mammals is catalyzed by a number of oxidases (Blaschko *et al*., 1958). Semicarbazide-sensitive amine oxidase (SSAO) encodes a wide family of enzymes named E.C.1.4.3.6 [amine:oxygen oxidoreductase (deaminating) (copper containing)]. SSAO catalyses the oxidative deamination of primary aromatic and aliphatic amines. Its catalytic action requires oxygen and generates ammonia, hydrogen peroxide (H_2O_2) and the corresponding aldehyde (Hernandez *et al*., 2005). All these enzymes are inhibited by semicarbazide (Lewinsohn. 1984; Precious and Lyles, 1988).

The physiological function of SSAO is not clear, but it was reported that SSAO is implicated in different pathways and plays important roles in the plurimetabolic syndrome (Boomsma *et al*., 2000). Although SSAO has been mostly regarded as being involved in the detoxification of amines, the products of the reaction (hydrogen peroxide (H_2O_2), formaldehyde and methylglyoxal) are more toxic than the amine substrates themselves (Lyles. 1996) and it also has been reported to lead the increase of oxidative stress, protein cross-linkage and cytotoxicity (Yu and Deng, 2000). The SSAO inhibitory activity is generally performed by measuring the H_2O_2 released from the conversion of benzylamine to benzaldehyde via SSAO.

1.3. Marjoram (*Thymus mastichina* L.)

1.3.1. The taxonomic aspects

Marjoram *Thymus mastichina* L. belongs to the genus *Thymus* which is one of the most important genera of the Lamiaceae family (Morales. 2005). The species of *Thymus* genus are herbaceous perennial shrubs, well identified as aromatic and medicinal plants (Amiri. 2012; Jia *et al*., 2010).

T. mastichina is endemic from the central Iberian Peninsula in Spain, except in East region, Cataluña and Aragón (Delgado *et al*., 2014; Morales. 2005). It‘s a specie widely

distributed along the peninsula with an excellent adaptation to numerous ecological ecosystems (Blanco. 2007).

Plant morphology

According to Blanco (2007), *T. mastichina i*s a perennial subshrub, up to 80 centimeters in length, erect and branched. Villous stems with retrorse hairs in all the four sides. Not over-wintering leaves, flat, stalked, elliptical to lanceolate, without cilium at the base, with yellow spherical glands; fasciculate over-wintering leaves, smaller, densely villous with very short hair and ashy appearance; those of the stem of the year, older, glabrescent, green, and entire or sometimes with crenate edges. Inflorescence in dense verticillastrate, up to 20 mm in diameter; the superiors, spiciform; the inferiors, usually globose, pedunculated or sub-sessile; equipped with bracts, usually ovate, not coloured, more or less villous with spherical glands.

The flowers have a goblet of 2.7-7.4 mm in length, villous, with long hairs, teeth usually ciliate (cilium up to 0,5 mm), the top of 2,5-4 mm and the bottom of 3,5-4,5 mm, totally covered by yellow spherical glands. The corolla is up to 12 mm in length, with a 5-8 mm tube; inferior lip with sub-equal three lobules; superior lip erect with short lobules and spherical white glands. Stamens, exserted with filaments up to 17 mm and white anthers up to 2 mm. Carpels up to 1 mm, globose with dark brown colour. 2n= 56, 58, 60. The florescence usually occurs from May to July but occasionally some specimens are found in April, August and September.

Ecology

T. mastichina growths in very different type of soils, and the texture or soil structure don't alter it (Blanco. 2007). However, it tends to prefer siliceous and decarbonised soils (Sánchez-Gómez *et al*., 2004). According to Blanco (2007), it can be found in stony mountains, in limestone crags and more frequently in areas of slate and quartzite, even if it has preference for more or less sandy soils. As regards climate, it withstands temperatures above 45 °C and is capable of surviving in snowy places. The regime of annual precipitation that supports ranges from 300 mm to over 1000 mm. The areas that occupies in altitude vary greatly, it can be found below the 200 MSL and up to more than 1500 MSL.

Ethnobotany

According to Grieve (1931), ancient Egyptians used thyme for embalming and ancient Greeks used it in their baths and burnt it as incense in their temples, believing it was a source of courage. The spread of thyme throughout Europe was thought to be due to the Romans, as they used it to purify their rooms and to "give an aromatic flavour to cheese and liqueurs". In traditional medicine, leaves and flowering parts of *Thymus* species were widely used as tonic and herbal tea, antiseptic, antitussive, and carminative as well as treating colds (Amiri. 2012). *Thymus* oils and extracts were widely used in pharmaceutical, cosmetic, and perfume industry, also for flavouring and preservation of several food products (Bauer *et al*., 1997).

T. mastichina was used traditionally in food preparations and was also used to make tea for stomach disease (Bento et al., 2006). According to Blanco (2007), above all, *T. mastichina* has been used since ancient times to aromatize, preserve, and facilitate the digestion of food because of it high concentration in essential oil.

1.3.2. Composition of marjoram plant

T. mastichina is an aromatic and medicinal plant principally characterized by its content in essential oils and phenolic compounds. The main components in the essential oil of *T. mastichina* are 1,8-cineole (30-68%) and linalol (3-48%), both are important compounds with therapeutic properties. These therapeutic properties include antiseptic, antispasmodic, anti-carcinogenic, digestive, diuretic and many other properties (Sengupta and Bhattacharjee, 2009).

Phenolic compounds are present in all parts of the plants such as seeds, leaves and roots and are one of the most important natural antioxidants (Pratt and Hudson, 1990). Phenolic compounds have, as a common characteristic, the presence of at least one aromatic ring hydroxyl-substituted (Morton *et al*., 2000). Another characteristic of these compounds substances is that they are presented commonly bound to other molecules, frequently to sugars (glycosyl residue) and proteins. The existence of phenolic compounds in free form also occurs in plant tissues. However, it is less common, possibly because they are toxic when present in the free state and detoxified, at least in part, when bound (Reis Giada. 2013).

According to their distribution in nature, phenolic compounds can be divided into three classes: shortly distributed (as simple phenols, pyrocatechol, hydroquinone, resorcinol,

aldehydes derived from benzoic acids that are components of essential oils, such as vanillin), widely distributed (divided in flavonoids and their derivatives, coumarins and phenolic acids, such as benzoic and cinnamic acid and their derivatives) and polymers (tannin and lignin) (Bravo. 1998). Flavonoids constitute the largest group of plant phenolics, accounting for over half of the eight thousand naturally occurring phenolic compounds (Harborne. 1999).

Phenolic compounds may also be classified according to their location in the plant (free in the soluble fraction of cell or bound to compounds of cell wall) together with their chemical structure as: soluble compounds (such as simple phenol, flavonoids and tannins of low and medium molecular weight not bound to membranes compounds) and insoluble compounds (essentially constituted by condensed tannins, phenolic acids and other phenolic compounds of low molecular weight bound to cell wall polysaccharides or proteins forming insoluble stable complexes). This classification is useful from the nutritional point of view, to the extent that the metabolic fate in the gastrointestinal tract and the physiological effects of each group will depend largely on their solubility characteristics. Insoluble phenolic compounds are not digested and may be partially or fully recovered quantitatively in the feces, while a part of the soluble can cross the intestinal barrier and be found in the blood, unchanged or as metabolites (Sánchez-Moreno. 2002).

The antioxidant activity of phenolic compounds depends mostly on their chemical structure (Scalbert and Williamson, 2000). Phenolic compounds may act by offering an hydrogen atom and an electron to free radicals which convert them into relatively harmless free radicals (Veskoukis *et al*., 2012). Furthermore, they act as metal chelators, mainly Fe and Cu, thus not allowing them to initiate Fenton and Haber–Weiss reactions (Nijveldt *et al*., 2001). And, among phenolic compounds, flavonoids are the most potent antioxidants from plants. However, their effectiveness decreases with the substitution of hydroxyl groups for sugars, being the glycosides less antioxidants than their corresponding aglycons (Rice-Evans *et al*., 1996).

Polyphenols are reported to exert a variety of valuable bioactivities including anti-inflammatory properties (Bakker *et al*., 2010, Nair *et al*., 2010; Siriwardhana *et al*., 2013). Investigations into the mechanism of action of polyphenols have shown that these molecules modulate cellular signaling processes during inflammation or may themselves serve as signaling agents (Rahman. 2007).

Flavonoids may counteract oxidative stress-induced endothelial dysfunction and platelet aggregation, which are the main causes of cardiovascular disease (Rahman. 2007; Dashwood. 2007). Furthermore, they play an important role in reducing the development of atherosclerotic plaque formation (Pandey and Rizvi, 2009), the risk of neurodegenerative disease (Dashwood. 2007; Vauzour *et al*., 2010) and the risk of coronary mortality (Mink *et al*., 2007). Besides, current research regardless cancer prevention has shown that flavonoids may interrupt various stages of the cancer process (Mitjavila and Moreno, 2012; Chahar *et al*., 2011). And among flavonoids, the dietary intake of quercetin was reported to be associated with lower coronary heart disease mortality due to the reduction on the systolic, diastolic and mean arterial pressures with protective effect regardless cardiovascular risks (Russo *et al*., 2012).

In the past, studies were carried out on various species of *Thymus* but little is known as regards *T. mastichina* (Blanco. 2007). Indeed, *T. mastichina* is scarcely studied despite of its crop growing potential, sustainability and profitability as source of antioxidant compounds that can give an add value to the products without additional costs for the farmers.

Studies carried out by Nieto *et al*. (2010, 2011) demonstrated the antioxidant effect of *Thymus zygis* and *gracilis*, included in ewes' diet on fresh lamb meat and cooked lamb meat without any detrimental effect on animal metabolism. Recently, *T. mastichina* starts to arouse interest because of the therapeutic properties of their essential oils (Miguel *et al*., 2004; Delgado *et al*., 2014), herbs and spices (Albano and Miguel, 2011). But, data still is very scarce concerning the whole plant.

1.4. Canola (*Brassica napus* L.)

1.4.1. Taxonomic aspects

Canola (*B. napus* L.) is an ancient crop plant that belongs to the Cruciferae (*Brassicaceae*) family, also known as the mustard family. Canola was bred naturally from rapeseed at the University of Manitoba, Canada, in the early 1970s and had a different nutritional profile in comparison with rapeseed. Rapeseed was characterized by its high content in toxic or undesirable factors such as glucosinolates which were considered as sharp-tasting compounds and consequently not particularly appealing to livestock (Mazhari *et al*., 2009; Bell. 1983). For those reasons, the use of canola seed as substitute of rapeseed was encouraged due to its low level of glucosinolates further to its

low content in erucic acid (monounsaturated omega-9 fatty acid), which was related to heart cardiopathogenic effects (Bellenand *et al*., 1980). Canola is generally referred to as Rapeseed 00 or Double Zero to denote both low glucosinolates and low erucic acid.

Plant morphology

B. napus L. belongs to the cruciferae family, and this name crucifer comes from the shape of flowers, with four diagonally opposite petals in the form of a cross. *B. napus* has dark bluish green foliage, glaucous, smooth, or with a few scattered hairs near the margins, and partially clasping. The stems are well branched, although the degree of branching depends on variety and environmental conditions; branches originate in the axils of the highest leaves on the stem, and each terminates in an inflorescence. The inflorescence is an elongated raceme, the flowers are yellow, clustered at the top but not higher than the terminal buds, and open upwards from the base of the raceme.

Ecology

The oleiferous *B. napus*, a cool-season crop, is widely adapted, and performs well in a range of soil conditions, providing that moisture and fertility levels are adequate. Air and soil temperatures influence canola plant growth and productivity. The optimum temperature for maximal growth and development is just over 20°C, and it is best grown between 12°C and 30°C. After emergence, seedlings prefer relatively cool temperatures up to flowering; high temperatures at flowering will hasten the plant's development, reducing the time from flowering to maturity. Weeds can be one of the most limiting parameters in rapeseed production and must be controlled early to avoid yield loss due to competition. Moreover, oilseed rape should not be grown on the same field more often than once every four years, to prevent the buildup of diseases, insects, and weeds.

1.4.2. Composition of canola seed

Typically canola seed contains a minimum of 35% protein at 13% moisture and has an oil content ranging from 35 to 45% (but can fall outside this range depending upon variety and environmental factors) (Raymer. 2002). Canola seed is a source of lipids and has generally high content in unsaturated fatty acids and low content in saturated fatty acids.

The lipids can be classed as simple lipids, compound lipids and derived lipids. Simple lipids are the esters of fatty acids with various alcohols. The compound lipids are the

esters of fatty acids and alcohols and possess additional groups (non-lipid substances) such as phospholipids and glycolipids. And, the derived lipids result from hydrolysis or enzymatic breakdown of simple and compound lipids and include fatty acids, monoglycerides and diglycerides etc. A fatty acid is a carboxylic acid with a long aliphatic tail (chain), which is either saturated or unsaturated. Fatty acids that have carbon–carbon double bonds are known as unsaturated and those without double bonds are known as saturated. CLA is a term that refers to a mixture of positional and geometric isomers of linoleic acid with 2 conjugated double bonds in four geometric forms (*cis/trans, trans/cis, cis/cis or trans/trans*) located at various positions of the carbon chain (De La Torre *et al*., 2005).

During the last years, the main emphasis in public health has been the reduction in total dietary fat and the replacement of dietary saturated fatty acids (SFAs) and trans fat with polyunsaturated fatty acids (PUFA) in order to decrease risks of degenerative diseases such as cardiovascular and coronary heart pathologies (Erkkilä *et al*., 2008; Parodi. 2005). Both polyunsaturated fatty acids, n-6 and n-3 PUFA, have been reported, separately, to be pro-inflammatory, pro-thrombotic, to have anti-arrhythmic effects and are associated with lower incidences of degenerative diseases (Christen *et al*., 2013; Erkkilä *et al*., 2008; Guesnet *et al*., 2005; Zampatti. 2013). However, an excess of omega-6 fatty acids (linoleic acid and arachidonic acid) and a deficiency of omega-3 fatty acids may be involved in the development of numerous diseases (Seaman. 2002).

Moreover, growing evidence suggests that conjugated linoleic acid (CLA), which is principally derived from ruminant products, has numerous potential health benefits (Bhattacharyaa *et al*., 2006). Results, over the last decade and mainly from animal models and *in vitro* studies, have shown CLA to be anticarcinogenic. It was reported to be effective with mouse skin carcinogenesis, mouse stomach tumorigenesis, and rat mammary tumorigenesis (Belbury. 1995). It seems to act in a dose dependent manner as demonstrated *in vitro* with breast cancer cells (Shultz *et al*. 1992), and *in vivo* with chemically induced mammary tumors in rats (Ip *et al*., 1994). Ip *et al*. (1999) found that butter enriched in CLA, by including sunflower oil in the diet of dairy cows, was able to inhibit rat mammary tumor yield by 53%. Moreover, other researches indicate that CLA may have other physiological effects such as a role in reducing atherosclerosis (Lee *et al*., 1994; Nicolosi *et al*., 1997; McLeod *et al*., 2004) and in the treatment of diabetes (Houseknecht *et al*., 1998).

Most of the studies on canola were carried out in dairy cows (Chichlowski *et al*., 2005; Delbecchi *et al*., 2001; Neves *et al*., 2009) and less information was available as regards dairy sheep. Further to its low content in erucic acid which made the diet more healthy, the inclusion canola in dairy cow's diet was reported to increase the levels of desirable monounsaturated and polyunsaturated fatty acids in the milk (Komprda *et al*., 2005) and could also be responsible of an increase in the milk trans-18:1 concentration greater than other oilseed supplements rich in PUFA (Glasser *et al*., 2008).

1.5. Effect of diet on animal performance and milk composition

1.5.1. Dietary antioxidant

Intensive research is being carried out on the extraction, characterization and utilization of natural plant with antioxidant properties. According to Greathead (2003) the most efficient method to take advantage of the bioactive compounds present in natural plants would be to grow the relevant plants in fields and then let the animals graze them in a controlled manner, assuming they are palatable. However, the problem would be the no-control on dosage of the secondary plant metabolites content due to the harvest variation (Greathead. 2003). Therefore, a solution could be the incorporation of the whole plant as additive or as part of the animal diet, which will permit the control of the intake without additional costs. Furthermore it's already known that phenolic compounds are widely distributed in plants (Bennett and Wallsgrove, 1994; Hyder *et al*., 2002), which makes the use of the whole plant an interesting way to take benefit from the phenolic compounds distributed in the different parts of the plant. However, according to Jouany and Morgavi (2007) the practical limitation for the continued use of the whole plant as supplement is the extreme variation in the quality and the quantity of the phenolic compounds offered due to changes such as vegetative stages and environmental conditions. Moreover, bioactive ingredients can lose their antioxidant properties during storage.

Many studies has been carry out in the inclusion of essential oils and phenolic compounds from plant. However, few studies have investigated the effect of the inclusion of dietary phenolic compounds through the inclusion of the whole plants in on animal diet.

It has been reported that feeding cattle a gossypol diet rich in phenols, increased milk yield, fat content and non-casein nitrogen content (Blauwiekel *et al*., 1997). It has also been shown that providing a moderate source of proanthocyanidins in the diet of sheep, by supplementing feed with *Lotus corniculatus* (birdsfoot trefoil), increased the yield of milk, protein and lactose (Wang *et al*., 1996). It was suggested that the increased yield of protein and milk may be related to the fact that in the reticulorumen (which has a pH of 6.0–7.0), phenolic compounds interact with proteins, thereby inhibiting the utilization of protein in the rumen by indigenous micro-organisms (it has been estimated that the reticulorumen microflora degrade up to 75% of ingested protein, Figure 1.2) but once the phenol–undegradable protein complex passes into the abomasums (pH 2.5–3.5), the complex breaks down and the released protein is degraded and utilized by the ruminant (Wilkins and Jones, 2000; O'Connell and Fox, 2001). It has also been shown that phenols reduce the incidence of subclinical helminth infections in ruminants, and in so doing increase milk yield and protein utilization.

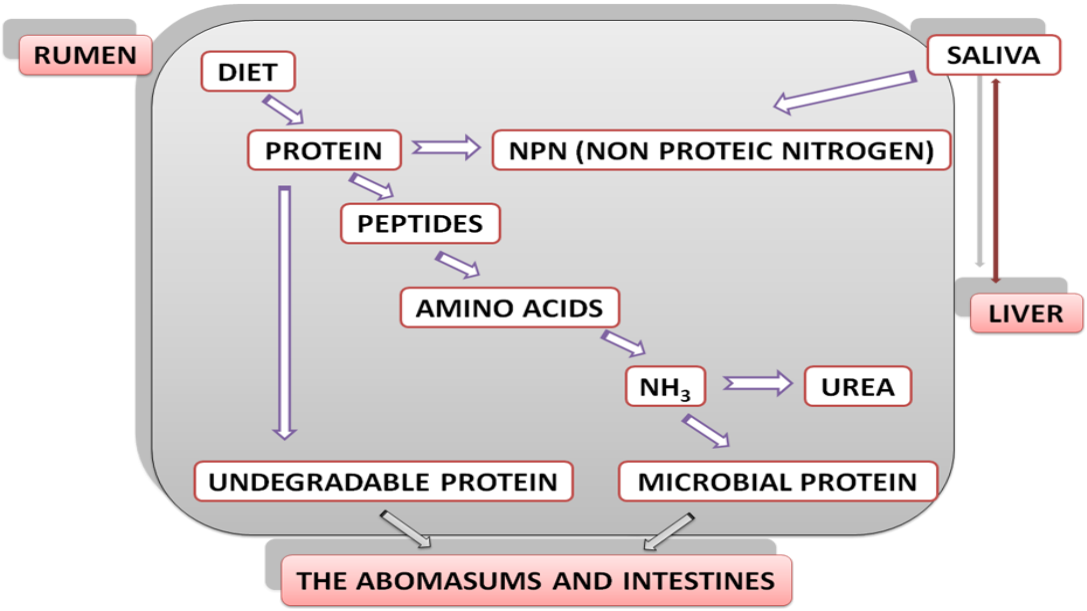


Figure 1. 2. Degradation of protein (adapted from Jayawardena. 2000)

However, other studies have showed that the supplementation of dairy cattle feed with concentration of 20% of decaffeinated tea waste, which has a high content of polyphenols, did not show any effect on milk yield or quality (Konwar *et al*., 1990).

Kim *et al*. (2001) and Ko *et al*. (2006) observed that the digestibility of nutrient (crude protein and crude fiber) increased with the addition of dried wormwood (*Artemisia sp*.) on sheep diet. Kim *et al*. (2001) also reported a significant increase in intestinal

bifidobacteria and a decrease in *clostridia* and *E. coli*. Moreover, recent research carried out by Hristov *et al*. (2013) in lactating dairy cows, using leaf material from *Origanum vulgare* L. as single dose, did not affect negatively rumen fermentation, total-tract apparent digestibility of dietary nutrients, milk yield and composition. And, by using aromatic plant extracts, Chiofalo *et al*. (2012) report that ewes, fed high dose of rosemary extract, yielded more milk than those of the control group. Moreover, the supplementation increased significantly the daily milk production of fat, protein, casein and lactose. The protein, casein and lactose yield followed the linear increment of milk yield as rosemary extract supplementation increased.

1.5.2. Dietary fat

Fat is an important energy component in the diet of ruminants (Bauman *et al*., 2003). Moreover, it becomes a common practice to increase the fat content and to change the fatty acid composition of milk (Chilliard *et al*., 2007; Shingfield *et al*., 2008).

Lipid digestion in ruminants is unique because feed lipids are placed into a hydrolytic and reductive environment after the ingestion process; the glycerol from triacylglycerols and phospholipids are fermented to volatile fatty acids and the unsaturated fatty acids are hydrogenated to mostly saturated fatty acids (Nafikov and Beitz, 2007).

The pathways of the ruminal biohydrogenation process in the rumen are the principal factors affecting the passage of fatty acids from the diet to the milk. The pathways of biohydrogenation of the major dietary polyunsaturated fatty acids (PUFA); linoleic acid (18:2n-6) and linolenic (18:3n-3) acids, as reported by Kim *et al*. (2009), were established in classical studies conducted during the 1960s through to 1980s and were reviewed by Palmquist *et al*. (2005), Bauman and Lock (2006) and Jenkins *et al*. (2008).

When dietary lipids enter the rumen, two important steps occur in the lipid metabolism; the initial step is the hydrolysis of the ester linkages in triglycerides, phospholipids, and glycolipids and the second is the biohydrogenation process of the unsaturated fatty acids which is the major transformation that dietary lipids can undergo in the rumen (Baumon *et al*., 2003) as shown in the Figure 1.3.

The first stage of the biohydrogenation pathway involves an isomerisation reaction that converts the *cis*-12 double bond of the linoleic acid to a *trans*-11 isomer. The next stage is the hydrogenation of the *cis*-9 double bond, by a microbial reductase, with the formation of *trans*-11 fatty acid. And, the final step is a further hydrogenation of the

trans-11 double bond producing the stearic acid (linoleic and linolenic acid pathways) or the *trans*-15 18:1 (linolenic acid pathway) (Harfoot and Hazlewood, 1997; Bauman *et al*., 2003).

The two predominant isomers of CLA are *cis*-9, *trans*-11 (namely rumenic acid, RA) and *trans*-10, *cis*-12, with RA being the most abundant (~75 to 90%) (Kim *et al*., 2009). The CLA found in ruminant milk appears to originate either directly from the rumen or by tissue desaturation of rumen-derived *trans*-11 C18:1 (namely vaccenic acid, VA) (Bauman *et al*., 1999). However, little *cis*-9, *trans*-11 CLA accumulates in the rumen and the majority of milk CLA is derived from vaccenic acid in the mammary gland by the action of delta-9 -desaturase (Griinari *et al*., 2000).

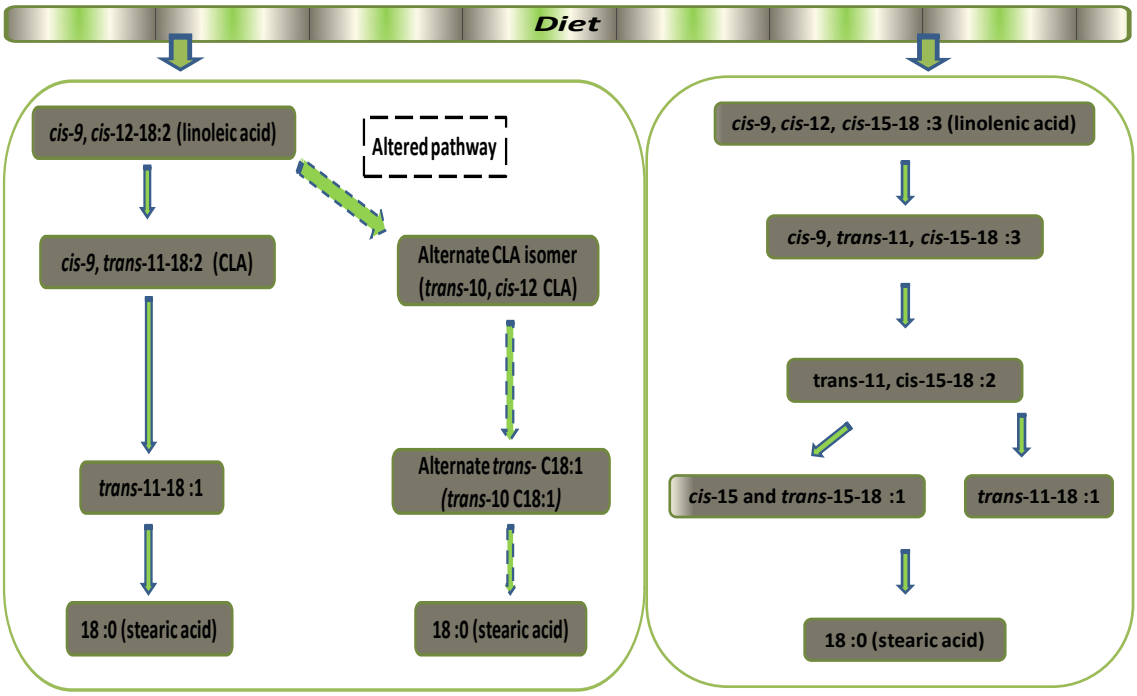


Figure 1. 3. Biohydrogenation pathways and altered biohydrogenation pathway of linoleic acid and linolenic acid in the rumen (adapted from Harfoot and Hazlewood, 1997 and Harvatine et al., 2009)

Shingfield *et al*. (2008) demonstrated that the major pathway of ruminal linoleic acid biohydrogenation involves the formation of vaccenic acid as an intermediate metabolite. However, trans-10 C18:1 could be also obtained by an alternative biohydrogenation pathway from trans-10, cis-12 C18:2 (Griinari *et al*., 1998, Figure 1.3). And, the increase of the concentration of this intermediate in milk fat is generally attributed to alterations in the rumen environmental conditions as a result of dietary changes (Bauman and Griinari, 2001; Chilliard and Ferlay, 2004; Palmquist *et al*., 2005).

The responses to lipid supplements differ between species (Chilliard *et al*., 2003; Pulina *et al*., 2006) and even among small ruminants (Sanz-Sampelayo *et al*., 2007; Tsiplakou and Zervas, 2008). Authors reported the effect of fat supplementation on milk yield, composition and fatty acid profile in dairy cows (Glasser *et al*., 2008). However, few data are available for dairy sheep, but recently; new researches showed that lipid supplementation in dairy ewe diets could be an effective nutritional strategy for improving the milk production and composition as well as some specific fatty acid profile.

The incorporation of free oils in cow's diets has proved to be an efficient procedure for increasing CLA content in milk (Glasser *et al*., 2008) showing better results than diets with whole seeds (Chilliard *et al*., 2009; Doreau *et al*., 2009). But, the use of high doses was frequently discouraged (Garnsworthy. 1997; Palmquist *et al*., 2005) because it can interfere with microbial fermentation in the rumen and might consequently affect the production and the composition of milk (Jenkins and McGuire, 2006). Indeed, whereas oils in free form tend to depress milk fat percentage, supplementation with oilseeds maintains or increases milk fat content (Glasser *et al*., 2008). The reason could be associated to oilseeds are generally accepted to provide some level of rumen protection from biohydrogenation due to the nature of their outer seed coats (Jenkins and McGuire, 2006) and also because oil in seeds may have been released slowly during ruminal digestion, possibly reducing the accumulation and amount of C18:1 *trans* fatty acids leaving the rumen, thus reducing the potential for milk fat depression (Gómez-Cortés *et al*., 2008a). However, in dairy ewes, recent studies, has shown that diets highly supplemented with vegetal oils modified the FA profile in dairy ewes (Gómez-Cortés *et al*., 2011a,b; Bodas *et al*., 2010) with no detrimental effects on animal performance (Gómez-Cortés *et al*., 2008a; Hervas *et al*., 2008; Castro *et al*., 2009). And, this may be the reason why there is more studies carried out with oilseeds in cows than in ewes. Another reason for the discrepancy between cows and ewes might be related to differences in lipid metabolism among ruminant species (Chilliard *et al*., 2003; Reynolds *et al*., 2006) or to the ability of ewes to ruminate and have normal rumen function, even when fed finely ground diets that cause rumen acidosis in cows (Pulina *et al*., 2006).

Also the limited use of oilseeds in dairy sheep diets is the fact that the results with vegetal oils are better than those with oilseed supplementation. Indeed, Zhang *et al*.

(2006b) reported an increase in VA and RA levels with sunflower seed but it was lower than that observed with sunflower oil by Hervas *et al*. (2008). And, the same pattern was observed with linseed administrated as seed or as oil by Gómez-Cortés *et al*. (2009) and Bodas *et al*. (2010).

In dairy sheep, oilseed supplementations induce changes in milk yield and in the major milk constituents (Table 1.2). Variations in milk yield depend on the diet composition. Indeed, Mughetti *et al*. (2012) reported an increase with an incorporation of 10% of extruded linseed and a decrease when the level was doubled. According to Sarrazin *et al*., (2004) moderate levels of fat inclusion in the diet may increase milk yield by improving feed efficiency, while higher inclusion levels may reduce feed intake (Petit *et al*., 2004) and milk yield by depressing ruminal function (Gonthier *et al*., 2005). However, this pattern was different with flaxseed, where the highest level of supplementation (26%) induces the highest milk yield in comparison with the other levels of supplementation (9 and 18%) (Zhang *et al*., 2006a).

Different authors have reported results regarding milk fat content; decrease (Luna *et al*., 2005), increase (Zhang *et al*., 2006b) or no difference (Mughetti *et al*., 2012). Moreover, Zhang *et al*. (2006a) observed that oilseeds with high ruminal degradability (e.g., sunflower seed) might have more detrimental impacts on milk fat proportion than oilseeds with relatively low ruminal degradability (e.g., flaxseed). For protein content, Zhang *et al*. (2006b) observed a decrease while other authors reported no changes (Zhang *et al*., 2006a; Gómez-Cortés *et al*., 2009; Mughetti *et al*., 2012) with oilseeds administration. The fatty acid composition of milk fat was as well affected by the dietary oilseed supplementations. The concentrations of short-chain fatty acids decreased (Zhang *et al*., 2006a,b; Mughetti *et al*., 2012), while the total PUFA increased (Zhang *et al*., 2006a,b; Mughetti *et al*., 2012).

The CLA content was positively influenced by the oilseeds supplementation. The differences in CLA level is mainly attributed to differences in *trans*-11 C18:1 level, which is the main precursor of CLA in the rumen (Zhang *et al*., 2006a; Gómez-Cortés *et al*., 2009). However, the differences in CLA concentrations in the study carried out by Zhang *et al*. (2006b) was as well associated with the levels of C18: 2 in the diet, with the CLA being an intermediate product in the biohydrogenation process of C18:2.

Table 1. 2. Effect of the inclusion of oilseeds in the diet of dairy sheep on milk yield, composition and fatty acids profile

Reference	*Plant seeds*	*Rate of inclusion*	*Milk yield (g/d)*	*Chemical composition of milk (%)*		*Fatty acid composition of milk (g/100g of total fatty acids)*				
				Fat content	Protein content	SCFA[1]	PUFA[2]	Trans 10 C18:1	VA[3]	RA[4]
Gómez-Cortés *et al*. 2009	Control	0%	534 [b]	6,51 [a]	5,89 [a]	21,43	-	0,53 [a]	1,55 [c]	0,73 [c]
	Low extruded linseed	6%	910 [a]	6,10 [a]	5,38 [a]	18,43	-	0,53 [a]	3,7 [b]	1,35 [b]
	High extruded linseed	12%	824 [a]	6,12 [a]	5,36 [a]	16,62	-	0,5 [a]	5,76 [a]	2,33 [a]
Zhang *et al*. 2006a	Control	0%	816 [b]	7,54 [b]	6,39 [a]	22,4 [a]	8,4 [c]	-	2,3 [d]	1,1 [d]
	Flaxseed	9%	848 [ab]	7,78 [b]	6,5 [a]	21,8 [b]	9,5 [b]	-	3 [c]	1,2 [c]
	Flaxseed	18%	844 [ab]	8,62 [a]	6,6 [a]	19,8 [b]	9,7 [b]	-	3,8 [b]	1,3 [b]
	Flaxseed	26%	868 [a]	8,5 [a]	6,29 [a]	18,9 [b]	11,8 [a]	-	4,2 [a]	1,9 [a]
Zhang *et al*. 2006b	Control	0%	1104 [a]	6,67 [a]	4,76 [a]	19 [a]	4,4 [c]	-	0,9 [b]	1 [c]
	Flaxseed	6,7%	1197 [a]	6,52 [ab]	4,71 [ab]	11,1 [c]	7,1 [b]	-	1,5 [a]	1,5 [b]
	Sunflower seed	5,9%	1062 [b]	6,41 [b]	4,65 [b]	12,5 [b]	8,2 [a]	-	2,3 [a]	2,3 [a]
Mughetti *et al*. 2012	Control	0%	1362	5,91	4,81	22,74 [a]	4,39 [c]	-	-	-
	Linseed	10%	1404	6,07	4,81	18,39 [b]	5,22 [b]	-	-	-
	Linseed	20%	1218	6,10	4,9	15,28 [c]	5,85 [a]	-	-	-

[1] short chain fatty acids: (C4:0-C12:0); [2] Poly-unsaturated fatty acids; [3] Vaccenic acid: trans-11 C18:1; [4] Rumenic acid: cis-9 trans 11

1.6. Situation of the dairy sheep sector

The world's dairy sheep flock increased substantially during the last five decades as shown in the Figure 1.4. By continents, in 2011, Asia showed more dairy sheep flock with 51%, followed by Africa with 33%, following by Europe which exceeds 15% and finally by the United States of America (USA) with less than 1% (FAO. 2013).

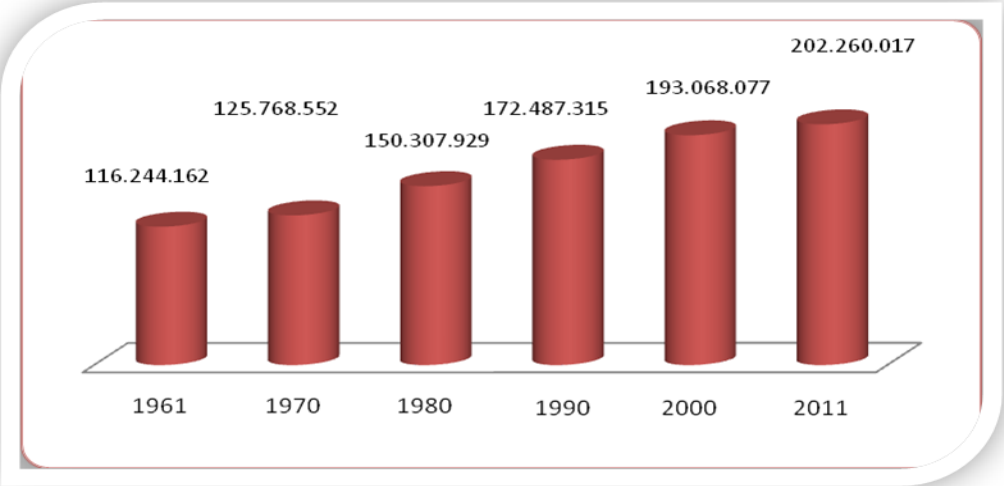


Figure 1. 4. The evolution of the world's dairy sheep flock (heads) (Adapted from FAO, 2013)

According to the FAO (2013), the sheep milk production around the world has suffered a spectacular rate of increase (92%), between 1961 and 2011, which coincides with the growth of sheep flock (74%). This positive relationship between flock and milk production occurred in Asia, Africa and United sates of America but not for Europe. Indeed, in Europe, an increment of the milk production was reported (Table 1.3) in spite of the decrease of flock.

The increase in milk production was thereby attributed to the enhancement of production per milking ewe, which was mainly associated to the improvement of the systems of production. The individual production oscillated from 57.9 kg/ewe in 1961 to 98.7 kg in 2011 with fluctuation from a maximum of 98.7 kg/ewe in Europe until a minimum of 32 kg/ewe in Africa, which is an indicator of the large specialization of dairy sheep management in Europe with respect to the other continents.

Table 1. 3. The distribution and the evolution of the sheep milk production (tones) per continent and around the world

	1961	*1970*	*1980*	*1990*	*2000*	*2011*
Africa	545.469	720.137	1.247.826	1.531.887	1.709.968	2.147.317
Americas	21.980	27.010	31.550	34.854	35.120	40.607
Asia	2.136.871	2.254.415	2.922.537	3.465.782	3.533.964	4.543.499
Europe	2.396.088	2.495.868	2.620.808	3.027.324	2.880.886	3.038.316
World	**5.100.408**	**5.497.430**	**6.822.721**	**8.059.847**	**8.159.939**	**9.769.739**

Source: Adapted from FAO (2013)

The main producers of sheep milk in Europe, in 2011, were in this order; Greece with 25.1% of the total milk produced in Europe followed by Romania (20.5%), Spain and Italy with 16.7% and 13.5% respectively, France with 8.9% and finally Bulgaria, Albania and Portugal with more than 2.0% each. As a whole, these 8 countries concentrate 92.7% of the total milk of sheep produced in Europe (Figure 1.5).

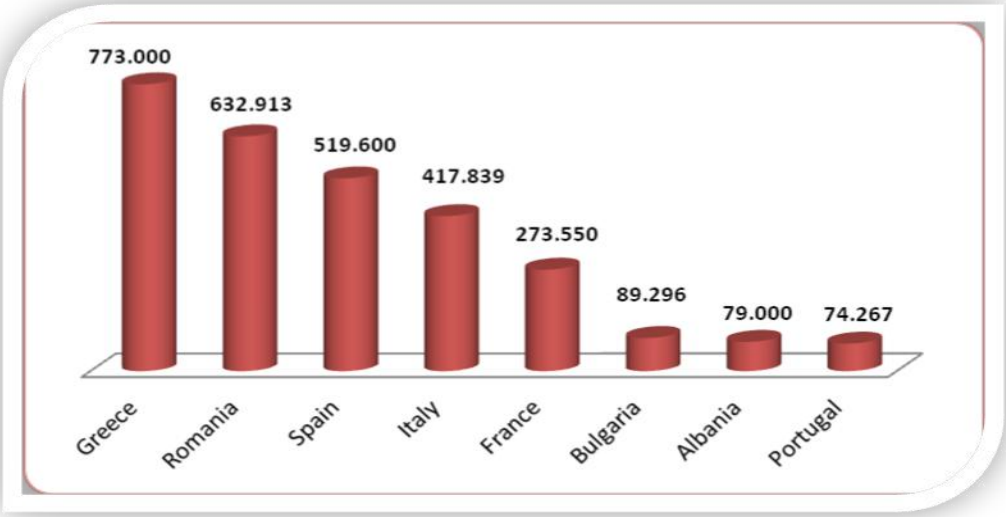


Figure 1. 5. The production of the sheep milk (t×1000) in the European countries (2011) (Adapted from FAO, 2013)

The study of the milk production per ewe in the main European countries, in 2011, shown that France holds the first place in the production of sheep milk with almost 211 kg/ewe followed by Spain and Portugal with almost 186 and 184 kg/ewe respectively (FAO. 2013).

The sheep sector in Spain has experimented an important role due to cultural and ecological reasons and also because of its contribution to the socio-economical life of the country despite of its relative importance in comparison with the bovine sector.

The distribution of the milk production in Spain, in 2011, has shown that 87% of the milk produced came from cows, 7% from ewes and finally 6% from goats (Figure1.6).

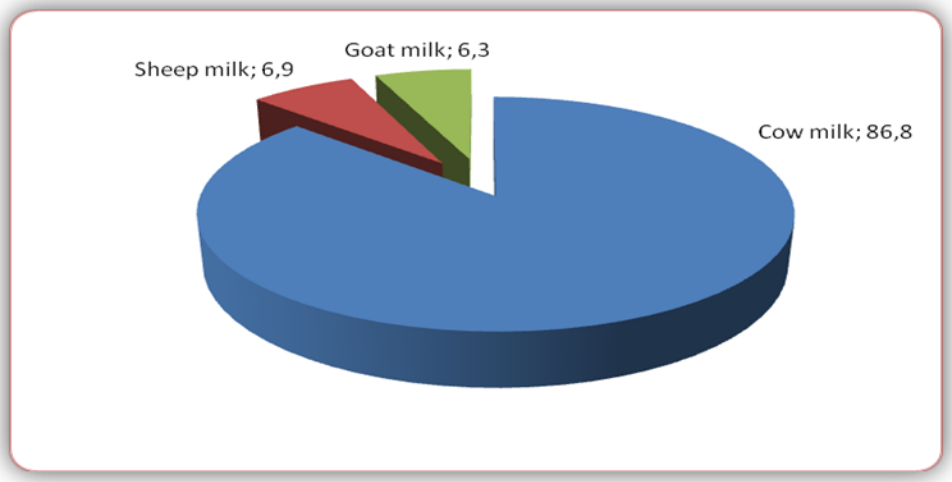


Figure 1. 6. The distribution of the milk production (%) in Spain according to the specie (2011) (Adapted from MAGRAMA, 2012)

The sheep flock in Spain had shown significant changes during the last five decades (Figure 1.7). An important decrease was observed between the sixties and the eighties with a reduction of 35.7% of registered sheep. From 1980's to 2000's, there was a recovery with almost 10 million animals, which was the result of the incorporation of Spain into the European Union (EU) in 1986. However, the spanish livestock start to decrease again in the years 2000 to 2012, due mainly to the reforms imposed by the Common Agricultural Policy (CAP), the changes in consumer tastes and the rise of prices of raw materials and animal health treatments, which decrease the interest of farmers as to this activity (Rodríguez. 2013).

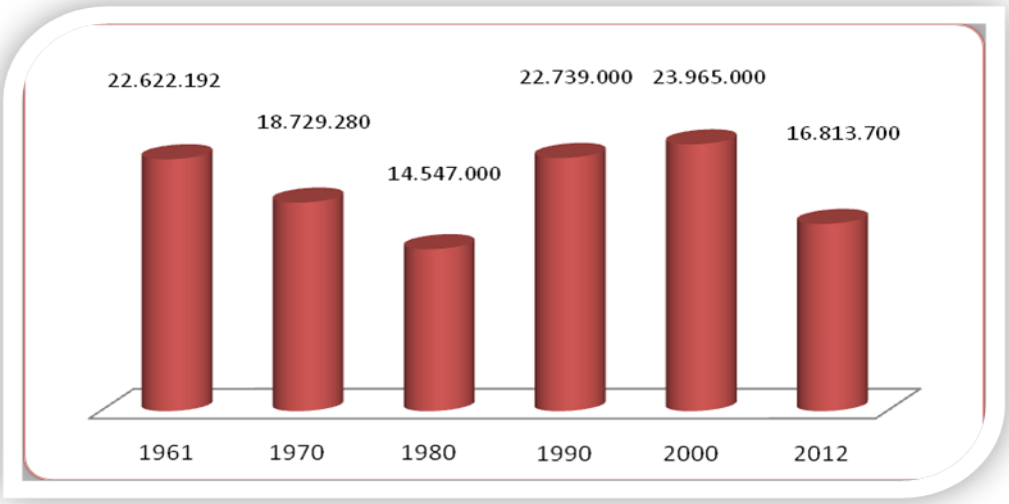


Figure 1. 7. The evolution of the sheep flock in Spain (heads) (Adapted from FAO, 2013)

In the other hand and inspite of the changes observed in sheep folck, milk production had seen a huge increase between 1980 and 2011 (Figure 1.8).

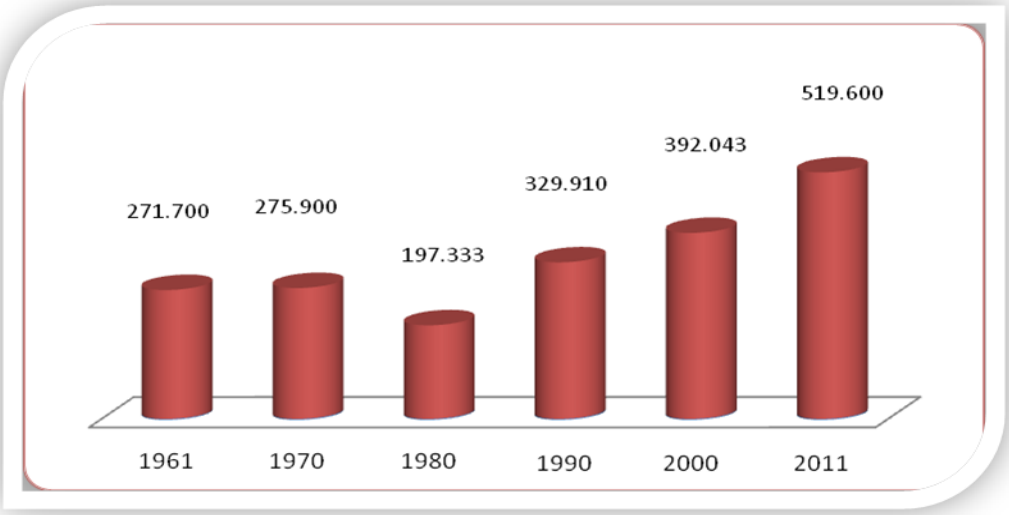


Figure 1. 8. The evolution of the sheep milk production in Spain (t×1000) (Adapted from FAO, 2013)

The reason for such increase was attributed to the enhancement of productivity per ewe. The individual milk production increased, from 66.4 kg/ewe in 1987 to 185.6 kg/ewe in 2011. And, this improvement in productivity was due to the intensification of sheep farms by the introduction of new breeds specialized in milk production (Ugarte *et al*., 2001; Caja and Rancourt, 2002), the improvement of feeding systems and reproduction and health management. Those efforts for intensification were the results of the increase of sheep milk price due to the growing demand for sheep dairy products (cheese, curds, etc.) (Rodríguez-Ruiz. 2013).

In Spain, the largest sheep flocks occur in Extremadura, Castilla y León, and Castilla La Mancha and then followed by Andalucía and Aragón, with nearly 80% of the total (Figure 1.9).

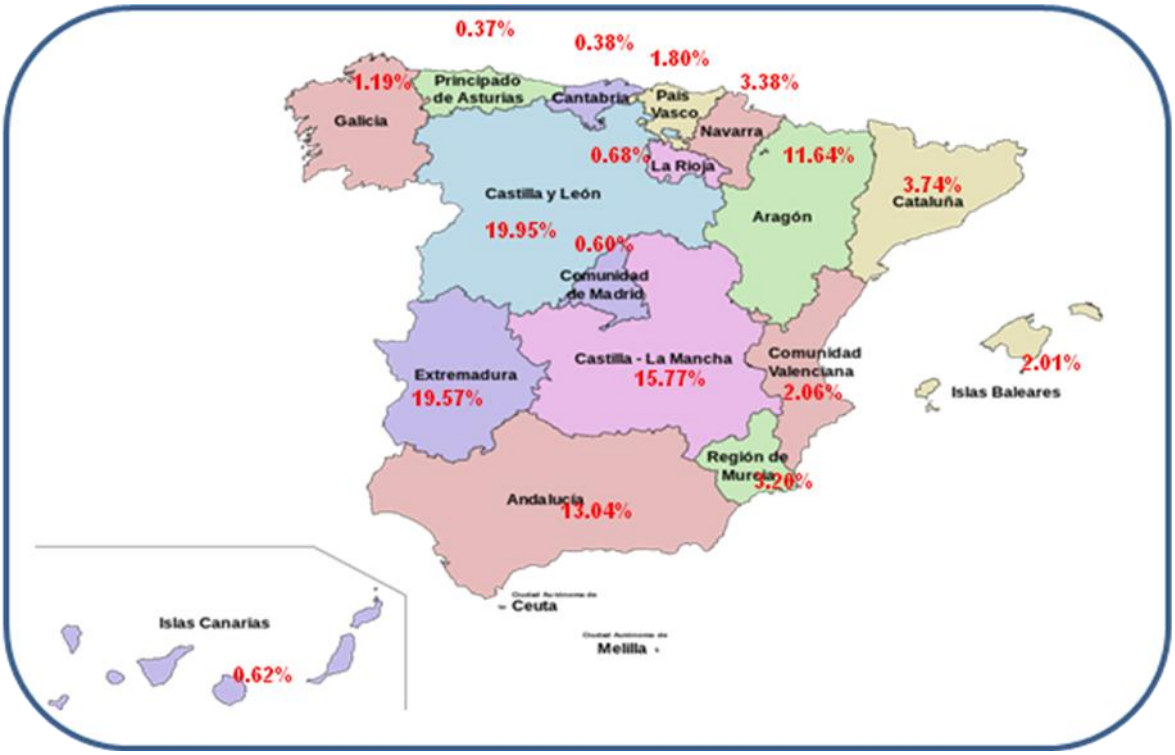


Figure 1. 9. The distribution of the sheep livestock in Spain (%) (Adapted from MAGRAMA, 2012)

At national level, Castilla y León is the community with the highest percentage of sheep with 19.95% of the total number of animals in comparison with the rest of communities in Spain (Figure 1.10).

The sheep flock in Castilla y León increase after the incorporation of Spain into the European common market (1980-2000). However, in the last 10 years, a decrease was observed, due to the changes in the policy of the EU in relation to subsidies.

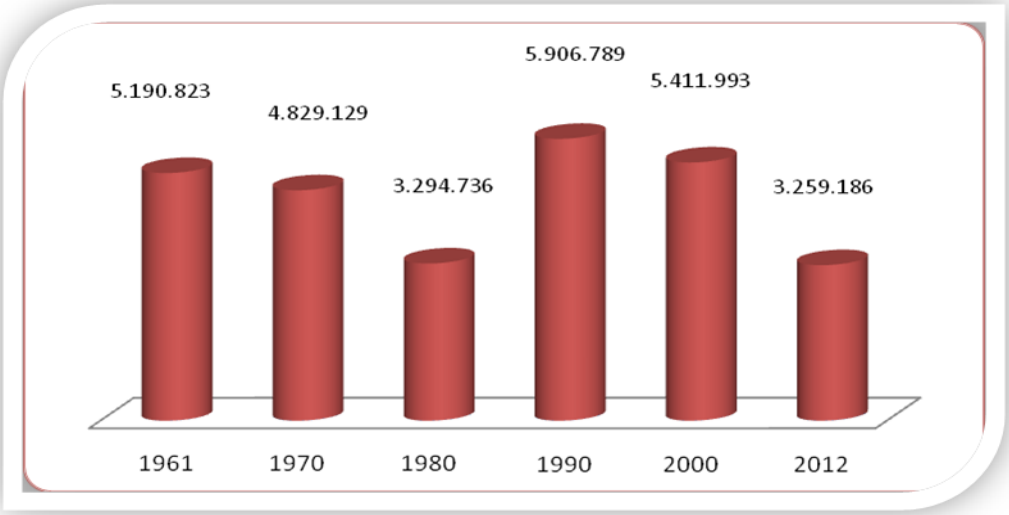


Figure 1. 10. The evolution of the sheep flock in Castile and León (heads) (Adapted from MAGRAMA, 2012)

During the last five decades, the region of Castilla y León has seen a continuous growth in sheep milk production (Figure 1.11).

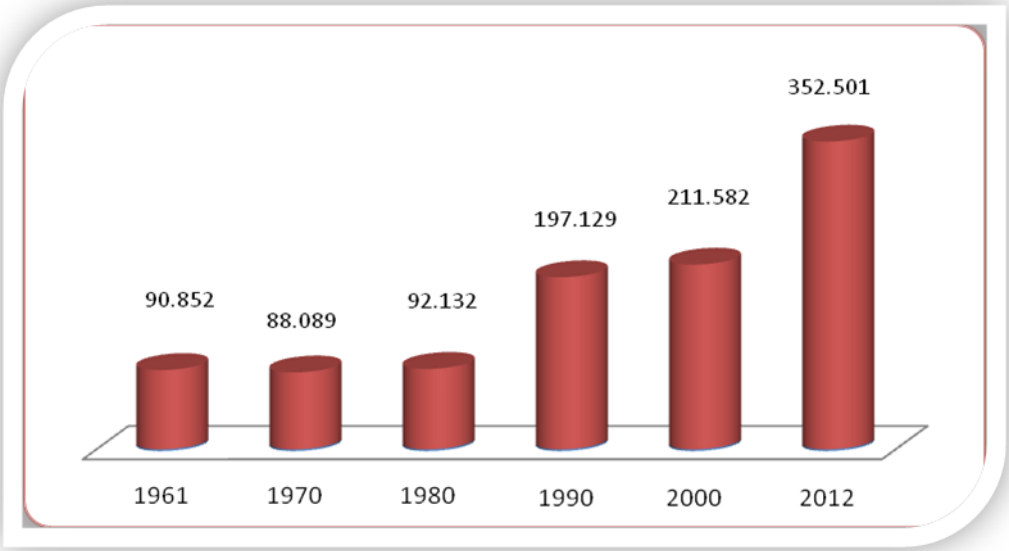


Figure 1. 11. The evolution of the sheep milk production in Castilla y León (tones) (Adapted from MAGRAMA, 2012)

This increase can be associated to the increase of the productivity per ewe mainly due to the genetic improvement of breeds (mainly Assaf, Awassi and Lacaune), the intensification of the systems of production and the increase of demand for sheep milk by the dairy industries. According to MAGRAMA (2011), the milk produced by ewe in Castilla y León presents an average of 312 liters/ewe which is more than the double of the individual production in Castilla la Mancha (128 litres/ewe). And values are even lower in the case of Pais Vasco, Navarra and Extremadura from 59, 52 and 23 litres per year and ewe, respectively.

1.7. Microbiological and physico-chemical properties of sheep milk

The microbiological and physico-chemical properties of milk are essential for a successful development of dairy products and for consumers' health protection.

According to the Regulation (EC) No 853/2004, raw milk is defined as:

"Raw milk means milk produced by the secretion of the mammary gland of farmed animals that has not been heated to more than 40 °C or undergone any treatment that has an equivalent effect"

The Regulation (EC) No 1662/2006 amending the Regulation (EC) No 853/2004 lays down specific hygiene rules for food of animal origin. The germ content in raw milk, from species other than cow, used for the manufacture of dairy products, has to be inferior to 1.5 10^5 UFC/ml at 30ºC for products manufactured without heat treatment and 1.5 10^6 UFC/ml at 30ºC for heat-treated products.

The physical parameters of milk are essential markers for the monitoring of milk quality; the freezing point allow the identification of fraud due to the addition of water (Antunac *et al*., 2001) and the pH can be used as a pointer to the hygienic qualities. Furthermore, the physical properties of milk affect its technological properties such as the acidic coagulation (Ould Eleya *et al*., 1995). Sheep milk shows higher viscosity, acidity and pH than goat and cow milk while no differences were observed between species concerning density and freezing point (Park *et al*., 2007).

Milk always contains the same basic components (carbohydrates, protein, fat and minerals). However, there are some differences, as shown in the Table 1.4. Sheep milk contains higher total solids and higher fat content than goat and cow milk (Hilali *et al*.,

2011; Pandya and Ghodke, 2007; Park *et al*., 2007) as well as the highest energy value (Barłowska *et al*., 2011).

Fat and protein in sheep milk represent a higher percentage of the total solids in comparison with goat and cow milks, which is important because it determines the cheese yield that can be obtained from each milk (Hilali *et al*., 2011). However lactation milk yield and lactation length of milking ewes is lower and shorter than goat or cows which is negatively perceived by farmers (Haenlein. 2002).

Table 1. 4. Chemical composition of milk from sheep, goat and cow milk

	Sheep	**Goat**	**Cow**
Total solid (g/100g) [a]	17.0	11.1	12.2
Fat (g/100g) [b]	6.99 ± 1.23	4.07 ± 0.76	4.09 ± 0.46
Protein (g/100g) [b]	5.73 ± 0.61	3.26 ± 0.46	3.42 ± 0.35
Lactose (g/100g) [b]	4.75 ± 0.35	4.51 ± 0.26	4.82 ± 0.21
Casein (g/100g) [c]	4.2	2.4	2.6
Fatty acids (g/100g) [a]			
Saturated	3.8	2.3	2.4
Mono-unsaturated	1.5	0.8	1.1
Poly-unsaturated	0.3	0.1	0.1
Cholesterol (mg/100g)	10.0	10.0	14.0
Energy value (Kcal/100g)	95.0	60.0	66.0

Source: Tamime *et al*. (2011)[a], Barłowska *et al*. (2011)[b], Park *et al*. (2007) [c]

Carbohydrates

Lactose is the major carbohydrate in milk (Park *et al*., 2007). There is not important difference in lactose content between cow, sheep and goat. However, as the fat and protein in sheep milk is higher than in cow and goat milk, the proportion of lactose content in the total solids content is lower. The concentration of lactose in sheep milk as in other ruminants is lower at the beginning and towards the end of lactation, which is positively correlated with the milk yield (Pulina and Nudda, 2004; Haenlein and Wendorff, 2006).

From a nutritional point of view, lactose is a valuable nutrient, because it favors intestinal absorption of calcium, magnesium and phosphorus, and the utilization of Vitamin C (Hernández Ledesma *et al*., 2011). Moreover, Tamime *et al*., (2011) report

that sheep milks contain enough lactose to provide an energy source for the starter culture organisms.

Lipids

Lipids are one of the most important components of milk in terms of physical, organoleptic and nutritional properties that they impart to dairy products (Fontecha *et al*., 2011).

Milk fat is synthesized in the milk cells of the udder (Barłowska *et al*., 2011) and occurs as globules emulsified in the aqueous phase (87%) of milk (Jensen, 2002). Fat milk is composed by triglycerides (TAG; nearly 98%), diacylglycerides (0.25-0.48%), monoacylglycerides (0.02-0.04%), phospholipids (0.6-1.0%), cholesterol (0.2-0.4%), glycolipids (0.006%) and free fatty acids in milk (0.1-0.4%).

The average diameter of milk fat globules is less than 0.1 μm to approximately 18 μm (El-Zeini. 2006). In ewe milk, the fat globules are characteristically abundant in sizes less than 3.5 μm, which is similar to goat fat globules. According to Park (1994) smaller is the size of fat globules better is the digestibility and more efficient is the lipid metabolism. No appreciable differences have been reported as regards the mechanism of milk fat globule secretion in sheep and goats in comparison with cows (Park *et al*., 2007). Furthermore, structure and composition of the membrane is similar in the three species (Scolozzi *et al*., 2003).

In the other hand, the importance of the triacylglycerols species (TAG) contained in dairy fats is associated to their influence on technological aspects (melting point, crystallization behavior, etc.) and nutrition (action of lipolytic enzymes) (Fontecha *et al*., 2005). The triglyceride composition of goat, sheep and cow milk fat is different (Scintu and Piredda, 2007). According to Park *et al*., (2007), the percentage of triglycerides (TAG) with carbon number from 26 to 36 and 46 to 54 are higher in ewes in comparison with cows. However, Precht (1992) reported similarities in the chromatographic triglyceride profile between ewes and cows.

As regards fatty acid composition, the levels of short and medium chain fatty acids are significantly higher in sheep and goat in comparison with cow (Alonso *et al*., 1999; Goudjil *et al*., 2004). Furthermore, sheep milk, in general, present the highest quantities of *trans* fatty acids after cow and finally goat. However, the proportion of vaccenic acid in milk fat, which is the precursor in the synthesis of the conjugated linoleic acid (CLA)

(cis-9 trans-11C18:2), is similar in the three species (around 45–60%; Jensen. 2002; Goudjil *et al*., 2004). Concerning the total conjugated linoleic acid, the mean content seems to decrease in the following order: ewe > cow> goat milk fat, (1.08%; 1.01% and 0.65%, respectively) (Jahreis *et al*., 1999).

Phospholipids (PLs) are associated with the milk fat globule membrane (MFGM) and PLs species are expressed as % of total phospholipids. The most abundant PLs are phosphatidylethanolamine (PE) followed by phosphatidylcholine (PC) and sphingomyelin (SM) and then by phosphatidylserine (PS) and phosphatidyl-inositol (PI). Most of the studies reported values very similar between species (Andreotti *et al*., 2006; Garcia *et al*., 2013).

Protein

The main component of milk are proteins (Barłowska *et al*., 2011), which impact directly on the ability of milk to be processed and on the quality of dairy products (Albenzio and Santillo, 2011).

Milk proteins consist of caseins (αs1, αs2, β and κ) and whey proteins (βeta lactoglobulin and alpha-lactalbumin), and some proteins coming from blood, principally serum albumin and immunoglobulin (Scintu and Piredda, 2007).

The average protein content in sheep milk is higher than in goat or cow milk (Table 2.6). Proteins contribute approximately with 96% of the total nitrogen in sheep milk and 4% corresponds to non-protein nitrogen (NPN) (Hilali *et al*., 2011). Casein is the most important protein in milk, while the proportion of whey proteins are relatively lower (Barłowska *et al*., 2011). In comparison with goat and cow milk, sheep milk contains the highest content on whey proteins and also contains the highest content of casein (Dario *et al*., 2008), which provides a very good clotting ability to the milk (Park *et al*., 2007) and affects positively cheese yield and consistency and texture in fermented products (Guo. 2003).

The percentage of casein in the total protein (80.8–82.6%) remains almost stable over the lactation period, with a small decline close to the end of lactation (Pellegrini *et al*., 1994; Hilali. 2001), accompanied with an increase in whey proteins content. And, this increase has technological implications, such the production of curd with a weaker texture (Hilali. 2001). In general, the variations are more common in the casein fraction than in the whey protein fraction. The heterogeneity of caseins is determined by the

presence of genetic variants or by other factors such as a discrete phosphorylation level, variation in the extent of glycosylation of the κ- casein fraction, and the coexistence of proteins with different chain lengths (Park *et al*., 2007). The genetic polymorphisms of sheep milk proteins is very important because they are associated to quantitative and qualitative parameters in milk and milk derivates (yogurt, cheese, etc) (Albenzio and Santillo, 2011).

Minerals and vitamins

Sheep milk has around 0.9% total minerals compared to 0.7% in cow milk and 0.8% in goat milk (Park *et al*., 2007). Mineral content of sheep milk is significantly higher than that in goat or cow milk (Hilali *et al*., 2011), with the highest proportion of calcium, phosphorus, magnesium, iron, copper, iodine, selenium and zinc (Barłowska *et al*., 2011). In general, mineral contents of sheep milk seem to vary much more than those of cow milk (Rincon *et al*., 1994).

Milk is a valuable source of vitamins as well, both water-soluble and fat-soluble ones (Barłowska *et al*., 2011). The vitamin content is higher in sheep milk than in cow and goat milk, except for vitamine D and Biotin (Park *et al*., 2007). However, the carotene content of ovine and caprine milks is negligible compared with bovine milk and, as a consequence, the colour of the product will be whiter (Tamime *et al*., 2011).

1.8. Factors influencing the composition and the quality of milk

Many factors affect the composition and the quality of milk and can be classified as intrinsic (inherent to animal) or extrinsic (depend on external factors) (Ahmad *et al*., 2008; Aganga *et al*., 2002; Haenlein. 2002; Park *et al*., 2007).

1.8.1. Intrinsic factors

Genetics

There are important genetic differences associated to species, breeds and selected families (Haenlein. 2002). In the last decades, most of the genetic programs have been focused on the improvement of milk yield (Barillet. 1997). But, recently and by considering the fact that negative correlation exists between milk yield and fat and protein content, milk recording schemes, including the estimation of milk parameters such as fat, protein and total solid, were developed, in order to get a simultaneous

improvement of quantity and composition of milk (Petrović *et al*., 2005). Moreover, investigations have been carried out to develop indirect method to estimate cheese yield (Othmane *et al*., 1995, 2000).

Stage of lactation

Regardless of species or breed, the stage of lactation is an important factor that has a considerable influence on milk composition (Haenlein. 2002). Many components, especially fat and protein, are high in early lactation; much lower thereafter until they rise again markedly at the end of lactation; meanwhile the inverse occurs for milk yield. Mineral content also increased with stage of lactation.

Parity

The effect of parity on milk yield and quality in ewes is subject of controversy. An increase in milk yield as the number of lactation advanced was observed by Casoli *et al*. (1989), Hatziminaoglou *et al*. (1990) and Ubertalle *et al*. (1990), whereas Dell'Aquila *et al*. (1993) and Sevi *et al*. (2000) did not found any significant effect. In the other hand, an enhancement in protein and fat content with the increasing number of lactations has been reported by Casoli *et al*. (1989), Dell'Aquila *et al*. (1993) and sevi *et al*. (2000). However, an opposite trend as regards milk composition has been observed by Ubertalle (1989). According to Sevi *et al*. (2000), the increase of milk composition could be associated to the increase of body weight of ewes which leads to a better availability of body reserves for the synthesis of milk components, or to the better development of the udder glandular tissue resulting to an increase in the milk constituent's synthesis.

However, the interference with other factors can make difficult the analysis of results, such as the milk yield level (Casoli *et al*., 1989).

Physiological status

Nutritional physiology and endocrine status of the animal affects milk yield and composition over short or longer time period (Haenlein. 2002). This has been demonstrated by the variation of milk composition during oestrous cycle (Cowan and Larson, 1979). For example high somatotropin loads to the increase of milk yield, milk fat content, short-chain and medium-chain fatty acids significantly, while decreasing milk protein percentage, long-chain fatty acids and net energy balance (Disenhaus *et al*., 1995).

Udder health

Mammary involution and inflammation are known to negatively affect milk quality towards poor milk composition and altered coagulating properties of milk (Albenzio *et al*., 2002, 2003). Indeed, infected udders in comparison with healthy or doubtful udders decrease lactose and fat content, the casein index and were negatively associated with curd characteristics, by affecting clotting time and curd firmness (Bianchi *et al*., 2004).

1.8.2. Extrinsic factors

External factors such as season, milking system, diets affect directly the milk yield and composition. Moreover, extrinsic factors may exert and indirect effect; animals affected by external factors cannot express all its genetic potential (Arranz *et al*., 2001) and therefore milk yield and composition.

Season

There are significant seasonal differences in milk composition (Haenlein. 2002). Winter feeding is providing in general different proportions and qualities of grazing, hays, silage and supplements, which influence considerably milk composition. The greatest seasonal differences were measured in sheep milk, 1.28% in summer and 0.54% at the end of the winter period (Jahreis *et al*., 1999). Furthermore, the effect of photoperiod was as well reported in dairy ewes (Mikolayunas *et al*., 2008).

Milking system

A number of researchers have previously investigated the effects of milking systems (manual and mechanic), the interval between milking and the milking frequency on milk composition. De la Fuente *et al*. (1997) reported significant differences in milk fat and protein content between the manual and the mechanic milking system. Furthermore, the reduction of the milking frequency, in high-yielding ewes, from twice to once a day, decreased lactose concentration and increased protein and total solids content without any effect on fat content (Pulido *et al*., 2012). On the other hand, high milking intervals, superior o equal to 16 h, during mid- and late lactation, did not result in significant changes in milk composition for East Friesian crossbred dairy ewes (McKusick *et al*., 2002).

Nutritional control

Changes in milk composition is realized when one or more desired nutrients are incorporated into the diet, followed by absorption and transport of the nutrient to the mammary gland, and finalized with secretion of the nutrient in milk. The composition and the quantity of the daily diet in relation to the production requirements is the most important factor due to its correlation with changes in milk yield and composition (Moran-Fehr. 1981; Haenlein. 1995).

According to Jenkins and McGuire (2006), the most crucial component of milk to dietary manipulation was fat content, which could be changed over a range of 3%. It was clear that lactose content could not be manipulated by dietary changes, except under extreme and unusual feeding situations. Milk protein was more responsive to diet (over a 0.5-percentage unit range) than lactose, but less responsive than fat. The period from 1980 to 2005 has seen efforts at trying to alter the content or composition of those 3 components. As expected, the greatest changes were made in milk fat and fatty acid composition. Therefore, several studies have examined the modification of milk fat profile through the addition of dietary fat as discussed afterwards. However, the problem of modulating the fatty acid composition of milk was the fact that fatty acids are susceptible to oxidation, which can modify the nutritional and sensory properties of the derived dairy products (Boroski *et al*., 2012). So, including antioxidant started recently to be considered as strategy to inhibit the free radical autoxidation and thereby preserve polyunsaturated lipids from oxidative deterioration, as discussed below.

1.9. Dairy products

1.9.1. Cheese product

1.9.1.1. Definition, classification and regulation of cheese

Cheese is a derived milk product, produced in a wide range of flavours and forms through the world (Fox and McSweeney, 2004).

The original Codex General Standard for Cheese was adopted in 1963 and replaced in 1978. A further revision of the standard was completed in 1999 and amended in 2006. This contains the following definition of cheese relevant to all cheeses:

"Cheese is the ripened or unripened soft or semihard, hard or extra-hard product, which may be coated, and in which the whey protein:casein ratio does not exceed that of milk,

obtained by coagulating totally or partly the protein of milk, skimmed milk, partly skimmed milk, cream, whey cream or buttermilk, or any combination of these materials, through the action of rennet or other suitable coagulating agents, and by partially draining the whey resulting from such coagulation; and/or processing techniques involving coagulation of the protein of milk and/or products obtained from milk which give an end product with similar physical, chemical and organoleptic characteristics".

Cheese, as a stable and versatile food, which can be taken on many forms is very important from an industrial and consume point of view (Johnson and Law, 2010). Cheese technologists have attempted to categorize cheeses based upon various criteria. In Spain, the most relevant criterions, according to the Royal Decree (RD 1113/2006), were those based on milk origin and ripening time.

For cheeses, without certifications of mark (protected designation of origin (PDO), protected geographical indication (PGI) and traditional specialties guaranteed (TSG)) or certification of specific characters, the origin of milk (cow, sheep or goat) needs to be specified. Cheeses can be classified also in soft, semi-hard and hard according to their ripening time as shown in the Table 1.5.

Table 1. 5. Designation of cheeses according to the ripening time

Designation	**Weight >1.5 kg**	**Weight >1.5 kg**
	Minimum ripening time (days)	
Soft	7	
Semi-hard	35	20
Hard	105	45
Old	180	100
Mature	270	

Source: RD 1113/2006

As a product of animal origin, cheese is susceptible to pathogenic contamination, especially the raw-milk cheese. Consequently, Public Health Authorities (PHA) in many countries established basic requirements for producing raw-milk cheese. The first European directive dates from 1992 (92/46/EEC) and has been followed by a number of regulations (852/2004, 853/2004,2073/2005, 2074/2005). The production of raw-milk cheese was allowed, as long as certain minimum requirements were met; milk must come from animals that have no symptoms of infectious diseases that can be transmitted to humans through milk, that are healthy and that have not been given unauthorized

substances or products. Not processed milk must be stored in a clean place at a temperature below 8°C (in case of daily milking) or below 6°C (when milking is not carried out daily). Furthermore, the facilities must be clean, undergo regular maintenance and be kept in good conditions. And finally, microbiological characteristics of raw-milk cheese need to be guaranteed (absence of *Listeria monocytogenes, Salmonella, staphylococcal enterotoxins, Escherichia coli* and *coagulase-negative staphylococci*).

Cheese made from sheep milk

Cows' milk is the major milk used for cheese manufacture in both the world and Europe (92% and 94%, respectively) (FAO, 2013). However, significant quantities of cheese from sheep milk are also produced, especially in the countries with sheep-raising tradition. The main producers of cheese from sheep milk in Europe are Greece with 33.8% followed by Spain (16.9%), Italy and France with 15% and 14.3% respectively (FAO. 2012).7

In Spain, there is a large potential of production accompanied with various differentiated products. Spain provide a large variety of cheeses, many of them are protected by designation of origin (PDO) such as the "Idiazábal" from País Vasco, "Manchego" from Castilla-la-Mancha and "Zamorano" from Castilla y León, and few more in the different regions.

Castilla y León is a spanish community with an important cheese-making tradition, especially from sheep milk. Those cheeses are recognized because of its differentiated characteristics. This differentiation emanates from the model of production used to feed the flock, which is principally based on extensive grazing surfaces rich in natural plants. The cheese that characterizes the community of Castilla y León is the "Castellano cheese". It's a hard variety made from raw or pasteurizad ewes' milk throughout the year, with its peak production in spring months (Gaya *et al*., 2003). Externally, this cheese resembles to other ewes' milk hard spanish cheese varieties such as the manchego cheese.

Castellano cheese is protected at the national level by a collective mark but it still not protected by any of the three European Union schemes, known as PDO PGI and TSG. Efforts are made to obtain a certification of mark at the european level through providing evidences of the historical importance of the Castellano cheese in this

community and by demonstrating its value as a differentiated product. Actually, this recognition will not only help to the preservation of the traditional methods of production, but it will also encourage people to stay settled in rural areas by providing an economic incentive to produce traditional foods. In fact, in Castilla y León, dairy industry provides jobs for a total of 3.137 persons, which is more than the national average, which gives an idea of the importance of this sector at rural level.

The Castellano cheese, manufactured with raw milk, must respect the law 17/2001, the royal decree 687/2002 and the council directive 89/104/CEE, which establish a minimum of 60 days of ripening before consumption.

1.9.1.2. Cheesemaking steps

Johnson and Law (2010) reported that the technology of cheesemaking has two principal goals: firstly, to establish the parameters that make a given cheese desirable (flavour, texture, melt and stretch properties); and, second, to develop a manufacturing and ripening protocol that will routinely reproduce these parameters every time this cheese is made.

The fundamental operations to the production of a good quality cheese are:

Preparation of milk:

Preparation of milk consists on the selection, the standardization and the pasteurization of milk. The standardization is made by the addition of milk solids (condensed or milk powder) or by removing the cream (Johnson and Law, 2010).

Acidification:

It is now almost universal practice in industrial cheesemaking to add a culture (starter) of selected lactic acid-producing bacteria to raw or pasteurized cheesemilk (Fox and McSweeney, 2004). The choice of starter bacteria to use is based on tradition, flavour desired in the cheese and rate and extent of acid development desired during manufacture and in the finished cheese (Johnson and Law, 2010).

Coagulation:

The essential characteristic step in the manufacture of all cheese varieties is the coagulation of the casein component of the milk system to form a gel which entraps the fat, if present (Fox and McSweeney, 2004). The majority of cheeses are produced by

enzymatic (rennet) coagulation, traditionally from the stomach of young animals (claves, lambs, and buffalo).

Syneresis, Stirring and heating:

Gels formed from milk by renneting or acidification under quiescent conditions may subsequently show syneresis, because the gel (curd) contracts. Then, the curd is stirred and heated.

Pressing, salting and ripening:

Curds for most varieties are transferred to moulds where further drainage and acidification occurs (Fox and McSweeney, 2004). Salting cheeses contribute to synerisis and moisture content decrease. But, it should not be used as means of controlling the moisture content. Most cheese varieties undergo a period of ripening, which varies from 2 weeks to 2 years.

1.9.1.3. Nutritional and functional properties of cheese

Cheese is a nutritious, versatile dairy food, which nutrient content is influenced by the type of milk used, the manner of manufacture and, to a lesser extent, the degree of ripening (O'Brien and O'Connor, 2004). Most cheeses are potentially significant dietary sources of fat, which digestibility is in the range 88-94%. Furthermore, cheese contains high content of biologically valuable protein, which ranges approximately from 4-40% and is almost 100% digestible. Moreover, most cheeses can be consumed by lactose-intolerant individuals because it contains only trace amounts of lactose. Cheese is also an important dietary source of vitamins and minerals (O'Brien and O'Connor, 2004).

Cheese has positive effects on one or more functions in the body, beyond their nutritional properties (Norat and Riboli, 2003). According to Pintus et al. (2013), sheep cheese naturally enriched in α-linolenic, conjugated linoleic and vaccenic acids improves the lipid profile and reduces anandamide in the plasma of hypercholesterolaemic subjects.

Most of the bioactive peptides are hidden in the inactive state in the original parent protein structure in milk and may be released by proteolysis like in cheese during ripening. In several cheeses, bioactive peptide have been reported to inhibit the activity of the angiotensin I-converting enzyme (ACE) such in cheddar cheeses (Ong et al., 2007) and such inhibition mainly gives rise to antihypertensive effects but may also

modulate immuno-defense and nervous system activity (Korhonen and Pihlanto-Leppälä, 2004)

1.9.1.4. Organoleptic properties of cheese

Appearance, texture, and flavour are the three major components of cheese consumer acceptability (Bourne. 1978).

Appearance characteristics of cheese are assessed visually, usually prior to consuming the cheese. It include colour, presence of eyes or holes (or openness), mould, rind and visual texture (Delahunty and Drake, 2004). Colour is the first feature to be perceived and determines thereby the primary judgment on product quality (Fresno and Álvarez, 2012). Furthermore, the visual aspects of cheese may create a sensory expectation of how the cheese will "taste" or may have a strong influence on the perception of other characteristics. For example, many consumers believed that a couloured cheeses is more intensely flavoured than its uncoloured equivalent (Bogue *et al*., 1999). Moreover, appearance also includes the cheese market image such as size, shape and packaging.

The textural attributes, resulting from a combination of physical properties, are perceived by a combination of the sense of touch, vision and hearing. The cheese texture characteristics frequently described are those reported in the Table 1.6. The measurement is performed by tests involving the application of stress or strain under defined experimental conditions.

Table 1. 6. Textural properties of cheese and their definitions

Rheological properties	Definition
Firmness	The extent of resistance offered by the cheese, assessed during the first 5 chews using the front teeth
Adhesiveness	The degree o which the chewed mass sticks to mouth surfaces, evaluated after 5 chews
Cohesiveness	The degree to which the chewed mass holds together, evaluated after 5 chews
Springiness	Depress sample between thumb and finger, evaluate the total amount of recovery of the sample
Gumminess	The energy required to grind a semi-solid food when they are ready to be swallowed
Chewiness	The amount of energy required to masticate a solid food

Source: Delahunty and Drake (2004)

The flavour is most defined as the integrated perception of olfactory, taste and chemesthesis stimuli (trigeminal). Flavour perception begins prior to consumption when a consumer can smell a cheese, but is finally perceived during consumption when compounds that stimulate the olfactory system in the nose, the taste system in the mouth and the trigeminal system in the mouth and nose are released from the cheese and become available to receptors (Delahunty and Drake, 2004).

1.9.2. Fermented milk: Set yogurt

1.9.2.1. Definition, classification and regulation of yogurt

Yogurt represents a very important dairy product over the world in recent times. According to the Code of Federal Regulations of the FDA (CFR, 2013):

"Yogurt is the food produced by culturing one or more of the optional dairy ingredients with a characterizing bacterial culture that contains the lactic acid-producing bacteria, *Lactobacillus bulgaricus* and *Streptococcus thermophilus".*

The role of bacteria in yoghurt manufacture can be summarized as milk acidification and synthesis of aromatic compounds (Fadela. 2009). The milk may be homogenized and shall be pasteurized prior to the addition of the bacterial culture.

According to Tamime and Robinson (2000), yogurt is classified on the basis of chemical composition or fat content (full, semi-skimmed/medium or skimmed/low fat), on physical nature of the product (set, stirred or fluid/drinking), on the flavours (plain/natural, fruit or flavoured) or on the post-fermentation processing (vitamin addition or heat treatment).

According to the "Real Decreto "(271/2014) published in the "Boletin Oficial del Estado" number. 102, the lactic acid-producing bacteria must be viable and present in the final product at a minimum quantity of 10^7 colonies per grams o millilitres. The final pH value must be inferior o equal to 4,6. The specificity of the labelling of the yogurt expiration date is repealed, as well as the limit of sale of twenty-eight days from the date of manufacture and the dairy industry operators shall determine the appropriate date to each product type as well as its limit time.

Yogurt from sheep milk

The world production of yogurt has witnessed a remarkable increase during the last five decades (FAO, 2013). Spain is the second most important producers of yogurt and

fermented milks in the mediterranean area, after France, with 20.4% of the total produced in Europe (CAR/PL. 2002), by producing 820.100 tons of yogurts and fermented milk (FENIL. 2012). The production includes yogurts from several milks (cow, goat, and sheep), but the predominant starting material was the cow's milk. Actually, the use of sheep milk for yogurt production by the dairy industry is limited in comparison with yogurts from cow's milk.

In the last ten years, the consumption of yogurt and fermented milk has seen an important increase; from 14.05 in 2004 to 16.11 kg/person/year in 2011 (FENIL. 2012), probably due to the fact that consumers are more aware about the importance of healthy food. And, the decrease showed between 2012 and 2013, is mostly attributable to the economic crisis that affected negatively the purchasing power of the consumer (from 16.03 in 2012 to 15.5kg/person/year in 2013).

The dairy industry is one of the most active industries in Spain but the market of yogurt from sheep milk is not more organized than that of cheese from sheep milk. The main production of yogurt, from sheep milk, comes from artisanal farms. The producers defend a product that is natural, healthy and sustainable. For the most of them it's a way to develop the rural areas by taking advantage of the primary material available and promoting the economic movement in the zone.

1.9.2.2. Manufacturing process of yoghurt

Basic yogurt manufacturing processes generally use a dairy medium such as milk or a milk component as starting material. Other ingredients, such as various thickening agents/stabilizers (e.g., hydrocolloids such as starches or gelatins), and/or whey protein concentrates can optionally be added to adjust gel structure and/or consistency (Vandeweghe *et al*., 2001).

The main processing steps involved in the yogurt manufacture were described by Lee and Lucey (2010):

Milk Standardization:

This step is often realized to adjust the fat content. And, the methods employed for standardization are; the removal of part of the fat content from milk, the mixture of full cream milk with skimmed milk, the addition of cream to full fat milk or skimmed milk and the use of standardizing centrifuges, which is a process that may combine some of the methods mentioned above.

Homogenization:

This is an important processing step for yogurts containing fat because it reduces the fat globules to an average of less than 1 µm in diameter and assures a uniform distribution of the milk fat in the yogurt (Chandan and O'Rell, 2006). Consequently, no distinct creamy layer (crust) is observed on the surface of yogurt produced from homogenized mix, which involve an improvement in the consistency of the yogurt and greater stability of the coagulum against whey separation.

Heat treatment:

The temperature/time combinations for the batch heat treatments that are commonly used in the yogurt industry include 85°C for 30 min or 90-95°C for 5 min (Tamime and Robinson, 2000). However, very high temperature short time is also sometimes used (Sodini *et al*., 2004). Heat treatment influences the physical properties and microstructure of yogurt. Shah (2003) reported physical changes in the proteins as a result of heat treatment which have a profound effect on the viscosity of yogurt.

Fermentation process:

The milk base is cooled to the incubation temperature used for growth of the starter culture. Bacterial fermentation converts lactose into lactic acid, which reduces the pH of milk from 6.7 to ≤4.6.

According to Lee and Lucey (2010), an optimum temperature of the *thermophilic lactic* acid bacteria, i.e., *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, is around 40- 45°C. Lower temperature range or higher temperatures (>45◦C) can produce wheying-off problems, grainy texture, harsher flavours and over-acidification (Chandan and O'Rell, 2006).

Cooling:

When yogurts reach close to pH 4.6, yogurts are partially cooled (~20°C). Yogurt products are often blast chilled to <10°C (e.g., 5°C) in the refrigerated cold store to reduce further acid development (Tamime and Robinson, 2000).

1.9.2.3. Nutritional and functional properties of yogurt

As a healthy food, the health effects of yogurt are divided into two groups: nutritional function and physiological function (Song. 2011). The nutritional attribute is expressed as the function of supplying nutrition sufficiently, such as the source of lactose,

proteins, vitamin and calcium. The physiological function refers to prophylactic and therapeutic functions beyond nutritional function, like antimicrobial activity, gastrointestinal infections, anticancer effects reduction in serum cholesterol and immune system stimulation (Adolfsson *et al*., 2004, Ashraf and Shah, 2011, Meydani and Ha, 2006).

Yogurt contains more protein, calcium, and other nutrients than milk, reflecting the extra solids-not-fat content (Chandan and Shah, 2006; Tamime and Robinson, 2000). The microbial load content and the products of the lactic fermentation further distinguish yogurt from milk. Furthermore, yogurt have shown higher concentration of conjugated linoleic acid (CLA) than does the milk from which the yogurt was processed and this increase was mostly attributed to the microbial cultures presents during fermentation (Kim and Liu, 2002, Sieber *et al*., 2004). Several properties of the CLA in yogurts and its effect on human health were already reported, such as anticarcinogenic properties and modulation of the immune system (Kemp *et al*., 2003; Pariza *et al*., 2001).

The protein content of yoghurt is often increased by concentration or addition of skimmed milk solids, which means that it is an even more attractive source of protein than liquid milk (Tamime and Robinson, 2000). It has been argued that protein from yogurt is more easily digested than is protein from milk, as bacterial pre-digestion of milk proteins in yogurt may occur (Chandan. 2013). This argument is supported by evidence of a higher content of free amino acids, especially proline and glycine, in yogurt than in milk. Moreover, during fermentation, both heat treatment and acid production result in finer coagulation of casein, which may also contribute to better protein digestibility (Adolfsson *et al*., 2004). Proteins in yogurt are of excellent biological quality, because the nutritional value of milk proteins is well preserved during the fermentation process (Hewitt and Bancroft, 1985). Moreover, the formation of bioactive peptides partially explains the health-promoting properties of fermented milk products (Korhonen and Pihlanto-Leppälä, 2004).

In many countries yogurt has been continuously modified to obtain a product with better appeal, nutritional and functional effects. In Spain, the dairy companies collaborate with various investigation centers in order to develop products that provide attributes beyond nourishing properties, products that contain one or more compounds that promote welfare and health, or reduce the risk and protect from metabolic syndromes or

degenerative diseases. Basing on research, dairy industry developed products such as the milk enriched in calcium, Vitamin A and D and polyunsaturated fatty acids (OMEGA 3).

1.9.2.4. Organoleptic properties of yogurt

The evaluation of the appearance includes the surface of the product, colour, visible purity, presence of foreign matters, spots of mold, seepage of whey and phase separation (Karagül-Yüceer and Drake, 2013).

Texture is one of the most essential components of yogurt quality, it represents all the rheological and structural attributes perceptible by means of mechanical, tactile, and, when appropriate, visual and auditory receptors (Ozcan. 2013). Yogurt texture can be characterized by many attributes and these include both non-oral textural attributes such as spoon impression, clumpiness, thickness, smoothness, and oral texture attributes including thickness, stickiness, dairy film, meltdown rate, and fatty after mouthfeel (Grygorczyk *et al*., 2013). However, the most common sensory attributes relating to yogurt texture are thickness /viscosity, smoothness (opposite to lumpiness, graininess, grittiness) (Ozcan. 2013). Indeed, according to Lovely and Meullenet (2009) when consumers judged a yogurt to be too thick, too thin or lacking in smoothness, the overall liking score of the yogurt decreased significantly. Rheology and structure can be evaluated by instrumental methods and sensory tests (Foegeding *et al*., 2011; Fisher and Windhad, 2011) even if sensory and instrumental data are not always easily correlated (Sodini *et al*., 2011). High thickness, which is the amount of force required to slurp yogurt into mouth while making "O" shape with lips, is usually associated to higher amount of force (Grygorczyk *et al*., 2013).

Texture and flavour are the most important parameters for acceptability (Grygorczyk *et al*., 2013; Lovely and Meullenet, 2009; Jaworska *et al*., 2005). Also, flavour has the ability to push texture into the background but if the product's flavour lacks in distinctiveness, texture importance increases (Szczesniak and Kahn, 1971). Jaworska *et al*. (2005) explained that negative flavour attributes may become more prominent and overshadow the impact of texture on consumers.

Chapter 2: Objectives

2. Objectives

In recent years, the association between diet and health has received an increasing public attention, especially in the media (Mollet and Rowland, 2002). Although dairy products have historically enjoyed a widespread perception of being healthy food products, consumers have more recently been subjected to a wide variety of both negative and positive messages regarding the health effects of dairy consumption (Cash et al., 2005). It's generally known that milk is a complex mixture of lipids, protein and micronutrients, many of which have shown favorable or neutral effects on human health. However, the negative information associated to the saturated fat and its connection with chronic diseases has often harmed the reputation of milk products (Huth and Park, 2012). Such trends increased the need of consumer for secure and safe foods and promote the development of functional dairy products

The possibility to develop dairy products with low levels of saturated fatty acids is a very interesting approach to reduce the impact of saturated fatty acids on human health and to increase the popularity of these products. Furthermore, the opportunity to use dietary antioxidants, available in nature without additional costs, would be a very good strategy to reach that purpose, by reducing the oxidative deterioration in foods and also by developing foods rich in bioactive ingredients with the ability to reduce risks of chronic diseases.

In this context, the aim of this doctoral thesis has been the development of functional dairy products through the inclusion of *Thymus mastichina* L. in dairy sheep diets as source of natural antioxidant. The innovative aspect of this research emanates from the use of *Thymus mastichina* L., a wild endemic plant, and its inclusion in sheep diet as whole plant without further processing.

The specific objectives set out in this research work were as follows:

- Evaluation of *T. mastichina* L. as source of natural antioxidant, through the determination of its bioactive properties and the characterization of its phenolic compounds.
- Evaluation of the animal and lactation performances of the ewes, fed different modified diets (control, canola seed diet and marjoram diet), through

the analysis of the dry matter intake, nutrient digestibility, nitrogen balance and milk yield.

- Evaluation of physic-chemical properties of the dairy products, yogurt and cheese, manufactured from the milk of ewes fed different modified diets.
- Evaluation of the bioactive properties of the dairy products, yogurt and cheese, manufactured from the milk of ewes fed different modified diets.

Chapter 3: Materials and Methods

3.1. Evaluation of *T. mastichina* L. as source of natural antioxidant

3.1.1. Plant material

Cultivated and wild plant was used in the experiment. The marjoram (*T. mastichina* L.) was provided from two agriculture farms in the provincial city of Valladolid, Spain (Villabrágima en Ctra. Castromonte and San Pedro Latarce) where marjoram was cultivated for the first time from wild seeds, and also from a meadow as wild plant (Escuela de la Santa Espina). The cultivation of marjoram was initially performed in a nursery in order to ensure the growth of plants which were afterwards transplanted in fields.

Figure 3. 1. Marjoram plant (*T. mastichina* L.)

Marjoram was harvested in June 2010 when the plant had a height of 40 cm (Figure 3.1). The plant was dried at a temperature of 19.5ºC in a warehouse (80 m^2) with a natural ventilation system. This operation is essential and needs to be controlled because it is a plant rich in volatile compounds, and the final composition is significantly affected by the drying method used. The plants were kept in controlled conditions during 3 week in order to guarantee that the drying process was finished.

The plants harvested in the two farms and in the field were homogenized (93,6 % comes from the cultivated plants and 6,4% from the wild plants). The average of dry weight was 790g DM/kg for the cultivated plants and 973g DM/kg for the wild plants. And, the difference between both was basically attributed to the climatic changes during harvesting.

Marjoram was chopped into 1 cm of length with a grinding machine. The use of standard machines for the grinding and the harvesting operation implies that the machines required for the different operations on marjoram did not require additional

costs for the farmers which promote the use of marjoram as an ingredient in the animal diet. Finally, the chopped plant was packed and stored in darkness until further use. In fact, the bioactive properties of these plants are influenced by several factors such as the light and for this reason the plant was stored in darkness. The Figure 3.2 showed the different operations in the collection, removal, drying, hash and storage in the agriculture farms.

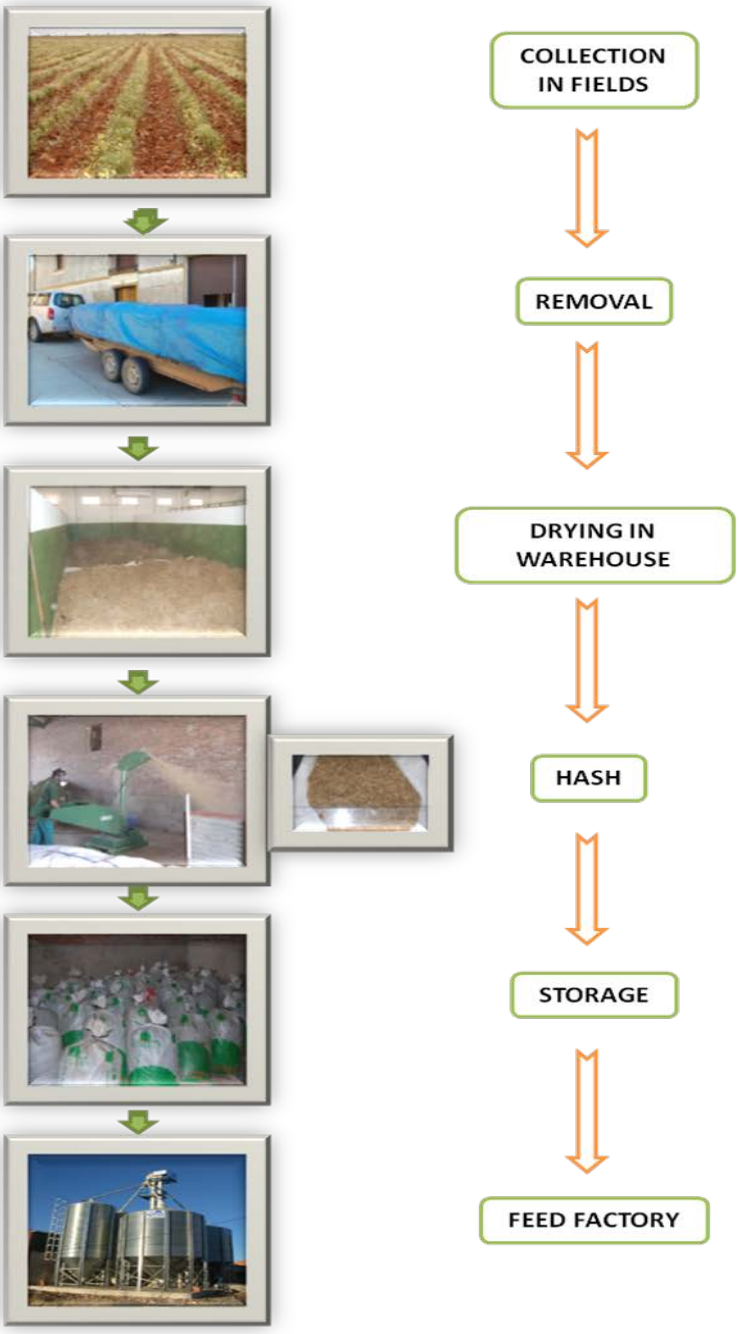


Figure 3. 2.Processing of marjoram plant

3.1.2. Evaluation of the bioactive properties of *T. mastichina*

3.1.2.1. Extraction of phenolic compounds

The extraction of phenolic compounds was performed using dried and ground plant. Samples were homogenized for 1 min at 24,000 rpm using an Ultra-Turrax T-25 Tissue homogenizer (Janke & Kunkel, IKA_-Labortechnik, Saufen, Germany). Two grams of the ground sample was homogenized in 100 mL of solvent. Three different solvents were used for the extraction (water, ethanol and methanol). The sample suspension was shaken at 175 rpm overnight (19h) in a Max Q 4000 Incubator and Shaker (Thermo Fisher Scientific, Millcreek Road, Marietta, Ohio, USA) at 25°C. After that, the sample suspension was centrifuged for 5 min at 4,000 g (ROTINA 380R, HettichLab, Tuttlingen, Germany) and the supernatant was filtered through 0.22 µm polytetrafluoroethylene (PTFE) filters (Sigma-Aldrich). The extracts were evaporated at 40°C in a rotary evaporator (Rotavapor RII, BÜCHI Laboratory, Flawil, Switzerland) and the extraction yield of each extract was estimated by weighing the material remaining after evaporation. Three replications of each extract were prepared and then stored at - 20 °C until subsequent analysis.

3.1.2.2. Total phenolic content (TPC)

The total phenolic content was determined using Folin-Ciocalteu Reagent (FCR) as described by Singelton *et al.* (1999) and gallic acid was used as standard. Folin-Ciocalteu reagent and gallic acid (GA) were purchased from Sigma-Aldrich (Wicklow, Ireland). In each replicate, 100 µL from the appropriately diluted sample extract, 100 µL methanol, 100 µL FCR and finally 700 µL sodium carbonate (Na_2CO_3) were added together and vortexed. The blend was incubated for 20 min in the dark at room temperature. After incubation the mixture was centrifuged at 13,000 rpm for 3 min (ROTINA 380R, HettichLab, Tuttlingen, Germany). The absorbance of the supernatant was measured at 735 nm by spectrophotometer. The TPC was expressed as mg of gallic acid /g dry weight extract (mg GAE/g DWE). The assay was performed in two batches which included three replications in each for both samples and standard.

3.1.2.3. The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

The capacity of the extracts to scavenge $DPPH^{\cdot}$ free radical was determined according to the method described by Sanchez-Moreno (2002). Briefly, 2,2-Diphenyl-1-picrylhydrazyl (Sigma-Aldrich, Wicklow, Ireland) was dissolved in methanol (0.238

mg/mL). 500 µL of the diluted samples, methanol as blank and Trolox dilutions as standards (Sigma-Aldrich, Wicklow, Ireland) were added to 500 µL of DPPH solution. Solutions were incubated in the dark at room temperature for 30 min. The absorbance was measured at 515 nm by spectrophotometer (Hitachi Double Beam Spectrophotometer. model U-2900 UV-VIS, Japan). The radical scavenging activity was expressed as mg trolox/g dry weight extract (g Trolox/100g DWE) and also as EC_{50}, which is the extract concentration providing 50% of radicals scavenging activity. All samples were replicated.

3.1.2.4. The Semicarbazide-Sensitive Amine Oxidase (SSAO) inhibitory assay

The SSAO inhibitory activity of *T. mastichina* was performed on water extracts. Standard curve was prepared with concentrations from 200 to 3.125 µM of SSAO. For the samples, 200 µl of the reaction solution was prepared using 50 µl of buffer as blank, standards and samples, which were preincubated with 50 µl of SSAO enzyme and then 100 µl of the cocktail reaction. The black flatbottom 96-well plate was incubated at 37ºC for 2 hours. The fluorescence of resorufin was recorded at 530 nm excitation/ 590 nm emission) during 35 seconds using a Fluostar Omega (Offenburg, Germany). The results were expressed as µM of semicarbazide. The analysis was performed in triplicate.

3.1.3. Characterization of the phenolic compounds of *T. mastichina*

3.1.3.1. Preparation of marjoram extracts

Solid-phase extraction (SPE) was used for the clean-up of extracts prior to the LC-MS separation and quantification of phenolic compounds. The method used in this study was described by García *et al*. (2004) with some modification. C18 Sep-Pak cartridge (Mildford, USA) was pre-conditioned with 6 mL of methanol followed by 6 mL of water without allowing the cartridge to dry out. Then, the dissolved extracts (water, ethanol and methanol) were passed through the cartridge. The eluates were then transferred to vials and evaporated to dryness with nitrogen. The residues obtained were dissolved in methanol. The distribution of the cleaned-up extracts was achieved with vortex agitation prior to the chromatographic analysis.

3.1.3.2. Characterization of phenolic compounds using the Liquid chromatography-mass spectrometry (LC-MS)

LC-MS analysis was performed on a Q-Tof premier mass spectrometer (Waters Corporation Micromass MS Technologies, Manchester, UK) coupled to Alliance 2695 HPLC system (Waters Corporation Milford, MA, USA). The Q-Tof Premier was equipped with a lockspray source where an internal reference compound (Leucine-Enkephalin) was introduced simultaneously with the analyte for accurate mass measurements.

Compounds were separated on an atlantis T3 C18 column (Waters Corporation Milford, USA, 100 mm x 2.1 mm; 3 µm particle size) using 0.5% aqueous formic acid (solvent A) and 0.5% formic acid in 50/50 v/v acetonitrile: methanol (solvent B). Column temperature was maintained at 40 °C. The gradient elution program applied was: 100% A for 15 min, 50% A: 50% B for 15 min and 80% A: 20% B for 50 min at a flow rate of 0.2 mL/min. Electrospray mass spectra data were recorded in the negative ionization mode for a mass range m/z 100 to m/z 1000. Capillary voltage and cone voltage were set at 3 kV and 30 V respectively. Collision induced dissociation or fragmentation (CID) of the analytes was achieved using 12-20 eV energy with argon as the collision gas. Identification of some of the phenolic compounds was carried out by comparing retention times and their masses with those of authentic standards. Five flavonoids standards, luteolin-7-O-glucoside, luteolin, naringin, apigenin and apigenin 7-O-glucoside were purchased from Extrasynthese, Genay, France and one hydroxycinnamic acid namely rosmarinic acid was purchased from Sigma-Aldrich, Wicklow, Ireland.. The purity of standards and solvents were in the range of 95-99.8%. Only luteolin-7-*O*-glucoside had 90% purity.

And, for the compounds for which no standards were available a tentative of identification was based on accurate mass measurements of the pseudomolecular [M-H]- ions in combination with collision-induced dissociation (CID) fragment ions. Quantification of each compound was performed on the basis of the relative area of an internal standard, kampferol. Results were expressed in µg kaempferol/mg DWE.

3.1.4. Statistical analysis

Data were analyzed using the General Linear Models procedure using the SPSS package (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA). Significant difference was

declared at $P<0.05$ and values with statistically significant differences were compared using Duncan's Multiple Range test and designed by different letters.

The correlation analysis was performed using the Pearson's correlation coefficient (r). A partial correlation was also used to measure the degree of association between two random variables (total phenolic content and DPPH free scavenging activity), with the effect of a set of controlling random variable removed (extraction solvent) in order to know the impact caused by this third variable on the relationship between the both primary variables.

3.2. Evaluation of animal and lactation performances of sheep

3.2.1. Experimental design

The experiments were conducted between January and March 2011. All animal handling practices followed the European Council Directive 86/6097EEC and recommendations of the European Commission (2007/526/EC) for the protection of animals used for experimental and other scientific purposes. Animals were divided in three experimental groups and offered experimental diets which consisted on a total mixed ration (TMR); including molasses to avoid selection of dietary components and based on dehydrated alfalfa hay and concentrate. The ingredients and chemical composition of all diets were shown in the Table 3.2. The content of essential oil in *T. mastichina* was 1.28% and the main components were 1,8-cineol (46.67%) and linalool (14.47%).

The control group (CO) was fed the TMR without any supplementation, the second group received the TMR supplemented with 2,85% of canola seed (CS) and both diets were formulated to be isoenergentic and isoproteic. The third group was fed a diet modified by a substitution of 7.5% of the CS diet by marjoram, which origin was described in the section 3.1.1.

3.2.2. Diet characterization

Procedures to determine dry matter (DM), organic matter (OM), total nitrogen (N), and ether extract (EE) were evaluated following the method described by AOAC (2006). And crude protein was calculated using the conversion factor 6.25. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined by the methods described by Goering and Van Soest (1970) and Van Soest *et al*. (1991), adding sodium sulphite to the solution. Neutral detergent fiber was assayed with α-amylase.

Table 3. 1. Ingredients and chemical composition of the experimental diets

Composition	Experimental diets[1]		
	CO	CS	TM
Ingredients(% of DM)			
Dehydrated alfalfa hay	27	27	25
Granulated alfalfa	7	7	6.5
Straw treated with glycerol	1.2	1.9	1.8
Corn grain	12	12	11
Oat	5	5	4.6
Wheat grain	4.8	6	5.6
Barley grain	4	4	3.7
Wheat bran	1.8	1.6	1.5
Beet pulp	3	3	2.8
Canola cake	5	5	4.6
Distillers dried grains (barley)	4.8	5.7	5.3
Canola meal	2.4	2.4	2.2
Corn gluten feed	1	1.2	1.1
Sunflower cake 28/30%	1.4	1.8	1.7
Soybean cake 44%	2.2	2.7	2.5
Soybean	3	3	2.8
Canola seed	-	2.85	2.6
Palm oil	0.12	-	-
Whole cotton grain	6	-	-
Molasses-urea-vitamin-Mineral	7.8	7.8	7.2
Marjoram			7.5
Nutrients[2]			
CP, %	17.10	18.67	17.60
NDF, %	29.93	30.48	32.06
ADF, %	19.03	21.05	21.16
EE %	4.27	4.92	5.06
EB (cal/kg DM)	4.39	4.38	4.42
Fatty acid composition, % total fatty acid			
C16:0	11.84	6.15	
C18:0	14.3	11.4	
C18:1	14.60	25.97	
C18:2	35.87	26.96	

[1]Refers to treatments: control diet (CO); canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by the marjoram)
[2]Refers to nutrients: crude protein (CP); neutral detergent fiber (NDF); acid detergent fiber (NDF); ether extract (EE) and energy balance (EB)

3.2.3. Control of animal performances of non-lactating ewes

3.2.3.1. Individual dry matter intake and body weight

A total of thirty dry (non-pregnant, non-lactating) assaf ewes (10 animals in each experimental group) were used to determine the individual feed intake. Offered and refusal diets were daily recorded for each animal during 5 consecutive days after an

adaptation period of 15 days. Samples of feeds and refusals were taken daily, pooled for each animal and dried at 60ºC to constant weight.

The body weight of each animal was recorded at the beginning and the end of the trial (5 days). The average of body weight of the control group, the CS group and the TM group, at the beginning of the trial, was 50.15 ± 4.19; 49.29 ± 4.25 and 47.33 ± 3.95 kg, respectively.

Figure 3. 3. The feeding system used for the measurement of the individual dry matter intake

3.2.3.2. Digestibility of nutrient and nitrogen balance

A total of twenty four Assaf ewes (8 animals in each experimental group) were used to determine the digestibility and the nitrogen balance. For 5 consecutive days, following 25-days adaptation period, the quantity of feed consumed and that of feces of each animal were registered. The individual cages permitted the total collection of feces separately from feed residues and urine. Samples of feces and urine, daily taken, were pooled and a representative sample (10 %) was kept frozen at -20ºC until subsequent analysis.

Figure 3. 4. The feeding system used for the measurement of the nutrient digestibility and nitrogen balance in dry ewes

3.2.4. Control of animal and lactation performances of lactating ewes

Thirty six multiparous Assaf ewes in mid lactation were randomly divided into three groups of equal size (12 animals in each) balanced for milk yield, live weight and body condition score. The batons presented average body weight (78.9 ± 2.01 kg), body condition score (2.19 ± 0.09) and milk yield (2.764 ± 0.144 kg). The experiment lasts 4 weeks.

Figure 3. 5. The feeding system used for the measurement of the dry matter intake and milk yield in lactating ewes

3.2.4.1. Dry matter intake and body weight

During 10 days-adaptation period (before commencing the trail), all animals received the control diet in order to stabilize the group. Fresh water was always available and diets were offered daily ad libitum. The intake therefore was recorded daily for each experimental lot by difference of weight between offers and refusals. The body weight of each ewe was recorded weekly during the experiment.

3.2.4.2. Milk production

Ewes were milked daily from 0830 to 1830 h in a 1 × 12 stall-milking parlor (DeLaval, Madrid, Spain). The individual milk production was recorded every day, both at morning and evening milking.

3.2.5. Statistical analysis

Data were analyzed using the SPSS package (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA). The data of the animal performances of non-lactating ewes was analyzed using the General Linear Models procedure in which the diet was considered as fixed effect plus the random effect of the animal. The data of milk yield and body

weight of lactating ewes was analyzed using the General Linear Models procedure with repeated measures in the time. The model accounted for the fixed effects of dietary treatment, time, and the interaction between treatment and time, plus the random effect of sheep. The Least square means were reported and significance was declared at $P<0.05$. Values with statistically significant differences were compared using Duncan's Multiple Range test and designed by different letters.

3.3. Evaluation of milk and dairy products properties

3.3.1. Experimental design

The milk used for the manufacture of dairy products was produced by the dairy ewes used in the preceding trial, described in the section 3.2.2.4. The bulk milk of each group of ewes (CO, CS and TM) was collected three times during the experimental period (beginning, middle and end), which lasts 4 weeks, and used for the manufacture of yogurts and cheeses.

From each group of ewes (CO, CS and TM), three batches of milk were transformed into yogurts at the beginning, middle and end of the experimental period (15 days of interval). The milk used for the yogurt manufacture corresponds to the morning milking. The milk of each group was collected separately to acquire batches of 5 L required for the traditional processing of yogurts.

A total of 12 cheeses were manufactured; two cheeses from each milk (CO, CS and TM) at the beginning and end of the experimental period with 20 days of interval. The milk used for the cheesemaking correspond to the afternoon milking of the day before, which was kept at 4ºC, mixed with the following morning milking in order to obtain the quantity of milk required for the manufacture of cheeses from each group of ewes (the manufacture of 1 cheese requires approximately 18 L of milk). The ripening has also been considered (2 and 4 months); one cheese was analyzed after 2 month of ripening and another one after 4 months. Data of cheeses at day 1 of manufacturing were excluded because "Castellano cheese", made from raw milk, cannot be consumed until 2 months of age for safety reasons as reported in the section 1.9.1.1.

3.3.2. Characterization of ewe's milk

Samples of bulk milk of each group of ewes were collected before each manufacturing process and stored at -20ºC until subsequent analysis. Milk samples for the bioactivity assay were freeze-dried.

3.3.2.1. Control of the physico-chemical composition of milk

The fat, protein, lactose and total solids content were determined by the infrared spectrometry method using the Milko-Scan 4000 FT+ (Foss Electric, Hillerød, Denmark). All the analyses were performed in triplicate.

Milk casein content was analyzed according to the ISO 17997-1: 2004. Precipitation of casein from a separate test portion of the same sample used for protein determination was performed by the addition of acetic acid and sodium acetate solutions such that the final pH of the mixture was approximately 4, 6. Then, the precipitated milk casein was removed by filtration and the remaining filtrate containing the non-casein nitrogen components was measured. The casein content is determined as the difference between the total nitrogen content and the non-casein nitrogen content.

Somatic cell content was determined according to the ISO 13366-2: 2006, by the fluoro-opto-electronic method using the Fossomatic 5000 (Foss Electric, Hillerød, Denmark).

3.3.2.2. Characterization of the lipidic profile of milk

3.3.2.2.1. Fat extraction

Milk fat extraction was carried out according to the ISO 14156: 2001 for fatty acids, CLA and triglycerides analysis. The milk sample was defrosted in a water bath to a temperature of 35-40ºC, mixed and cooled to 20ºC±2. 100 mL of the sample was mixed with 80 mL of ethanol and 20 mL of ammonia solution in a separating funnel. Then, 100 mL of diethyl ether was added and shaken vigorously for 1 min. After a phase separation, 100 mL of n-pentane was added and mixed carefully. And, after a second phase separation, the aqueous layer was discarded. 100 mL of sodium sulfate solution was added to the remaining contents and mixed carefully again. And, after a third phase separation, the aqueous layer was discarded. Finally, a second amount of 100 mL of sodium sulfate solution was added and mixed vigorously. Then, the aqueous layer was discarded after the phase separation.

The remaining organic layer was transferred to a conical flask and mixed with 5 to 10 gram of anhydrous sodium sulfate. The flask stood for 10 min, and then was filtered into a round-bottom flask. Using an R-205 Rotary Evaporator with Heating Bath B-490 (BÜTCHI Labortechnik AG, Flawil, Switzerland), the content of the flask was evaporated under reduced pressure in a water bath set at 50ºC until evaporation is visually completed. If the removal was not completed, the flask content was flushed with a stream of nitrogen for 1 min.

Milk fat extraction for phospholipids (PLs) analysis was carried out according to the Folch et al., (1957) extraction method with some modifications. 3 grams of the lyophilized sample was added in a volume of 22.5 mL of dichloromethane:methanol 2:1 (vol:vol). The mixture was vortexed for 1 min, shacked slowly for 30 min at room temperature in a rotating shaker, and finally centrifuged at 75000 rpm for 5 min at 0ºC. The lower dichloromethane layer was removed. The process was repeated adding 18 mL of dichloromethane:methanol 2:1 (vol:vol), except for the agitation step which lasts 5 min this time. Then, a volume of 4.5 mL water solution (0.8% NaCl) was added to the mixture, vortexed for 1 min and left overnight. The upper phase was removed and the intermediary phase was filtered through 0.45 µm polytetrafluoroethylene (PTFE) filters (Sigma-Aldrich) with dichloromethane and sodium sulfate anhydrous, and finally evaporated using nitrogen as nebulizing gas. Separated lipids were stored at -20ºC until analysis.

3.3.2.2.2. Preparation of methyl esters of milk fat

Methyl esters for fatty acids, CLA and triglycerides analysis analyses were prepared from the fat extracted from milk samples by base-catalyzed methanolysis of the extracted fatty acid fraction using 2N KOH in methanol as described by international standard method ISO 15884:2002.

3.3.2.2.3. Fatty acids (FAs) composition of milk

The obtained methyl esters were analyzed in a 6890 Agilent GLC (Palo Alto, CA, USA), fitted with MS detector (Agilent 5973N) operated in scan mode (50-550 Da) and a 100m CPSil-88 capillary column (100 m x 0.25 mm i.d. x 0.2 µm film thickness, Chrompack, Middelburg, Netherlands). The column temperature was maintained at 80ºC for 1 min after injection, then 7ºC/min to 170ºC, held for 55 min, 10ºC/min to 230ºC and held 33 min. Helium was the carrier gas with an inlet pressure set at 214 kPa (30 Psig) and a split ratio of 1:25. Reference butter fat BCR-164 was

used to obtain response factors (Rf). For quantitative purposes, tritridecanoin (C13; 2.16 mg/mL) was added to test samples and reference butter fat. The determinations were duplicated.

3.3.2.2.4. Conjugated linoleic acid (CLA) profile of milk

The obtained methyl esters were analyzed in a Silver ion (Ag^+)-HPLC separation of CLA methyl esters was carried out using an HPLC (model 1200, Palo Alto, CA, USA) equipped with a UV detector operated at 233 nm. Two chromSpher 5 lipid analytical silver-impregnated columns (250 mm × 4.6 mm i.d. stainless steel; 5 µm particle size; Varian-Chrompack Int., Middelburg, the Netherlands) were used in serie. The mobile phase was 0.1% acetonitrile in hexane, operated isocratically at a flow rate of 1.0 mL/min. 10 µL of the sample was injected. Pure and mixed CLA FAME isomers from Nu-Chek Prep were used as standards.

3.3.2.2.5. Triglycerides (TGs) profile of milk

For the analysis of triglycerides (TGs), 10 mg of the extracted fat was dissolved in 0.5 mL dichloromethane. The TGs analyses were performed on an Autosystem Gion 4072042 gas chromatograph (Perkin-Elmer, Beacons"eld, UK) equipped with an automatic injector (split/splitless) and programmed temperature. A capillary column (30 m × 0.22 mm i.d.), supplied by Restek (Bellefonte, PA), Rtx-65 TG (35% dimethyl, 65% diphenyl polysiloxane) (d_f = 0.10 µm) was used. Experimental chromatographic conditions were as follows: the initial temperature (280 ºC) was raised to 320º C at a rate of 15 ºC min^{-1} and then to 355 ºC at a rate of 7º C min^{-1} and then held at this temperature for 20 min. The injector and detector temperatures were 355 and 370º C, respectively and the flow rate was 0.8 mL min^{-1}.

3.3.2.2.6. Phospholipids (PLs) profile of milk

The samples of the fat extracted for phospholipids analysis were dissolved in dichloromethane prior to analysis, at a 0.1 mg/µL concentration. And, then the separation of phospholipids' classes was accomplished in a HPLC (model 1200, Palo Alto, CA, USA) coupled with an ELSD detector (SEDERE. SEDEX 85 model, Alfortville Cedex, France) using filtered air as the nebulizing gas at a pressure of 3.5 bar, at 60 ◦C and a column with 250mm×4.5mm Zorvax Rx-SIL column (Agilent Tecnhnologies, Palo Alto, CA, USA) with 5-µm particle diameter and a precolumn with the same packing was used.

The injection volume was 10µL and the column was equilibrated at 40ºC. The solvent gradient used is detailed in Rodríguez-Alcalá and Fontecha (2010).

3.3.2.3. Determination of the antioxidant capacity of milk

3.3.2.3.1. Extraction of the phenolic compounds of milk

The procedure followed for the extraction of antioxidants was the method described by Redeuil *et al*. (2009) with adjustments as regards the temperature of extraction. One gram of freeze-dried sample was mixed with 10 mL of two different solvents (water and methanol), then homogenised by Vortexing during 5 min. The mixture was left 1h at -20º C to allow protein precipitation and then centrifuged for 5 min at 4,000 g (ROTINA 380R, HettichLab, Tuttlingen, Germany). Finally, the upper fat layer was discarded and the supernatant was removed and used for antioxidant and anti-inflammatory activities determinations. Three replications sample were performed and then kept at - 20 °C until subsequent analysis.

3.3.2.3.2. The 2,2´azinobis(3 ethylbenzothiazoline-6-sulfonate) (ABTS) assay

The scavenging activity against the ABTS radical (Sigma-Aldrich, Wicklow, Ireland) was determined by the decolourization assay described by Re et al. (1999) and modified by Chen *et al*. (2003).

The ABTS radical cation (ABTS•+) solution was prepared by reacting 10 mL of ABTS solution (7 mM) with 10 mL of K_2SO_4 solution (2.45 mM) and allowing the mixture to stand in the dark at room temperature for 24 h before use. For each assay, the stock of ABTS•+ solution needs to be dissolved in methanol to reach an absorbance of 0.7 (±0.02) at 734 nm. Trolox was used as standard (Sigma-Aldrich, Wicklow, Ireland); the stock (2 mM/L) was dissolved in methanol (12,9 mg/mL) and then 1 in 10 dilutions were prepared in order to build the standard curve. 600 µL of samples and trolox dilutions were added to 6 mL of the diluted ABTS•+ solution and then incubated at 30°C for 1h. After that, the solutions were centrifuged for 6 min at 4,000 g (ROTINA 380R, HettichLab, Tuttlingen, Germany) at 30°C. Finally the absorbance (734 nm) was measured by a spectrophotometer (MODEL U-2900 UV-VIS, Japan). The results were expressed as mg trolox/g of dry weight extract (g Trolox/100g DWE).

3.3.3. Characterization of cheese and set-yogurt

After each manufacturing process, yogurts and cheeses were stored at 4ºC for organoleptic determinations, at -20ºC for physico-chemical composition and lipidic profile characterization and samples were freeze-dried for bioactivity assays.

3.3.3.1. Cheese product

3.3.3.1.1. Manufacturing process of cheese

For the cheesemaking, raw milk was heated to 30ºC and this temperature was maintained during the whole process of coagulation. A solution of $CaCl_2$ was added (0.06ml/L which means a concentration of 31.5 mg/L) and then two types of starters were used. The first starter was a mixture of *Lactococcus lactis* subsp. *Lactis* and *Lactococcus lactis* subsp. *cremoris* (Danisco CHOOZIT MA11 LYO-25 DCU, Sassenage, France) at a dose of 8.7±2.2 mg/L and the second kind which function was to give flavor to the cheese was composed by *Lactococcus lactis* subsp. and *Lactis biovar. diacetylactis* (Danisco CHOOZIT MD99 LYO-50 DCU, Sassenage, France) at a dose of 5.7±1.6 mg/L. Finally, 0.31 ± 0.05 ml/L of calf rennet (1:15000 strength, Arroyo Laboratories Santander, Spain) was added and mixed thoroughly.

Once the coagulation was obtained (60 min), the curd was cut into small cubes and heated to 38ºC in order to get an adequate whey separation. Curds were then placed into cheese moulds with clothes for pressing process in a vertical press (2.5 bar) during 3 h with a turn over each hour. After that, the curd was removed from the moulds and salted in saturated brine during 12 hours (19ºD and 9ºC for acidity and temperature, respectively). Finally, cheeses were transferred into a repining room at a temperature of 10-12ºC and a relative humidity of 85%, where they remained 2 months for the semi-cured cheeses and 4 months for the cured cheeses.

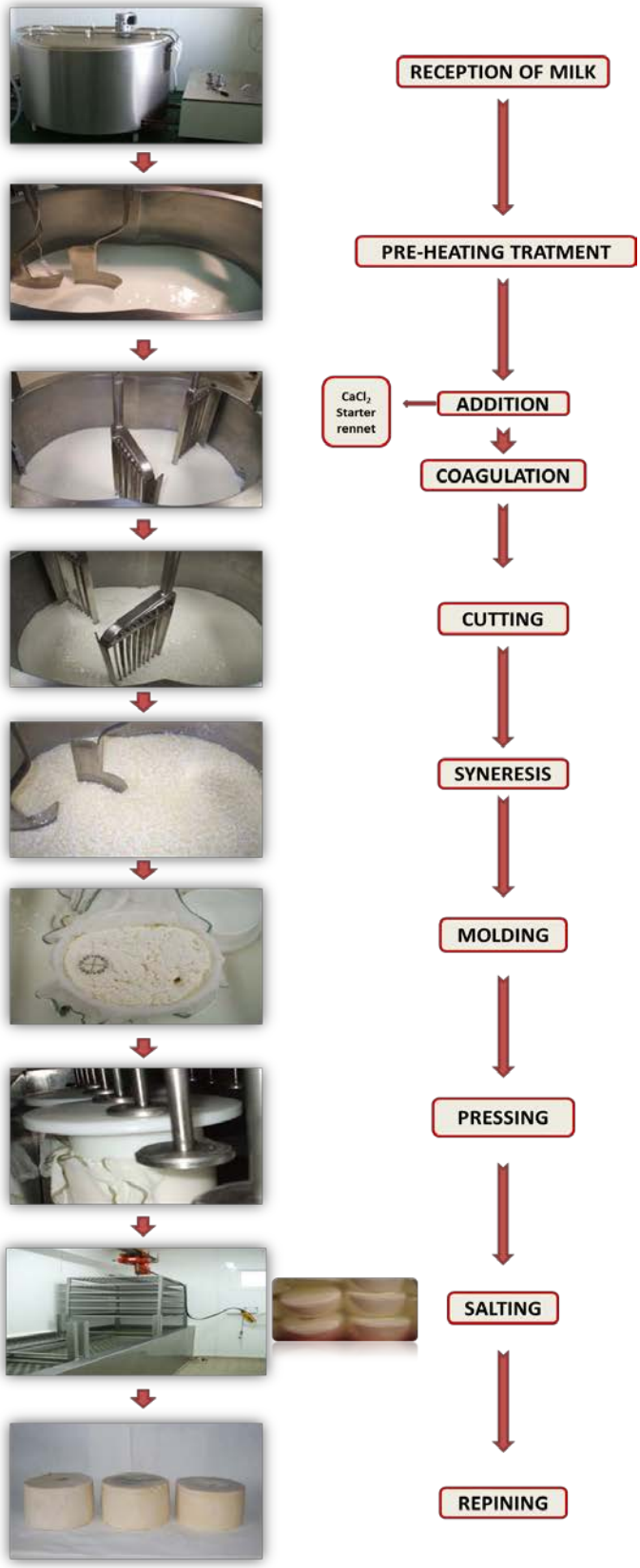


Figure 3. 6. The manufacturing process of cheese

3.3.3.1.2. Determination of the physico-chemical composition of cheese

Cheese samples were analyzed by following the procedures according to the International Organization for Standardization (ISO). Cheese samples were defrosted at 4º C during 24 h. All samples were performed in duplicate.

For fat content, samples were analyzed by following the ISO procedure 1735: 2004. In brief, 1g of cheese was digested with hydrochloric acid and then ethanol was added. The acid-ethanolic solution was extracted with diethyl ether and light petroleum and then evaporated using an R-205 Rotary Evaporator with Heating Bath B-490 (BÜTCHI Labortechnik AG, Flawil, Switzerland).

The protein content was determined by gravimetric method according to the ISO 8968-1: 2001. The method consists of heating the sample with sulfuric acid, which decomposes the organic substance by oxidation to liberate the reduced nitrogen as ammonium sulfate. The operation was performed in a SpeedDigester K-425/K-436 (BÜTCHI Labortechnik, Flawil, Switzerland). Chemical decomposition of the sample was completed when the initially very dark-coloured medium has become clear and colourless. The solution was then distilled with sodium hydroxide in a KjelFlex k-350 (BÜTCHI Labortechnik AG, Flawil, Switzerland), which converts the ammonium salt to ammonia. The amount of ammonia present, and thus the amount of nitrogen present in the sample, was then determined by back titration using a solution of boric acid and a solution of hydrochloric acid. The amount of nitrogen was then converted in protein through the multiplication by the factor (6.38)

For the total solids content, samples were analyzed according to the ISO 5534: 2000. 1 g of cheese was mixed with sand, and after dried by heating it in a drying oven at 102ºC. Dried samples were weighed to determine the loss of mass.

The pH was measured at room temperature (20ºC±2ºC) using the pH-MATIC 23 (CRISON INSTRUMENTS, Alella, Barcelona, Spain).

Sodium chloride was determined by a classic titrametric analysis as described by Cimiano (1999). Organic substances were destructed by potassium chromate and nitric acid and then the chloride content was determined by argentometric titration using a nitrate solution.

3.3.3.1.3. Characterization of the lipidic profile of cheese

3.3.3.1.3.1. Fat extraction

Cheese fat extraction was carried out according to the ISO 14156: 2001 for fatty acids and triglycerides analysis. A sample of 1g of cheese was mixed with sodium sulphate and then transferred to an extraction thimble. The extraction thimble was inserted into the chamber of the Soxhlet extraction apparatus. 250 mL of n-pentane was used for the extraction and the sample was extracted for 6h under reflux. Using the R-205 Rotary Evaporator with Heating Bath B-490 (BÜTCHI Labortechnik AG, Flawil, Switzerland), the content of flask was evaporated under reduced pressure in a water bath set at 50ºC until evaporation is visually completed. If the removal is not completed, the flask content was flushed with a stream of nitrogen for 1 min.

3.3.3.1.3.2. Preparation of methyl esters of cheese fat

The procedure followed for the preparation of the methyl esters for fatty acids and triglycerides analyses was described for milk samples in the section 3.3.2.2.2.

3.3.3.1.3.3. Fatty acids (FAs) composition of cheese

The procedure followed for the determination of the fatty acids composition of cheese samples was described for milk samples in the section 3.3.2.2.2.

3.3.3.1.3.4. Triglycerides (TGs) profile of cheese

The procedure followed for the determination of the triglycerides composition of cheese samples was described for milk samples in the section 3.3.2.2.4.

3.3.3.1.4. Evaluation of the organoleptic properties of cheese

3.3.3.1.4.1. Colour characteristics of cheese

Cheese colour was measured by using a colorimeter (Konica Minolta CR-410) with reference to illumination D65. The L*, a*, and b* colour measurements were determined according to the CIELAB colour space, were L* corresponds to light/dark chromaticity (changing from 0% dark to 100% light), a* to green/red chromaticity (negative values indicate green while positive values indicate red) and b* to yellow/blue chromaticity (negative values indicate blue and positive values indicate yellow) as it shown in the Figure 3.7.

This instrument was calibrated by with a white tile (L*= 94.4; a*= 0.3157, b*= 0.3323) before the measurements. All samples were triplicated.

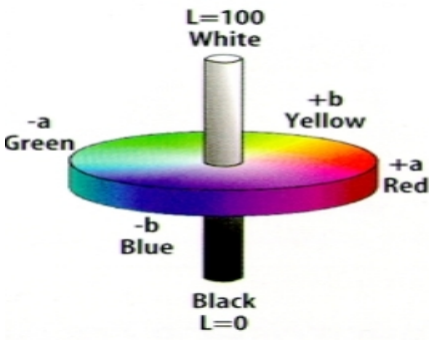


Figure 3. 7. The CIELAB* colour space (Image source: http://www.hunterlab.com)

3.3.3.1.4.2. Textural attributes of cheese

The texture analysis of cheeses was performed by a Texture Analyser (TA-XT2), calibrated by a 5 Kg load cell. Six representative samples of each cheese were cut into cylinders (1.5 cm of diameter and 2 cm of height). This test was performed at 20±2ºC using a plastic cylindrical probe (5.5 cm of diameter and 2.5 cm of height), a penetration depth of 6mm and a cross head speed of 2mm/s. From the force *vs* time, six parameters were obtained for compression: hardness (g) is defined as the peak force during the first compression cycle, adhesiveness (g.s) is the negative area under the curve obtained between cycles, springiness (s) is defined as the ratio of the time recorded between the start of the second area and the second probe reversal to the time recorded between the start of the first area and the first probe reversal, cohesiveness (dimensionless) is calculated as the ratio of the area under the second curve to the area under the first curve, gumminess (g) is defined as the product of hardness time's cohesiveness, and chewiness (g.s) is defined as the product of hardness time's cohesiveness time's springiness.

Firmness = F1
Cohesiveness= A2/A1
Adhesiveness= A3
Springiness= t2/t1
Chewiness= Firmness*Cohesiveness*Springiness

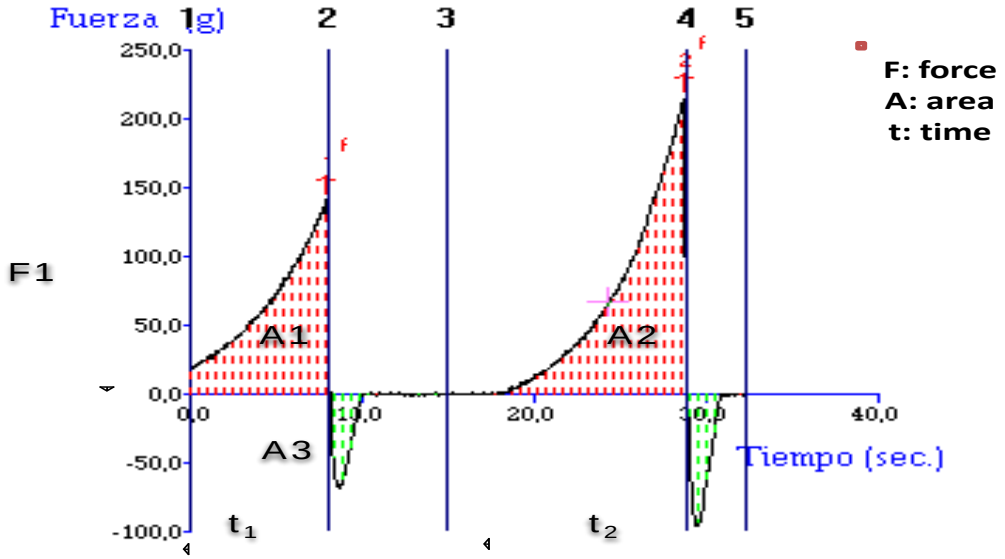


Figure 3. 8. The texture profile analysis (O´Callaghan and Guinee, 2004)

3.3.3.1.4.3. Sensory evaluation of cheese

This study utilized a multiple comparisons test for the sensory evaluation of cheeses (Lawless and Heymann, 2010). A total of 18 tests were performed by 9 trained panelists. The training for sensory analysis was performed according to the ISO (8586-1:1993). The panelists were provided with five (5) coded samples of the products being tested and three (3) coded reference samples and must pair each tested sample with one reference sample.

In this discriminative test, the objective was to reject the null hypothesis, which is the equality between the different groups of products (CO, CS, and TM). The Pearson chi-square is a statistic test that allows comparing a set of observed frequencies with a matching set of expected (hypothesized) frequencies (Lawless and Heymann, 2010). The level of significance was declared at $P<0.05$.

3.3.3.1.5. Evaluation of the antioxidant capacity of cheese

For the antioxidant assay, cheese samples correspond to those manufactured at the end of the experimental period.

3.3.3.1.5.1. Extraction of the phenolic compounds of cheese

The extraction procedure used for cheese samples was the method described by Meira *et al*. (2012) with slight modifications. The cheese extracts were prepared by mixing 1 gram of freeze-dried cheese with two different solvents (water and methanol). The mixture was then homogenized by gentle stirring at 150 rpm for 1 h at 40 °C with a Max Q 4000 Incubator and Shaker (Thermo Fisher Scientific, Millcreek Road, Marietta, Ohio, USA). After centrifugation, the upper fat layer was discarded and the extract was filtered through Whatman no. 2 paper.

3.3.3.1.5.2. The 2,2´azinobis(3 ethylbenzothiazoline-6-sulfonate) (ABTS) assay

The procedure followed for the determination of the antioxidant activity of the cheese samples was described for milk samples in the section 3.3.2.3.2.

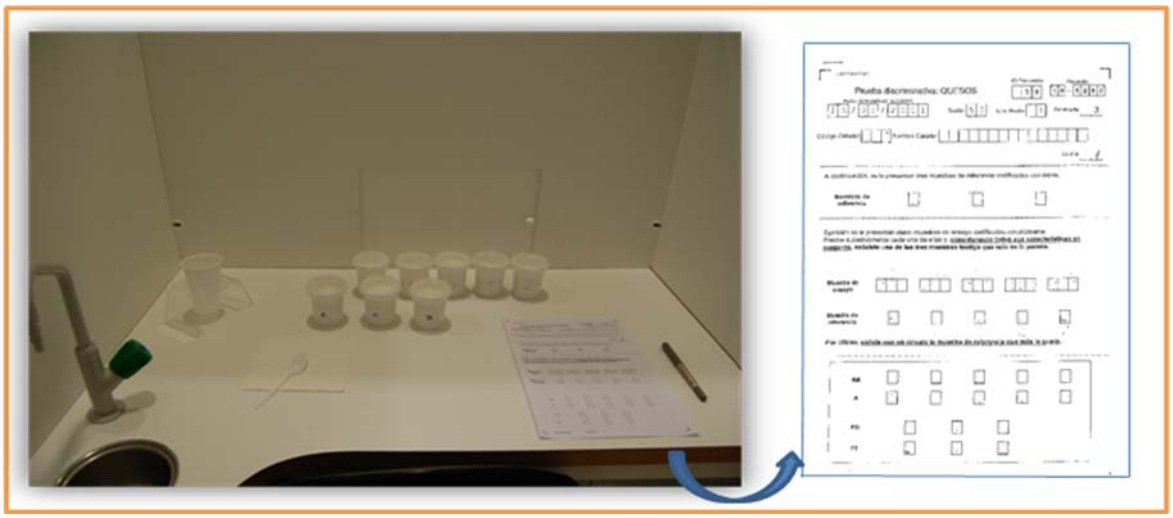


Figure 3. 9. Sensory booths (separate air-conditioning maintained at 20±2°C and RH 40±5%)

3.3.3.2. Fermented milk: Set-yogurt

3.3.3.2.1. Manufacturing process of yogurt

For yogurt processing, the filtrated milk was heated to 80ºC and for 30 min, cooled to 44-45ºC and then inoculated with starter culture (YO-MIX 300 LYO, Danisco, Danemark; 23 mg/L) that included *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*. The inoculated milk was transferred to plastic cups (125 mL, Alta Barrera SL, Barcelona, Spain) and incubated (PORTINOX ARGBT 700-1P,

Sevilla, Spain) at 42ºC until the yogurt reached a pH of 4.6. Finally, the yogurts were stored at 4ºC overnight.

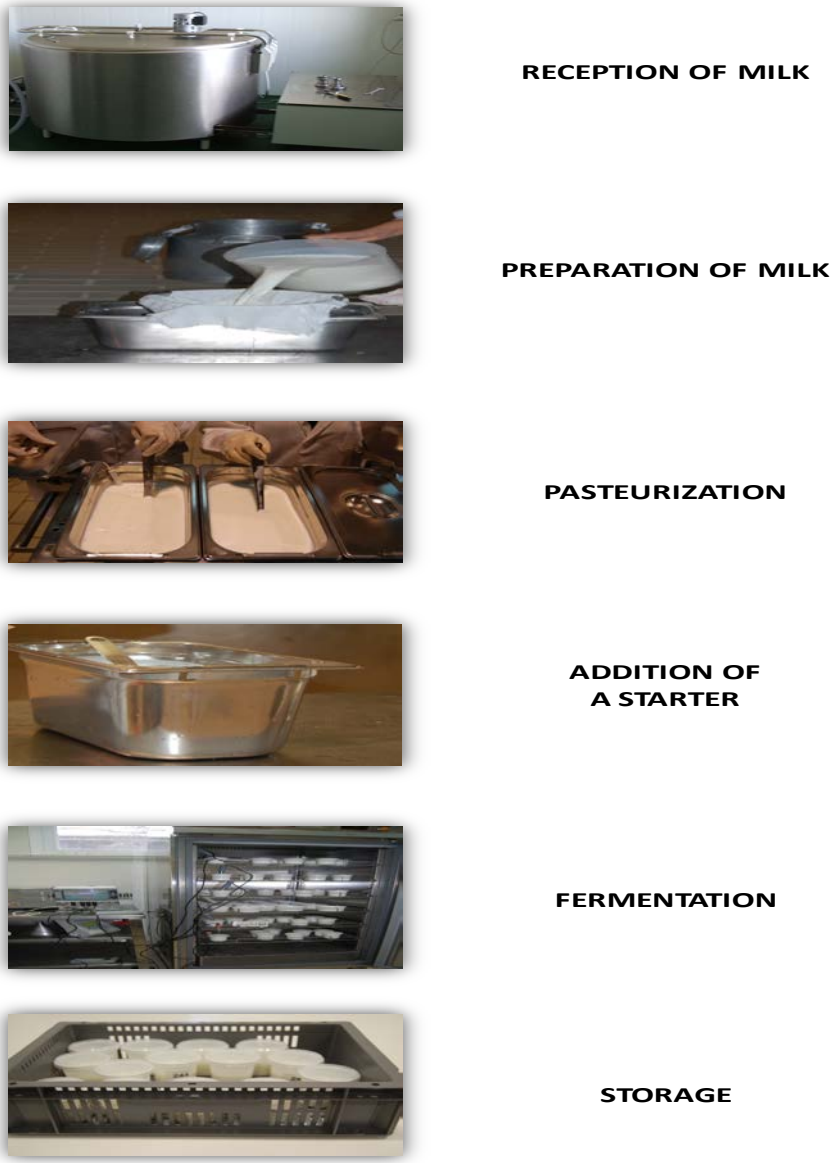


Figure 3. 10. The manufacturing process of yogurt

3.3.3.2.2. Determination of the physico-chemical composition of yogurt

Yogurt samples were analyzed by following the procedures according to the International Organization for Standardization (ISO). Yogurts were defrosted in a water bath to a temperature of 35-40ºC and then mixed and cooled to 20ºC±2. A sample of 2gr of yogurt was dissolved in 8ml of distilled water and then analyzed for fat, protein and total solids content. All samples were performed in duplicate.

Fat content was determined by gravimetric method according to the ISO 1211: 2010; an ammoniacal ethanolic solution of the sample was extracted with diethyl ether and light petroleum and then the solvents were evaporated by using an R-205 Rotary Evaporator with Heating Bath B-490 (BÜTCHI Labortechnik, Flawil, Switzerland). The mass of the fat extracted was subsequently determined.

For total solids content, the procedure used was the ISO 13580: 2005 and it consists in drying the sample by heating it in a drying oven at 103 ± 2ºC and then weighting the sample to determine the loss of mass.

The titrable acidity was determined according to the ISO 11869: 1997 using the TitroMatic 1S (CRISON INSTRUMENTS, Alella, Barcelona, Spain).

The procedure for the determination of protein content and pH values was described for cheese samples in the section 3.3.3.3.

3.3.3.2.3. Microbiological analysis of yogurt

The yogurts used for the microbiological analysis correspond to those manufactured at the beginning of the experimental period. Yogurt samples (two of each group) were analyzed at day 1, day 14 and day 28 after fabrications, we used the fabrication, fabrication 1), because in the time of the study, the consumption of yogurts was regulated by the RD 176/2013, in which the consumption of yogurts needs to be prior to the 28 days subsequent to the fabrication.

The control of the viability of lactic acid microflora in the yogurt was determined according to the ISO 7889: 2003. A 10 g sample of yoghurt was decimally diluted in sterile peptone water (0.1%) and dilution plated over the media. S. *thermophilus* were determined on M17 agar medium (supplemented with %5 sterile 10% w/v lactose) at 37ºC/48h with duplicate plates under microaerophilic conditions, L. *bulgaricus* were counted on MRS agar medium duplicate plates at 37ºC/72h which pH of agara medium was adjusted to 5.4. Plates were incubated under two different conditions; aerobic and anaerobic for S. *thermophilus* and L. *bulgaricus*, respectively, using the AnaeroGenTM gas generating kit (Oxoid Ltd, Basingstoke, Hampshire, UK).

The enumeration of S. *thermophilus* and L. *bulgaricus* was carried out by using the present formula showed in the Equation

Equation 3. 1

$$N = \frac{\sum C}{(n1 + 0{,}1\ n2)d}$$

Where N is the number of microorganisms per gram of sample, $\sum C$ is the sum of the colonies in all the plates, n_1 is the number of colonies counted in the first dilution, n_2 is the number of colonies counted in the second dilution and d is the mass in gram of the non diluted sample in the plate used as first dilution.

3.3.3.2.4. Characterization of the lipidic profile of yogurt

3.3.3.2.4.1. Fat extraction of yogurt

The yogurt samples were defrosted in a water bath to a temperature of 35-40ºC, mixed and cooled to 20ºC± 2. 100 mL of the homogenized defrosted yogurt was used for the extraction procedure.

3.3.3.2.4.2. Preparation of methyl esters of yogurt fat

The procedure followed for the preparation of the methyl esters for fatty acids and triglycerides analyses was described for milk samples in the section 3.3.2.2.2.

3.3.3.2.4.3. Fatty acids (FAs) composition of yogurt

The procedure followed for the determination of the fatty acids composition of yogurt samples was described for milk samples in the section 3.3.2.2.3.

3.3.3.2.4.4. Triglycerides (TGs) profile of yogurt

The procedure followed for the determination of the triglycerides profile of yogurt samples was described for milk samples in the section 3.3.2.2.5.

3.3.3.2.5. Evaluation of the organoleptic properties of yogurt

3.3.3.2.5.1. Colour characteristics of yogurt

The procedure followed for the measurement of the colour of yogurt samples was described for cheese samples in the section 3.3.3.6.1.

3.3.3.2.5.2. Textural attributes of yogurt

The texture analysis of yogurts was performed by a Texture Analyser (TA-XT2), calibrated by a 5 Kg load cell, and equipped with cylindrical flat-bottomed puncture

probe (P/25a). This test was performed at 10±2ºC using a penetration depth of 15mm and a cross head speed of 2mm/s. The textural parameters derived were: maximum force in compression (firmness, g), positive area of the curve (consistency, g) and the adhesiveness which is the negative force area when pulling the probe out of the yogurt sample (g.s) were determined through a single compression test. The apparent viscosity (in centipoises) was determined with the Brookfield Viscosimeter, model (DV-II+) by using the Spindle LV-4 (Brookfield Engineering Laboratories, Inc., 2000) at 1.1 rpm.

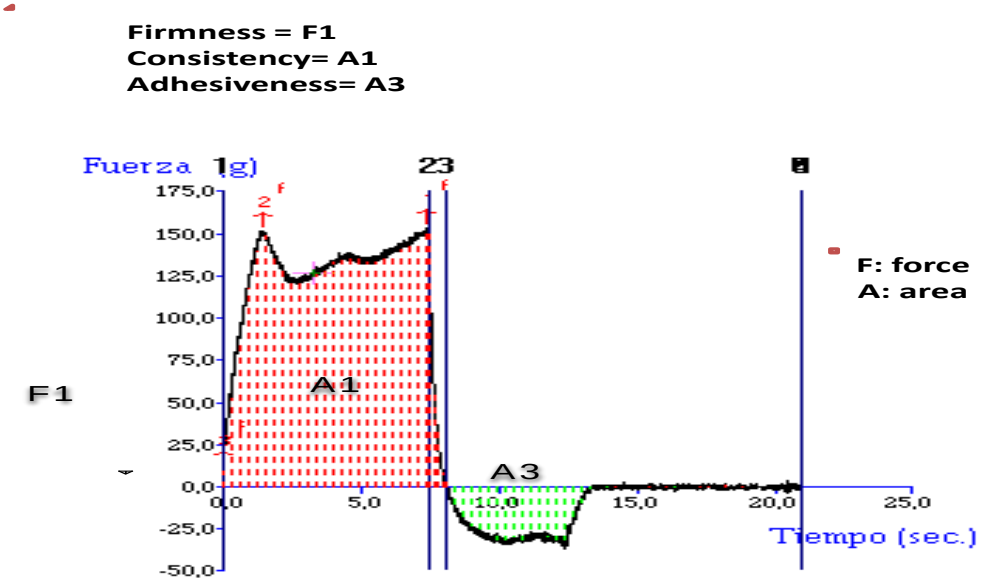


Figure 3. 11. The texture analysis (Ozcan. 2013)

3.3.3.2.5.3. Sensory evaluation of yogurt

The procedure followed for the sensory evaluation of yogurt samples was described for cheese samples in the section 3.3.3.6.3.

3.3.3.2.6. Characterization of the phenolic compounds of yogurt

The procedure followed for the characterization of yogurt extracts was described for milk samples in the section 3.1.3.2.

3.3.3.2.7. Evaluation of the bioactive properties of yogurts

3.3.3.2.7.1. Extraction of the phenolic compounds of yogurt

The first part of the extraction procedure was the same as that described for milk samples in the section 3.3.2.3.1, for the collection of the supernatant. The second part consisted on a further washing of the pellet with 25 ml of solvent (water or methanol),

after the collection of the supernatant, followed by a centrifugation and finally by a combination of both supernatants as described by Serafini *et al*. (2009).

3.3.3.2.7.2. The 2,2´azinobis(3 ethylbenzothiazoline-6-sulfonate) (ABTS) assay

The procedure followed for the determination of the antioxidant activity of the yogurt samples was described for milk samples in the section 3.3.2.3.2.

3.3.3.2.7.3. The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

The procedure followed for the determination of the antioxidant activity of the yogurt samples was described for marjoram extracts in the section 3.1.2.3.

3.3.3.2.7.4. The Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay employs AAPH as peroxyl radical generator and fluorescein as target molecule to detect the ability of the added antioxidant to inhibit their interaction relative to Trolox standard according to Huang *et al*. (2005). The assay method provides an integrated and quantitative determination of the antioxidant capacity by employing the area under the curve (AUC) of the magnitude and time of inhibition of peroxyl radicals attack against fluorescein. Fluorescence at 485/520 nm was monitored using an automated BMG FLUOstar Omega microplate reader system (Offenburg, Germany). ORAC values were expressed as µM trolox equivalents (µM TE). All samples were replicated.

3.3.3.2.7.5. The Semicarbazide-Sensitive Amine Oxidase (SSAO) inhibitory assay

The procedure followed for the determination of the anti-inflammatory activity of the yogurt was described for cheese samples in the section 3.1.2.4.

3.3.4. Statistical analysis

Data were analyzed by using the SPSS package (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA). All Data was analyzed using the General Linear Models procedure. For the physico-chemical composition and lipídic profile of milk and yogurt and for the texture and color of yogurts, the model accounted for the fixed effects of diet, time, and the interaction between diet and time. For the data of the physico-chemical composition the lipídic profile, color and texture of cheese, the model accounted for the fixed effects of diet, time, ripening and the interaction between diet, ripening and time. For the data of the antioxidant activity, the effect of the solvent extractant was included in each

model, for milk, yogurt and cheese. For the microbiological data of yogurt, the model accounted for diet, self life and the interaction between diet and self life. For the data of the anti-inflammatory activity of yogurt the model o accounted for the effect of the diet. In sensorial analysis, the test used to decide whether or not to reject the null hypothesis that state that there is no detectable difference between the three products is the $\chi 2$ (Chi-squared). Values with statistically significant differences ($P<0.05$) were compared using Duncan's Multiple Range test and designed by different letters.

Chapter 4: Results and Discussion

Chapter 1

Evaluation of *T. mastichina* as source of natural antioxidants

4.1. Evaluation of *T. mastichina* as source of natural antioxidants

4.1.1. Evaluation of the bioactive properties of *T. mastichina*

4.1.1.1. Total phenolic content (TPC) of marjoram extracts

The Folin–Ciocalteu phenol assay was used to evaluate the total phenolic content present in the samples (Albano and Miguel, 2011; Dalar *et al*., 2012; Kerio *et al*., 2013; Roby *et al*., 2013). In the present study, the total phenolic content was expressed as gallic acid equivalent.

Total phenolic content of extracts varied significantly according to the solvent used for the extraction (Table 4.1). Methanol extracts had significantly higher phenol content than ethanol or water extracts (195.71 ± 4.07, 168.96 ± 4.48 and 152.30 ± 5.44 mg GAE/g dry extract, respectively ($P< 0.05$), Table 4.1). Those results were in agreement with results reported by Barros *et al*. (2010) for *T. mastichina*, in which methanol extracts showed the highest values in comparison with ethanol and water extracts (165.29 ± 1.11, 109.09 ± 0.46 and 59.93 ± 0.06 mg GAE/g dry extract, respectively). The total phenolic content in the present results was higher than values reported by Barros *et al*. (2010), especially for water extracts. Those differences might be associated to the origin of the plants (Spanish *vs* Portuguese) since the edapho-climatological conditions were different in both areas.

Other studies were carried out on *T. mastichina,* from the Spanish and the Portuguese regions, by using different procedures for the extraction of phenolic content, different to that described in the present study and also different parts of the plant (flowers, leaves..). Delgado *et al*. (2014) used the arterial parts (leaves and flowers) of the Spanish T. *mastichina*. The methanolic extracts were obtained by the application of a preliminary extraction with petroleum ether in order to eliminate chlorophyll and fats. The total phenolic content determined for twenty *T. mastichina* samples ranged between 2.90 and 9.15 mg GAE/g dry extract. For *T. mastichina* from the Portuguese region, Albano and Miguel (2011) performed the analysis on the flowering aerial parts of the plant and used two different procedures for the extraction; the first one consisted of reextraction with methanol of the phenols from the dried plant material after the hydrodistillation for essential oils isolation and the second involved the maceration of

the chopped dried plant material with ethanol, suspension of the crude extract in distilled water and finally the extraction with diethyl ether, ethyl acetate, and n-butanol. Regardless of the extraction procedure and the solvent used, the total phenolic content ranged between 0.78 and 26.18 mg GAE/mL.

The differences between results might mainly be associated to the solvent used, the extraction method and also the part of the plant on which the analyses were performed.

Table 4. 1. Extraction yield and total phenolic content of marjoram extracts

Sample	**Total phenols (mg GAE[a]/g dry extract)**
Water extract	152.30 [c]± 5.44
Ethanol extract	168.96 [b] ± 4.48
Methanol extract	195.71 [a] ± 4.07

[a] GAE, Gallic acid equivalent
Values (mean ± SD, n=3) in the same column a different letter are significantly different ($P< 0.05$)

Since *T. mastichina* belongs to the *Lamiaceae* family, results of total phenolic content will also be compared to species that belong to the same family or if data is available to the *Thymus* genus. Roby *et al*. (2013*)* reported that methanol was more efficient solvent in extracting phenols from the leaves of *Thymus vulgaris L.* than ethanol, diethyl ether and hexane (8.10; 7.30, 6.15 and 4.75 mg GAE/g of dry material, respectively). The best values of methanol extracts were observed for thyme (*Thymus vulgaris* L.) in comparison with sage (*Salvia officinalis* L.) and marjoram (*Origanum majorana* L.) and ranged from 8.1 to 3.9 mg GAE/g dry material. Similarly, results of Lagouri *et al*. (2009) revealed that the highest TPC was observed on leaves of *Thymus vulgaris*, extracted with methanol, in comparison with hexane and dichloromethane extracts (148.31; 11.97 and 7.35 mg caffeic acid/g, respectively) and also in comparison with methanol extracts of *Origanum onites, Origanum dictamnus, and Rosmarinus officinalis* (148.31; 128.58; 129.52 and 129.51 mg caffeic acid/g, respectively).

Rababah *et al*. (2010) reported that the TPC determined in the methanol extracts of the *Thymus capitatus* leaves was higher than that of ethanol extracts, independently of the temperature used for the extraction (2419.9 vs 2323.6 mg GAE/100 g of dry material at 60ºC and 1066.4 vs 219.8 mg GAE/100 g of dry material at 20ºC, respectively).

In the other hand, results reported by Kırca and Arslan, (2008), where analyses were performed on methanolic extracts prepared from various parts of some selected plants

from Turkey belonging to the *Lamiaceae* family, showed that the highest values were observed in the flower extract of *Origanum vulgare* (92.70 ± 0.78 mg of GAE per g dry weight extract) and the lowest value was reported for seeds from *Sideritis trojana* (13.08 ± 0.07 mg of GAE per g dry weight extract). Moreover, Turumtay *et al*., (2014) working on the methanolic extracts of Anzer tea (*Thymus praecox* Opiz subsp. *caucasicus* var. *caucasicus*) showed that the lowest total phenolic content was detected in stem extracts whereas the highest concentration was found in flower extracts (11.95 ± 0.196 and 24.59 ± 0.091 mg GAE per g dry weight, respectively).

The values of the water extracts in the present study (152.30 ± 5.44) were similar to the values reported by Hinneburg *et al*. (2006) and Dorman *et al*. (2004) for Basil (*Ocimum basilicum*) and savory (*Satureja cuneifolia* Ten.) (147 ± 1.60 and 151 mg GAE/g dry extract, respectively) although different methods of drying were used. In the present study air-dried extracts were used whereas in the study carried out by Hinneburg *et al*. (2006) samples were freeze-dried.

Besides the solvent used for the extraction, the part of the plant on which the analysis was performed as described above, the specie can also be responsible to some extent for the variability of the total phenolic content. Certainly, although similar phenolic compounds can be found in diverse plants when they belong to the same family (Yanishlieva *et al*., 2006), other phenolic compounds are as well specific to the species (Albano and Miguel, 2011). Furthermore, differences may also be associated to the wild origin of the plant.

4.1.1.2. Radical scavenging activity (DPPH) of marjoram extracts

The DPPH free radical scavenging activity assay was used to measure the ability of the samples to reduce the free radical DPPH and this method has been extensively used for plants and spices (Albano and Miguel, 2011; Delgado *et al*., 2014; Kırca and Arslan, 2008; Lagouri *et al*., 2009). DPPH radical scavenging activity was expressed as Trolox equivalents per 100 grams of dry extract or as the extract concentration providing 50% of radicals scavenging activity (EC_{50}).

The DPPH free radical scavenging activity was measured in marjoram extracts (methanol, ethanol and water extracts) and followed a concentration-dependent pattern (Figure 4.1). As expected, an increase of the extract concentration (0.015-0.06 mg/mL) improved the scavenging activity; exponentially for the water and the ethanol extracts

(R^2 = 0.999; R^2 = 0.999, respectively) and in a polynomial fashion for the methanol extracts (R^2 = 1). Those results were in agreements with those reported by Delgado *et al*. (2014), Lagouri *et al*. (2009) and Rababah *et al*. (2010). However, the scavenging activity of methanol extracts increased significantly (P<0.05) above concentration 0.03 mg/mL since the optimum concentration at which it was suitable to compare the different solvent extracts was the central point which corresponded to 0.03 mg/mL. At this concentration, the DPPH free radical scavenging activity of methanol extracts was higher than that of water and ethanol extracts (58.85 ± 0.52, 35.12 ± 0.20 and 39.63 g Trolox/100 g of dry weight extract, respectively). The DPPH scavenging activity presented for the methanol extracts was in agreement with the results reported by Hossain *et al*. (2008) for the synthetic antioxidant (BHT) (80.85 g Trolox/100gDW).

The DPPH free radical scavenging activity expressed as $EC_{50,}$ showed as well that methanolic extracts had higher antioxidant activity than ethanolic and water extracts (0.028, 0.044 and 0.052 mg/mL, respectively). Those results were in agreement with those reported by Barros *et al*. (2010), in which the DPPH radical scavenging activity of the methanolic extracts of *T. mastichina* was higher than those extracted with ethanol and water (0,69, 0,94 and 2,57 mg/ml). In general, regardless of the solvent used, values of the DPPH free radical scavenging activity in the present study were higher than those reported by Barros *et al.* (2010).

As for phenolic content, the discrepancy between the present results and those observed in other studies on *T. mastichina* (Albano and Miguel, 2011; Delgado *et al*., 2014) might be associated to the fact that different parts of the plants were used for the determination of the antioxidant activity and to the difference between the extraction procedures. Delgado *et al*. (2014), in a study carried out on twenty Spanish *T. mastichina*, found that the DPPH scavenging activity of the methanolic extracts, expressed as EC_{25}, ranged between 0,90 and 3.45 mg/mL. Albano and Miguel (2011) found, by using two different procedures and several solvents, as described for phenolic content, that the DPPH scavenging activity of the Portuguese *T. mastichina*, ranged between 2,7 and 8.3 µg/mL.

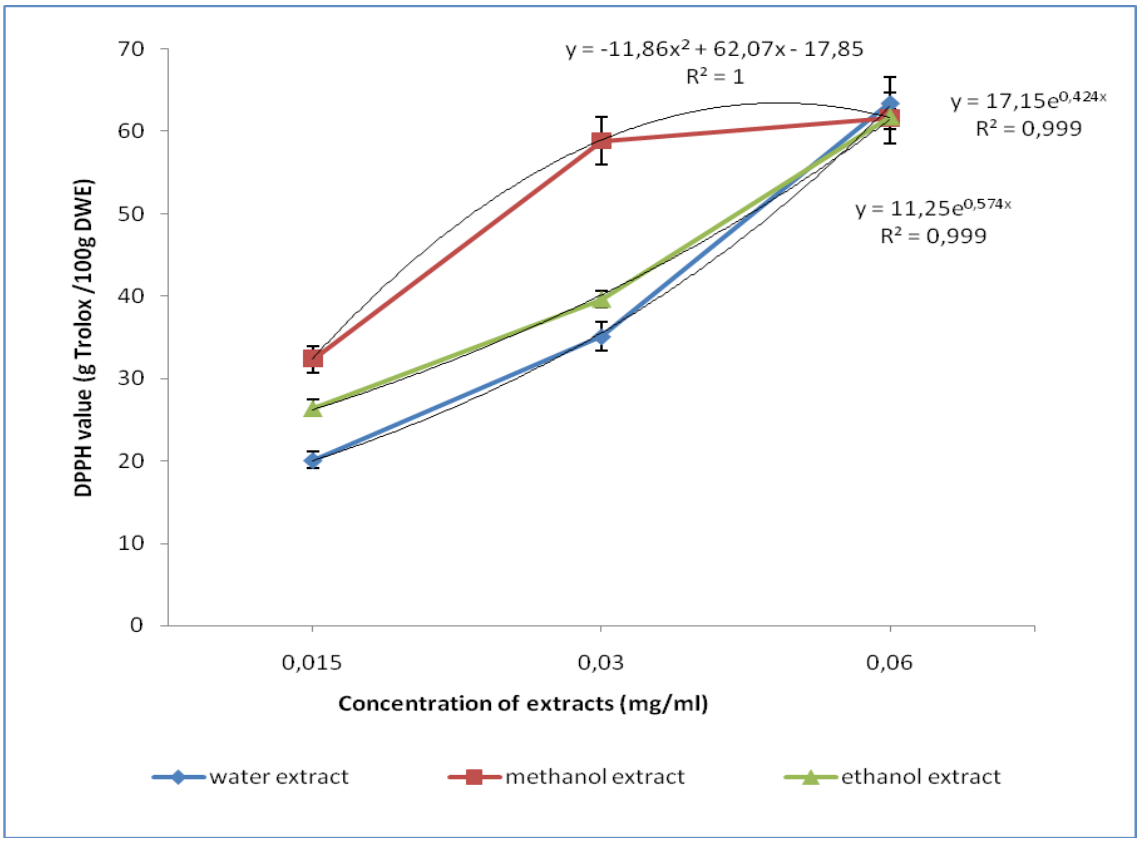


Figure 4. 1. DPPH radical-scavenging effect of methanol, ethanol and water extracts of marjoram

In comparison with other species that belong to the *Lamiaceae* family, Rababah *et al*. (2010) reported that the DPPH free radicals scavenging activity of the methanolic extracts of *T. mastichina*, expressed as $EC_{50,}$ was higher than in oregano and thyme (0.119 ± 0.01 mg/mL). Moreover, the antioxidant activity of the methanolic extracts of *T. mastichina* was two times lower than that EC_{50} for the synthetic antioxidant (BHT) (0.019 mg/mL) (Rababah *et al*., 2010). Other studies have investigated the DPPH scavenging activity of other species of *Thymus* (Nickavar and Esbati, 2012; Turumtay *et al*., 2014) by using other solvents or other parts of the plant (flower, leaves and stems), different to that used in the present study. The DPPH scavenging activity of the ethanolic extract of *T. kotschyanus*, and *T. pubescens*, observed by Nickavar and Esbati (2012), was similar to that observed for *T. mastichina* (0.0472, 0.0487 and 0.044 mg/mL, respectively). Turumtay *et al*. (2014) used different parts of the Anzer tea (*Thymus praecox* Opiz subsp. *caucasicus* var. *caucasicus*) to measure the DPPH scavenging activity of the plant and found that the antioxidant activity of the flowers was the highest in comparison with leaves and stems (0.060, 0.087 and 0.139 mg/mL, respectively) .

A significant correlation (r=0.976) was observed between the content of total phenols (mg GAE/g dry weight extract) and the DPPH scavenging activity (equivalent Trolox) of marjoram extracts. As expected, the DPPH scavenging activity of marjoram was higher following the same pattern as that observed for phenolic contents since extracts that contain high amount of polyphenols also exhibit high antioxidant activity (Wong *et al*., 2005). This linear correlation between antioxidant capacity and total phenolic content of aromatic plants and spices was also reported by Rababah *et al*. (2010), Turumtay *et al*. (2014), Hossain *et al*. (2010) and Liu *et al*. (2011) who reported coefficients of correlation between 0.852 to 0,965. However, other authors as Wong *et al.* (2005) and Kırca and Arslan, (2008) reported a low linear correlation (r = 0,566-0,548 and 0.560, respectively). This lack of correlation might be associated to the fact that some plants contain bioactive components, others than phenolic compounds, with high antioxidant activity.

Also in order to determine the effect of solvent as third variable, a partial correlation was performed. As result, the correlation between the primary variables (total phenolic content and DPPH scavenging activity) did not decreased (r= 0.996) in comparison with the Pearson's correlation (r=0.976), which means that the solvent used (methanol, ethanol and water) did not affect significantly the relationship between the total phenolic content and the DPPH scavenging activity. However, when the correlation included only the methanol and the water extracts, the effect of solvent became significant (r = -0.071). Those results were in agreement with those mentioned before where a positive correlation was reported between antioxidant capacity and total phenolic content and also with those in which the effect of other factors, such the solvent used, could influence significantly this correlative relationship.

As observed, the high antioxidant capacity of *T. mastichina* was mostly associated to its high phenolic content. As it shown below (Table 4.2), *T. mastichina* was rich in flavonoids (luteolin, quercetin and apigenin). Several study found that luteolin and quercetin has high antioxidant properties (Materska. 2008; Özgen. 2011) with quercetin being the compound with the highest radical scavenging activity in comparison with luteolin and apigenin (Majewska *et al*., 2011). 1,8-cineol, which is the main chemotype detected in the essential oil of *T. mastichina* L. was as well reported as a compound with high antioxidant activity (Blanco Salas. 2012).

4.1.1.3. SSAO inhibitory activity of marjoram extracts

The inhibition of SSAO activity represents a target for anti-inflammation because SSAO plays a key role in inflammation process through its ability to produce catalytic products such as hydrogen peroxide and other reactives aldehydes species.

The SSAO inhibitory activity of the water extracts of *T. mastichina* was compared with that of semicarbazide (positive control). The standard curve of semicarbazide was used to quantify the inhibitory activity of marjoram, which were 22.70, 28.13 and 57.27 µM of semicarbazide for 0.015, 0.3, and 0.9 mg/ml, respectively.

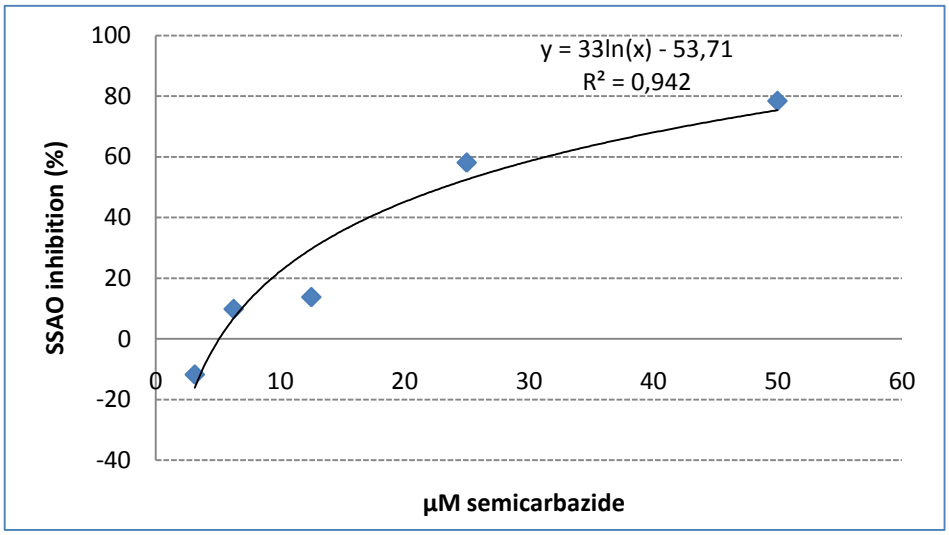


Figure 4. 2. Semicarbazide-sensitive amine oxidase (SSAO) inhibition curve

T. mastichina was found to exhibit dose-dependent SSAO inhibitory activities of 37.67%, 45.88% and 100%, respectively, for 0.15, 0.3 and 0.9 mg/mL of Marjoram (Figure 4.3). The EC_{50} of *T. mastichina* was much lower than that of semicarbazide (0.39 mg/ml and 3.53 µM which corresponds to 393,63 mg/ml, respectively). *T. mastichina* was found to be a strong SSAO inhibitor, and its EC_{50} was 1/1009 lower than EC_{50} for semicarbazide, which means that *T. mastichina* was more than one thousand time stronger than semicarbazide. The high value of EC_{50} might be associated to the fact that the SSAO assay was performed on the water extracts of the whole plant (*T. mastichina*) and not on an isolated compound such as in the results found by Lin et al. (2008), in a study performed on geranin isolated from *P. niruri*, in which geranin was reported as a strong SSAO inhibitor (about 1/5.2 that of semicarbazide).

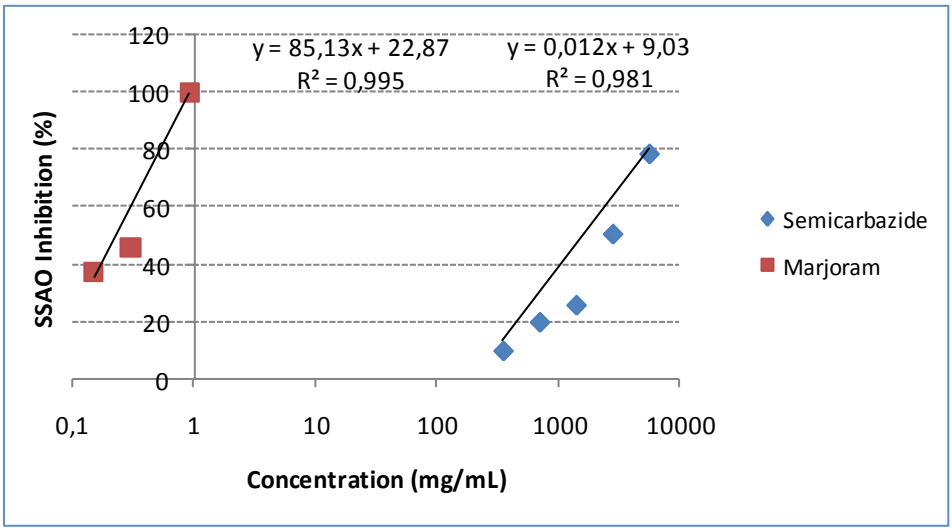


Figure 4. 3. The inhibitory activities of marjoram (0.15, 0.3 and 0.9 mg/mL) and semicarbazide (3.125, 6.25, 12.5, 25 and 50 µM which corresponds to 348.5, 697.1, 1394.1, 2788.2 and 5576.5 mg/mL) on SSAO activities

Albano and Miguel (2011) in a study performed on the flowering aerial parts of *T. mastichina* used extraction procedures different to that described in the present study and used the 5-lipoxygenase assay to measure the anti-inflammatory activity of *T. mastichina*. In the same and in comparion with other species that belong to the *Lamiaceae* familly (*Origanum vulgare*, *Salvia officinalis*, *Thymbra capitata*, *Thymus camphoratus* and *Thymus carnosus*) and for four extracts from a total of six, *T. mastichina* was found to be the strongest inhibitor of 5-Lipoxygenase. Consequently, regardless of the extraction procedures, the part of the plant on which the assay was performed and the method used for the determination of the anti-inflammatory activity *T. mastichina* was found to exert high anti-inflammatory activity.

4.1.2. Characterization of phenolic compounds in *T. mastichina* L.

4.1.2.1. Context

Marjoram *T. mastichina* L. belongs to the Thymus species, which are well known as aromatic and medicinal plants (Jia *et al*., 2010), with antioxidant activities comparable to those of α-tocopherol and butylated hydroxytoluene [BHT] (Brewer. 2011). Their potent antioxidant properties have been mostly associated to the polyphenols present in the main composition (Hossain *et al*., 2010; Sørensen, *et al*., 2012). Many aromatic herbs and spices have been studied and to some extent their phenolic chemistry is known (Cai *et al*., 2004; Wojdyło *et al*., 2007) but few information is available in relation to the phenolic composition of *T. mastichina* L.

In the present study, the characterization of the polyphenols from *T. mastichina* L. was performed using the liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). Liquid chromatography coupled to mass spectrometry (LC/MS) was widely reported as an efficient, accurate and specific method for the analysis of polyphenols in many natural products (Abad-García *et al.* 2009). Indeed, LC-ESI-MS/MS is recognized as a powerful analytical tool due to its high sensitivity, short run time, less use of toxic organic solvents compared to reversed phase HPLC coupled with diode-array detector and also for its ability to differentiate compounds with same nominal mass but different exact masses (Hossain *et al*., 2010; Liu *et al*., 2005; Rauter *et al*., 2005).

4.1.2.2. Mass spectrometric conditions for polyphenols of *T. mastichina L.*

Polyphenols except polymeric tannins are usually small molecules and those are identified using mass spectral data in the mass range of m/z 100 to m/z 1000. The negative ionization mode is more suitable than the positive ionization mode for the acquisition of MS data from polyphenols, because they contain one or more hydroxyl and/or carboxylic acid groups (Hossain *et al*., 2010). The methanolic extracts showed the highest extraction yield, phenolic content and DPPH scavenging activity comparing to ethanol and water extracts. For these reasons phenolic characterization of marjoram was performed only on the methanol extract. Several studies have also used methanol rather than other solvents to extract the phenolic components from different aromatic plants (Hossain *et al*., 2010; Roby *et al*., 2013). Figure 4.4 showed the total ion current (TIC) chromatogram of marjoram extracted with methanol. The identification of each peak is specified in Table 4.2.

A total of eighteen different polyphenols were identified and quantified calculating the accurate mass. Most of the identified compounds belong to the flavonoids class (seventeen) and one compound is a hydroxycinnamic acid derivative (rosmarinic acid). Since the amount of polyphenols is dependent on the extraction method, the use of the solide phase extraction in this study may had promoted the presence of flavonoids at the extent of the phenolic acids.

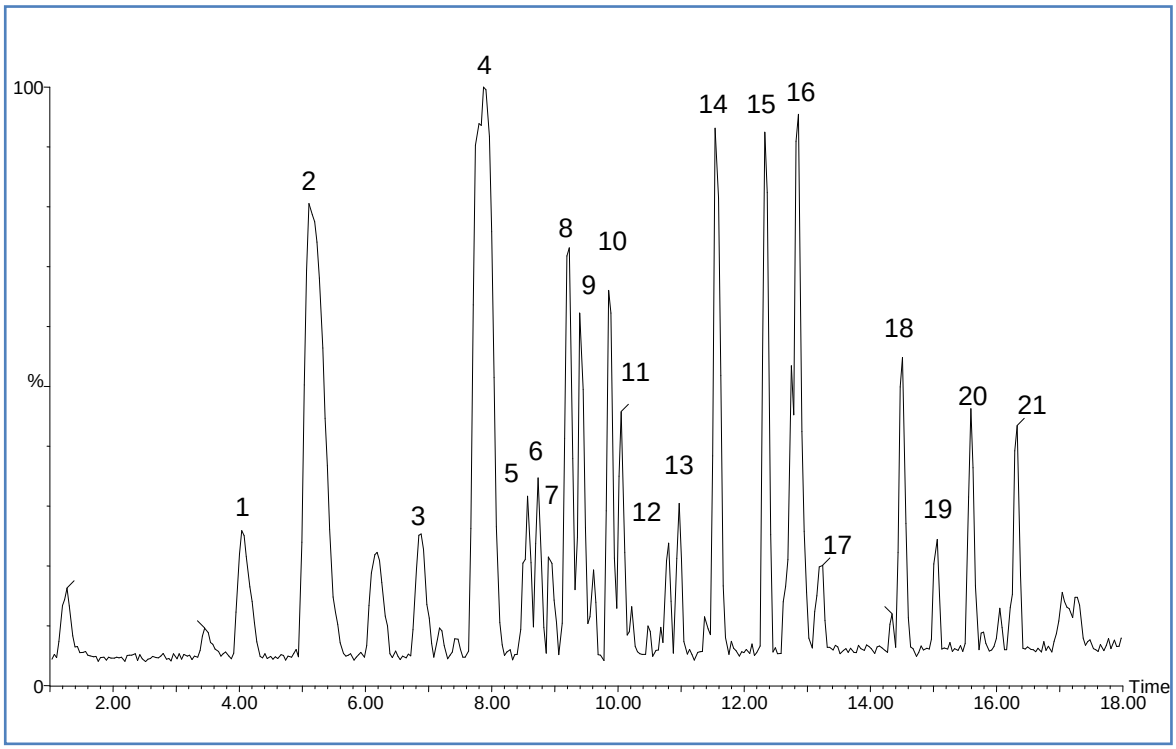


Figure 4. 4. Phenolic analysis of marjoram extract by LC-MS

The largest number of polyphenols identified by LC-MS, in this study performed for *T. mastichina* L., belonged to the flavonoids class (Table 4.2). Seventeen flavonoids were identified through the use of reference standards and accurate mass measurement data. Reference standards were used to identify 5 flavonoids; luteolin-7-O-glucoside (4), luteolin (14), naringin (15), apigenin (16) and apigenin 7-O-glucoside (9). For the twelve remaining flavonoids, for which there were no 'in-house' standards, their identifications were based on accurate mass measurements and MS/MS experiments. The twelve flavonoids identified were dihydroquercetin-O-rhamnoside (1), quercetin glucoside (2), naringin glucoside (3), quercetin-O-arabinoside (5), isorhamnetin -O-hexoside (6), quercetin- dihexoside (7), eriodictyol (8), apigenin 7-O-glucuronide (10), quercetin-hexoside-glucuronide (12), luteolin/-hexoside-glucuronide (13), kaempferide (17) and cirsimaritin (19).

The fragmentation of the deprotonated molecular ion provided the aglycones as prominent fragments and the mass difference between the deprotonated molecular ion and the aglycone fragment determined the molecular mass of the glycoside substituent. The majority of flavonoids reported were present as glycosyl or /and glucuronyl conjugates.

Other studies also reported the presence of the glycosyl and glucuronyl conjugates (Justesen. 2000; Hossain *et al*., 2010), but not bounded to the same aglycone, as in this study. Among the glycosylated flavonoids, the glucoside conjugates were the most commonly observed. Delgado *et al*. (2014) and Preira and Cardoso (2013), observed that species of *Thymus* usually comprise distinct glycosidic forms of the flavonoids luteolin, apigenin and narginin. The MS/MS experiments revealed that the $[M-H]^-$ ions at *m/z* 463.09 eluting at 4.33 min, *m/z* 447.09 eluting at 5.16 min, *m/z* 433.11 eluting at 6.02 min and *m/z* 431.10 eluting at 9.06 min were quercetin-glucoside, luteolin-7-O-glucoside, narginin-glucoside and apigenin-7-O-glucoside, respectively. Moreover, isorhamnetin-O-hexoside and quercetin-dihexoside had similar fragmentation with the loss of one or two hexose moieties (162u), respectively. Glucoronide derivatives were also identified. Subsequent CID of apigenin-7-O-glucuronide showed the loss of a glucoronic acid (*m/z* 176.0) and produced the predominant fragment at *m/z* 269.0 corresponding to the aglycone apigenin. The apigenin-glucuronide has been described for the first time by Kaiser *et al*. (2013) in marjoram (*Origanum majorana* L.). Furthermore in the daughter scan mode (MS/MS), the spectra presented fragments at *m*/*z* 301.0 and at *m*/*z* 285.0 corresponding to the deprotonated molecules quercetin and luteolin, respectively, after the loss of both glucosyl and glucuronyl moieties. In agreement with our findings the following glucosides and glucuronides were reported in oregano, rosemary and marjoram as apigenin-7-O-glucoside (Hossain *et al*., 2011; Lin *et al*., 2007), in sage as luteolin-7-O-glucoside and luteolin-7-O-glucuronide (Lu and Foo, 2001; Yanishlieva *et al*., 2006) and in thyme as luteolin-7-O-glucuronide (Dapkevicius *et al*., 2002).

Only one arabinoside derivative was detected in the *Thymus mastichina* L. extract. This compound eluting at 8.13 was identified as quercetin-O-arabinoside (Table 4.2). The CID of quercetin-O-arabinoside showed the loss of a pentose (*m/z* 132) and produced the predominant fragment at *m/z* 301.0 corresponding to deprotonated quercetin.

Furthermore in the MS/MS experiment, cirsimaritin (*m/z* 313.1) lost two consecutive methyl groups, which resulted in two fragment ions at *m/z* 298.0 and at *m/z* 283.1. Hossain *et al*. (2010) had previously reported similar results using LC-ESI-MS/MS on cirsimaritin in rosemary, oregano, sage, basil and thyme extracts.

The quantities of each compound were expressed in µg kaempferol/mg dry weight and, luteolin-7-O-glucoside was the most abundant phenolic compound (6.22 µg

kaempferol/mg DWE) followed by quercetin glucoside (5.46 µg kaempferol/mg DWE) and luteolin (5.13 µg kaempferol/mg DWE). Many authors report that aglycones exhibit higher antioxidant activity than their glycosidic counterparts (Bravo. 1998; Kim and Lee, 2004; Rice-Evans et al., 1997). In addition, apigenin, naringin and eriodictyol were also present in significant amounts (3.76, 3.03 and 2.87 µg kaempferol/mg DWE, respectively). Those results were similar to values reported by Wojdyło *et al*. (2007) in which quercetin, luteolin and apigenin were the predominant flavonoids observed in 32 aromatic plants. Moreover, Delgado *et al*. (2014) detected the presence of apigenin and luteolin in *T. mastichina* and those values were the highest in Salamanca and Soria provinces (0.15 to 0.91 mg/g and 0.35 to 1.85 mg/g, respectively). Additionally, Roby *et al*. (2013) reported that apigenin was the principle flavonoids (more than 70% of the total flavonoids) in marjoram leaves. Narginin was also reported to be abundant in salvia species growing in different habitats (Ben Farhat *et al*., 2013).

Rosmarinic acid was the unique polyphenol from the hydroxycinnamic acid derivatives category identified with the LC-MS. It was recognized by comparing the retention time and the characteristic MS spectral data with that of the authentic standard (Table 4.2). Accurate mass measurements and fragmentation pattern during CID further confirmed their structural composition. The fragments produced, during CID analysis of the deprotonated ion at m/z 359.08 identified as rosmarinic acid, are caffeic acid at *m/z* 179.0 and the 2-hydroxy derivative of hydrocaffeic acid at *m/z* 197.0. Similar pattern of fragmentation of rosmarinic acid has been already observed in other studies, analyzing different extracts of Lamiaceae spices (Herrero *et al*., 2010; Justesen, 2000; Shen *et al*., 2010).

The rosmarinic acid in the current study was present at high levels (1.94 µg kaempferol/mg DWE) but not as the main constituent. This result was in agreement with values published by Kaiser *et al*. (2013) in marjoram (*Origanum majorana* L.) extracts. However it contrast results, in Lamiaceae family or in Thyme genus, in which rosmarinic acid was confirmed to be the major constituent (Delgado *et al*., 2013; Herrero *et al*., 2011; Nagy *et al*., 2011; Ramchoun *et al*., 2012; Roby *et al*., 2013).

Pseudomolecular ions at *m/z* 327.20 (observed exact mass 327,2171), *m/z* 320.20 (observed exact mass 329,2328) and *m/z* 343.10 (observed exact mass 343,0818) eluting at 14.31 min, 15.39 min and 16.12 min respectively could not be identified.

Table 4. 2. Phenolic compounds identified in marjoram by LC-ESI-MS/MS

Peak	Polyphenols	Emperical formula	Obsd m/z [MH]-	Calcd m/z [MH]-	Major fragments	ug kaempferol/mg DWE	Retention time (min)
1	Dihydroquercetin-3-O-rhamnoside	$C_{21}H_{22}O_{11}$	449,1100	449,1084	287	1,42	3,51
2	Quercetin glucoside	$C_{21}H_{20}O_{12}$	463,0898	463,0877	301	5,46	4,33
3	Naringin glucoside	$C_{21}H_{22}O_{10}$	433,1150	433,1135	271	1,50	6,02
4	Luteolin-7-O-glucoside	$C_{21}H_{19}O_{11}$	447,0939	447,0927	285	6,22	7,30
5	Quercetin-3-O-arabinoside	$C_{20}H_{17}O_{11}$	433,0784	433,7710	301	1,63	8,13
6	Isorhamnetin-3-O-hexoside	$C_{22}H_{21}O_{12}$	477,1039	477,1033	315	1,18	8,29
7	Quercetin 2-hexoside	$C_{30}H_{25}O_{15}$	625,1190	625,1193	301	0,69	8,53
8	Eriodictyol	$C_{15}H_{11}O_{6}$	287,0559	287,0556	259	2,87	8,83
9	Apigenin-7-O-glucoside	$C_{21}H_{19}O_{10}$	431,0992	431,0978	431	1,59	9,06
10	Apigenin-7-O-glucuronide	$C_{21}H_{17}O_{11}$	445,0785	445,0771	269	0,65	9,28
11	Rosmarinic acid	$C_{18}H_{15}O_{8}$	359,0788	359,0767	161	1,94	9,52
12	Quercetin-hexoside-glucuronide	$C_{31}H_{27}O_{15}$	639,1339	639,1350	301	1,28	9,78
13	Luteolin/-hexoside-glucuronide	$C_{31}H_{27}O_{14}$	623,1389	623,1401	285	0,63	10,51
14	Luteolin	$C_{15}H_{10}O_{6}$	285,0406	285,0399	285	5,13	11,30
15	Naringin	$C_{15}H_{11}O_{5}$	271,0618	271,0606	151	3,03	12,09
16	Apigenin	$C_{15}H_{19}O_{5}$	269,0454	269,0450	269	3,76	12,58
17	Kaempferide	$C_{16}H_{11}O_{6}$	299,0569	299,0556	299	1,61	12,99
18	Unknown	$C_{18}H_{31}O_{5}$	327,2175	327,2171	291	2,17	14,31
19	Cirsimaritin	$C_{17}H_{13}O_{6}$	313,0724	313,0712			14,80
20	Unknown	$C_{18}H_{33}O_{5}$	329,2343	329,2328		1,77	15,39
21	Unknown	$C_{18}H_{15}O_{7}$	343,0835	343,0818		1,44	16,12

4.1.3. Optimization of extraction and yield of marjoram extracts

The efficiencies of different solvents (water, ethanol and methanol) on the extraction of total polyphenols from *T. mastichina* L. were investigated. As shown in Table 4.3, the extraction yields varied significantly according to the extraction solvents used for extraction. In terms of total extraction yield, methanol was the most effective extractant solvent followed by ethanol and water (11.88, 8.51 and 7.91%, respectively).

Those results were in agreement with other results published previously by Hossain *et al*. (2008) and Shan *et al*. (2005) where methanol was reported to be a highly efficient solvent for the extraction of phenolic antioxidant. According to Roby *et al*. (2013) the polarity of solvents is correlated with the degree of extraction. Higher polarity solvents were more efficient in extracting phenolic compound from plant material than lower polarity solvents such as ethanol and water.

Table 4. 3. Extraction yield of marjoram extracts

Sample	**Extraction yield (%)[a]**
Water extract	$7.91^{b}\pm 0.52$
Ethanol extract	$8.51^{b}\pm 0.27$
Methanol extract	$11.88^{a} \pm 0.77$

[a] Extraction yield (%) = ((sample extract weight/sample weight) *100)
Values (mean ± SD, n=3) in the same column a different letter are significantly different ($p< 0.05$)

Chapter 2

Evaluation of the animal and lactation performances of sheep

4.2. Evaluation of the animal and lactation performances of sheep

4.2.1. Evaluation of the animal performances of non-lactating ewes

4.2.1.1. Control of the individual dry matter intake (iDMI) and body weight

Results of body weight and individual dry matter intake (iDMI) were showed in the Table 4.4. Significant differences in the average of body weight, during the experimental period, were observed between the three groups of ewes ($P<0.001$). Marjoram group (TM) had the lowest body weight in comparison with the control (CO) and the canola seed group (CS). The values for body weight were between 40.80 and 63.80 kg for all the animals during the experimental period.

The individual dry matter intake differed between groups ($P<0.05$). No significant difference was observed between the CS group and the CO group. These results were in accordance with those reported by Huang *et al*. (2009) in which the DM intake of non-lactating ewes was not affected by the fat supplementation. However, the TM group had lower intake in comparison with the CS group (Table 4.4), but it need to be mentioned that once the individual dry matter intake was expressed as g/kg of body weight the difference between both groups became not significant which means that the difference observed for the body weight could partly explain the difference observed for the dry matter intake.

Table 4. 4. Effect of feeding treatments on individual dry matter intake and body weight

Item	Treatments[1]			SEM[2]	*P*<[3]
	CO	CS	TM		D
iDM intake (g/d)	2069 [ab]	2218 [a]	1950 [b]	40,66	0.022
Body weight (kg)	50.93 [a]	50.21 [a]	48.17 [b]	0.359	<0.001
DM intake(g/kg BW)	40.80	44.18	40.28	0.777	0.079

[1]Refers to treatments: control diet (CO); canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by the marjoram)
[2]SEM: standard error of least square means
[3]Probability of significant effects due to the experimental diets (D)

4.2.1.2. Control of digestibility and nitrogen balance

No significant difference in the total tract digestibility of DM, CP, NDF, ADF and EE was observed between ewes fed with control diet and ewes fed with CS diet ($P>0.05$). These results were in agreement with values reported by Zhang *et al*. (2007) in lactating ewes in spite of the differences between both studies, such as the level of inclusion of canola seed (2.85% vs. 7.3%, respectively). Similar results were also found in dairy cows fed with canola seed as oilseed supplementation (Neves *et al*., 2009). However, Enjalbert *et al*. (2004) reported a decrease in the total tract nutrient digestibility in sheep fed with ruminal infusion of soy oil or calcium sodium chlorides of soy oil fatty acids. The difference between studies may be related to the amount and nature of the supplemental fat and also to the nature of the basal diet (Doreau and Chilliard, 1997). Indeed, according to Jenkins *et al*. (2008) when fatty acids especially unsaturated fatty acids are provided in excess, ruminal microbial metabolism depressed. However, Gomez-Cortés *et al*. (2008), reported that using a diet with 6% of soybean oil, contrast the widespread idea that an excess of polyunsaturated fatty acids can alter ruminal metabolism, even if the mechanisms involved still not clear. Regarding fat from oilseeds, Palmquist (1990) reported that to limit the negative effects of unsaturated fat on ruminal fermentation, the supplementation needs to be limited to about 2 ± 3% of DM. In the present study the amount of canola seed used was 2.85% on a dry matter basis, which could explain, partially, the observed results.

Table 4. 5. Effect of feeding treatments on apparent digestibility and nitrogen balance

Item	**Treatments**[1]			**SEM**[2]	***P*<**[3]
	CO	**CS**	**TM**		**D**
Apparent digestibility, %					
Dry matter (DM)	74,38[a]	75,27[a]	72,36[b]	0,387	0.003
Crude protein (CP)	77,84[a]	77,85[a]	75,64[b]	0,393	0.02
Neutral detergent fiber (NDF)	54,64[ab]	56,83[a]	52,52[b]	0,765	0.06
Acid detergent fiber (ADF)	54,12[ab]	57,12[a]	50,76[b]	0,884	0.007
Ether extract (EE)	71,79[b]	71.53 [b]	77,29[a]	0,899	0.007
Nitrogen balance (g/day)					
Nitrogen Intake	22.53±2.68	24.60±2.92	23.05±2.74	0.573	0.995
Retained nitrogen	10.03±2.71	10.06±2.37	10.17±3.45	0.562	0.321

[1]Refers to treatments: control diet (CO); canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by the marjoram)
[2]SEM: standard error of least square means
[3]Probability of significant effects due to the experimental diets (D)

The inclusion of canola seed had no effect on crude protein digestibility (P>0.05) and, no difference (P>0.05) was observed in nitrogen intake and in retained nitrogen between the CO group and the CS group. The results were in agreement with values found by Huang *et al*. (2009) in sheep fed with 1% CLA as free acid or as calcium sodium chloride. However, other authors reported an increase in the apparent digestibility of nitrogen in ewes (Castro *et al*., 2009; Appeddu *et al*., 2004), which may related to variations in dietary nitrogen and not to the lipid supplementation because endogenous nitrogen passing through the duodenum does not vary to a large extent when lipids are added to the diet (Doreau and Ferlay, 1995). Martínez- Marín *et al*. (2011) also reported that the effect of lipid supplementation on ruminal and total protein digestion is not relevant.

No differences regardless of fat digestibility were observed in the present study, which was in agreement with other results reported in sheep fed different sources of fat by Castro *et al*. (2009) and Appeddu *et al*. (2004). However, Reddy *et al*. (2003) found an increase in fat digestibility in Deccani sheep fed dietary protected lipids. Doreau and Chilliard (1997) advanced that even if it is often considered that fat supplementation increases fat digestibility in ruminants, this increase is an underestimation of fat intake in diets non-supplemented with fat, in which FA, incorporated in cell structures, are not easy to extract by classical methods, whereas supplemental lipids, which are generally triacylglycerols, are easily extractable. For these reasons the author concluded that results are questionable and should be used only to compare the digestibility of different sources of lipid given in the same amounts.

The substitution of 7.5% of the CS diet by the marjoram plant induced changes on animal responses. A significant decrease (P<0.05) was observed in DM, CP, NDF and ADF digestibility, meanwhile a significant increase was observed for lipid digestibility (P<0.01). The observed decreases might be associated to the high content in NDF and ADF of TM diet in comparison with CO and CS diets (Table 3.1) and also to the inhibition of protozoal, cellulolytic bacteria and/or fungal growth in the rumen, as result of the antioxidant compounds supplementation (Ferme *et al*., 2004 and Sivakumaran *et al*., 2004). Furthermore, polyphenols had adverse effects because they bind and precipitate macromolecules, such as dietary protein, carbohydrate, and digestive enzymes reducing thereby food digestibility (Bravo. 1998). However, the present results were in contrast with values reported by Kim *et al*., (2002), in which the digestibility of

all the nutrients increased with the addition of a whole aromatic plant (*wormwood; Artemisia sp.*) in sheep diets. Moreover, Ko *et al*. (2006) reported a significant increase of EE digestibility with the addition of whole aromatic plant (*wormwood; Artemisia sp.*) in sheep diets. The observed increase was associated to the presence of 6,7-dimethylesculetin, capillarisin and coumarin in the plant which would stimulate the secretion of bile sodium chlorides and lipolytic enzymes in the small intestine.

In the present study, the increase of the fat digestibility could be related to the phenolic compounds present in marjoram plant, which acted as scavengers of free radicals and chelators of metal ions that were capable of catalyzing lipid peroxidation (Bravo. 1998) which may preserved the lipids in the diets from oxidation and made them more available for the microorganisms in the rumen. In the other hand, the fact that marjoram did not improve the digestibility of DM, CP, NDF and ADF could be explained by the fact that antioxidants compounds only improve the negative effect of excessive unsaturated fatty acids (Vázquez-Añón and Jenkins, 2007; and Hino *et al*., 1993). And, in the present study no excessive unsaturated fatty acids was provided and no negative effect on the digestibility of DM, CP, NDF and ADF was observed with canola seed supplementation, which means that no negative effects were needed to be improved by TM diet. Moreover, the increase of fat digestibility with MP diet sustains this observation because among the nutrients digestibility, the fat digestibility was the only parameter negatively affected by the canola seed supplementation.

Vázquez-Añón and Jenkins (2007), in order to obtain positive results, used a highly lipophylic quinoline antioxidant compounds which under an aqueous solution such as rumen fluid, these compounds stayed within the lipid moieties, protecting the fatty acids from further peroxidation and reducing the toxic effect of unsaturated fatty acids. Additionally, *in vitro* experiments showed that the polyphenols concentration should not exceed 20-30 mg/g feed in order to protect rumen feed degradability and microbial growth (Makkar *et al*., 2007; Alexander *et al*., 2008; Hart *et al*., 2008).

In the present study, the percentage of 7.5% of *Thymus mastichina* was the same as that used by Nieto *et al*. (2010, 2011) in sheep fed other type of *thyme* (*Thymus zygis ssp. gracilis*) and where no negative effects were reported on animal metabolism. Furthermore, the total phenolic content in marjoram diet (TM), which was 7.5% of the total phenolic content in marjoram plant (11.42-14.68 mg GAE/g feed, Table 4.1) did not exceed the range reported in the studies mentioned above.

4.2.2. Evaluation of animal and lactation performances of lactating ewes

4.2.2.1. Control of dry matter intake (DMI)

The average of voluntary dry matter intake, recorded diary for each group of ewes during 4 weeks, was 3618 ± 36.5; 3356 ± 25.9 and 2815 ± 19.9 g/d for the CO group, the CS group and the TM group, respectively. Significant differences were found between all the groups (P<0.05). The highest value was observed for the control group and the lowest for the marjoram group. No significant changes in the dry matter intake of the different group of ewes were reported throughout the experimental period (P>0.05).

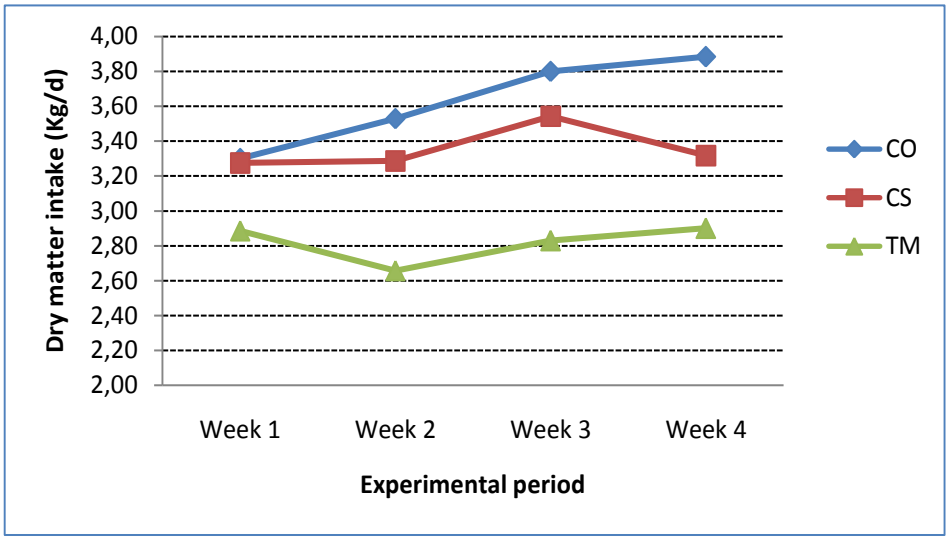


Figure 4. 5. Evolution of the dry matter intake during the experimental period

The inclusion of canola seed affected negatively the DMI and this result contrast that reported by Zhang *et al*. (2007) in which DM intake of lactating ewes was not affected by canola seed supplementation. This decrease might be associated to the amount of fatty acids included in the diet (Wang *et al*., 2012) or to the degree of unsaturation of those fatty acids (Eastridge and Firkins, 2000; He and Armentano, 2011).

In the present study, the decrease of the DMI with the TM diet could be associated to its high content in fiber (NDF and ADF) in comparison with the CO and CS diets. This result was in contrast with others results in dairy cattle in which the inclusion of antioxidants in diets enriched with polyunsaturated fatty acids did not affect feed intake

(Wang *et al*., 2010; He and Armentano, 2011) or did increase the DM intake (Vázquez-Añón and Jenkins, 2007; Vázquez-Añón *et al*., 2008).

No significant differences were observed for body weight regardless of diet and no significant changes were reported for body weight throughout the experimental period (Table 4.6).

4.2.2.2. Control of milk production

No significant differences were observed for milk yield regardless of diet (P>0.05). No significant modifications were found throughout the experimental period (Table 4.6).

Table 4. 6. Effect of feeding treatments on body weight and milk yield

Item	Treatments[1]			SEM[2]	P<[3]		
	Control	CS	MP		D	T	D×T
Body weight (kg)	84.86	79.12	78.33	1.170	0.361	0.051	0.881
Milk yield (g/d)	2211	2506	2220	73.88	0.541	<0.001	0.190

[1]Refers to treatments: control diet (CO); canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by the marjoram)
[2]SEM: standard error of least square means
[3]Probability of significant effects due to experimental diets (D), time on diet (T) and their interaction (D×T)

The addition of canola seed did not affect (P>0.10) the daily milk yield (on average 2.31 kg/d). Those results were in agreement with several studies in which supplemental fat does not influence milk production (Gomez-Cortés *et al*., 2011b; Appedu *et al*., 2004). Indeed, according to Zhang *et al*. (2006a) even if supplemental fat is usually fed to increase energy density of the diet in order to increase milk production, the production does not always respond positively. In agreement with the foregoing, Sanz Sampelayo *et al*. (2007) reported that in most cases, and independently of the food intake, milk production does not vary significantly. The main important factors responsible of the increase of the quantity and composition of milk production were the high energy supply of the diet (Gomez-Cortés *et al*., 2009) and the energy status of the animal (Sanz Sampelayo *et al*., 2007). In the present study no significant differences were reported regarding EB of the diets or body weight of the different group of animals (P>0.10) which can be an explanation for the unchanged results as regards milk yield.

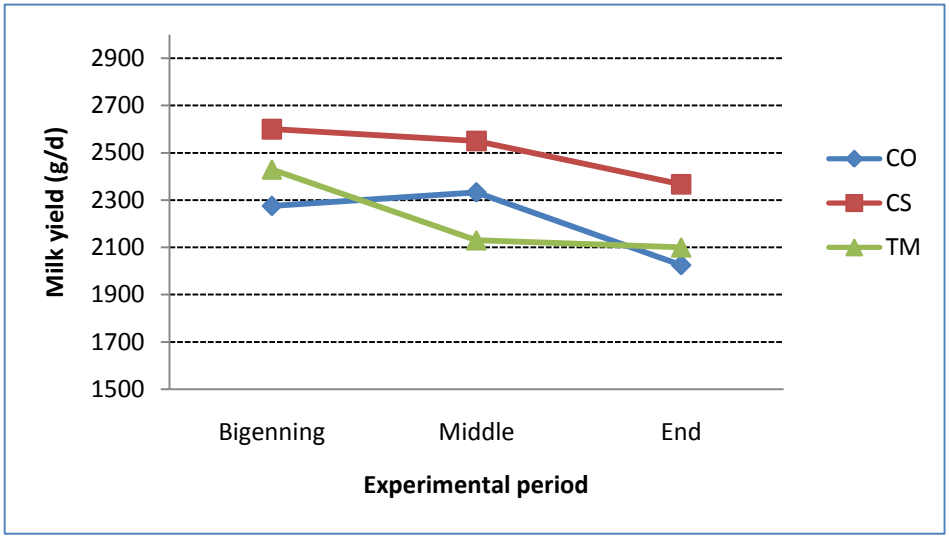


Figure 4. 6. Evolution of milk yield during the experimental period

As with canola seed diet, no significant differences (P>0.10) were reported either for milk yield with the marjoram diet. Those results were in agreement with other studies in which milk yield was not affected by the inclusion of an antioxidant in the diet; Vitamin E (Smith *et al*., 2003), 0,025% DM Agrado Plus (He and Armentano, 2010) and β-carotene (De Ondarza *et al*., 2009). However others studies reported a significant improvement of the milk yield (Vázquez-Añón *et al*., 2008; Wang *et al*., 2010; Wang *et al*., 2012). Wang et *al*., (2012) associated the increase of milk yield in cows fed antioxidant to the anti-oxidative status of the cows and not to the increase of DMI. In the other hand, Vázquez-Añón *et al*. (2008) related the increment of the milk yield to the increase of DM, OM and fiber digestibility of the diet. In the present study, the decrease of the DM and fiber digestibility did not affect negatively the milk yield.

Chapter 3

Characterization of milk and dairy products

4.3. Characterization of milk and dairy products

4.3.1. Characterization of ewe's milk

4.3.1.1. Determination of the physico-chemical composition of milk

Physicochemical composition of milk was analyzed in order to compare the effect of diets during the experimental period (Table 4.7). The results showed significant differences between treatments (CO, CS and TM). Significant changes were reported for all the physicochemical parameters throughout the experimental period, except for lactose.

Table 4. 7. Effect of diets on the physicochemical composition of milk

Item	Treatments[1]			SEM[2]	P<[3]		
	Control	CS	MP		D	T	D×T
Composition							
Fat (%)	5.68 [a]	5.43 [b]	5.31 [c]	0.039	<0.001	<0.001	<0.001
Protein (%)	4.82 [b]	4.80 [c]	4.91 [a]	0.013	<0.001	<0.001	<0.001
Casein (%)	3.77 [ab]	3.73 [cb]	3.80 [a]	0.030	0.033	0.008	<0.001
Lactose (%)	4.97	4.96	4.96	0.013	0.085	<0.001	0.078
Total solids (%)	16.37 [a]	16.10 [b]	16.06 [c]	0.037	<0.001	<0.001	<0.001
Somatic cells (10^6/mL)	1.33 [c]	1.85 [b]	2.53 [a]	0.891	<0.001	<0.001	<0.001

[1]Refers to treatments: Control diet (CO); Canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of CS diet by marjoram)
[2]SEM: standard error of least square means
[3]Probability of significant effects due to experimental diets (D), time (T) and their interaction (D×T)

A significant decrease was observed for fat, protein and total solids content between CO and CS milk. For TM milk in comparison with CS milk, a significant decrease in fat and total solids content was found whereas an increase in protein and casein content was observed (P<0.05).

Milk fat, protein, casein and total solids contents were significantly affected by the addition of canola seed (P<0.05). The fat content decreased by the addition of canola seed and the results were in agreement with the values reported by Zhang *et al*. (2006a) for flaxseed and sunflower seed (6.7 and 5.9%, respectively) in which fat content decreased substantially. However, other studies did not observe any change (Gomez-Cortés *et al*., 2009; Gomez-Cortés *et al*., 2011a, b; Mughetti *et al*., 2012), reported increase (Zhang *et al*., 2006b; Hervás *et al*., 2008) as result of fat supplementation. Those differences might be associated to the type of fat incorporated in the diet (vegetal oils, oilseeds or protected fat) (Palmquist. 2006). The decrease of fat in milk might be

related to the combination of several factors. Actually, in the present study, ewes were supplemented with a low-fiber diets with a large proportions of readily-fermentable carbohydrates (starch), which might decrease the production of acetate and butyrate in the rumen and consequently limit the milk fat synthesis (Bauman and Griinari, 2001; Palmquist. 2006; Pulina *et al*., 2006). Moreover, the level of unsaturated fatty acids in CS diet might act as an inhibitor of the *de novo synthesis* of milk and consequently contribute to milk fat depression (Zhang *et al*., 2006a). The ruminal degradability of the extruded canola seed used might also affect the fat content by improving the rate of release of PUFA into the rumen that might increase the amount of trans-fatty acids intermediates leaving the rumen and consequently the risk of milk fat depression (Bauman and Griinari, 2001). Furthermore, the dry matter intake in ewes fed CS diet decreased in comparison with CO ewes which could increase the delivery and uptake of long chain fatty acids from the body fat mobilization and consequently inhibit *de novo synthesis* of milk and the mammary gland's ability to synthesize milk fat (He and Armentano, 2011).

On the other hand, the inclusion of marjoram in CS diet induced a significant decrease of milk fat content ($P<0.05$). The present results were in contrast with other studies where the inclusion of antioxidants in animal diet did not affect milk composition (He and Armentano, 2010; Wang *et al*., 2010; Wang *et al*., 2012), or studies where an increase was reported when plant extracts or commercial antioxidants were included (Chiofalo *et al*. 2012; Vázquez-Añón *et al*., 2008, respectively). However, it needs to be mentioned that the mechanism by which antioxidant improve milk fat content is not clear yet (Vázquez-Añón *et al*., 2008). In the present study, the decrease of milk fat in TM milk in comparison with CS milk might be associated to the fact that TM, as natural antioxidant, protected the unsaturated fatty acids from oxidation before its absorption (Vázquez-Añón *et al*., 2008) and consequently inhibited the *de novo synthesis* of milk which led to the decrease in fat proportion. Moreover, the dry matter intake of ewes fed TM diet decreased substantially which might inhibit the *de novo synthesis* of milk.

A significant decrease in protein content was found in ewes fed with canola seed diet in comparison with ewes fed with the control diet, and this decrease was in agreement with results reported by Zhang *et al*. (2006a) in which milk from ewes fed flaxseed and sunflower seed and also with other studies that used different vegetal oils such as soybean oil and sunflower oil (Mele *et al*., 2006; Hervàs *et al*., 2008; Gomez-Cortés *et*

al., 2011b). The decrease in casein content was in agreement with the results reported by Osuna *et al*. (1998) in lactating ewes that received calcium soaps of fatty acids alone or in combination with whole sunflower seed. This reduction in protein and casein contents might be related to the insufficient availability of amino acids to the mammary gland for the increased milk yield stimulated by fat supplementation (Gaynor *et al*., 1994; Pulina *et al*., 2006). In fact, in the present study, milk yield increased numerically with canola seed supplementation (2211 *vs* 2505 g/d). The involvement of impaired metabolism of insulin or glucose in the mammary gland reducing milk protein proportion when fats are fed to lactating ruminants might also be associated to this reduction (Dhiman *et al*., 1995). However, the results were in contrast with others in which feeding fat to lactating ewes had no effect on protein content (Zhang *et al*., 2006b; Gomez-Cortés *et al*., 2008; Castro *et al*., 2009; Gomez-Cortés *et al*., 2009; Bodas *et al*., 2010; Gomez-Cortés *et al*., 2011a).

In contrast ewes fed with marjoram showed a difference in the values of protein. The observed results were in agreement with values reported by Chiofalo *et al*. (2012) and Wang *et al*. (1996) but in contrast with results reported by Vázquez-Añón *et al*. (2008) in which the milk protein content decreased substantially. The increase in protein content might be related to the fact that in the reticulorumen, phenolic compounds interact with proteins which inhibit its utilization in the rumen by indigenous micro-organisms and reduce its degradation (Chiofalo *et al*., 2012; Hart *et al*., 2008). But, once into the abomasums, the complex of phenolic compound–undegradable protein breaks down and the released protein is degraded and utilized by the ruminant (Wilkins and Jones, 2000; O'Connell and Fox, 2001) which enhance the amino acids availability in the small intestine (Jouany and Morgavi, 2007) and subsequently in the mammary gland for the synthesis of milk protein.

The somatic cell counts (SCC) are used as an indicator of animal health and milk quality in the dairy industry (Boutoial *et al*., 2013). Maximum levels in milk are set by health officials to assure milk quality supply from dairy farmers to milk and dairy product consumers. However, in the European Union (EU), there is no legal limit for the SSC in goats and sheep milk and the legal limit for cow's milk is 400.000 ml^{-1} (Directive 92/46ECC). The values determined for somatic cells in the three batches of milk were 1.33 10^6 mL^{-1}; 1.85 10^6 mL^{-1} and 2.53 10^6 mL^{-1} for CO, CS and TM milk, respectively.

The effect of time on milk composition was showed in the Table 4.8, in which significant differences were reported. During the experimental period, the milk fat and total solids content decreased substantially in all milks (P<0.05) whilst the protein content did not change for CO and CS diet and increased for TM diet.

Table 4. 8. Effect of time on the physicochemical composition of milk

Diets [1]	**Time** [2]	**Fat (%)**	**Total solids (%)**	**Protein (%)**
CO	Beginning	5.80 ± 0.01 [a]	16.58 ± 0.02 [a]	4.80± 0.02 [a]
	Middle	5.70 ± 0.01 [b]	16.33 ± 0.03 [b]	4.83± 0.04 [a]
	End	5.54 ± 0.02 [c]	16.19 ± 0.03 [c]	4.83± 0.01 [a]
CS	Beginning	5.83 ± 0.01 [a]	16.53 ± 0.02 [b]	4.71± 0.01 [a]
	Middle	4.98 ± 0.04 [b]	16.73 ± 0.03 [a]	4.96± 0.21 [a]
	End	5.49 ± 0.02 [c]	16.03 ± 0.03 [c]	4.73± 0.01 [a]
TM	Beginning	5.39 ± 0.01 [b]	16.26 ± 0.02 [a]	4.86± 0.01 [b]
	Middle	5.48± 0.01 [a]	15.99 ± 0.03 [b]	4.83± 0.04 [b]
	End	5.06 ± 0.01 [c]	15.91 ± 0.02 [c]	5.04± 0.01 [a]

[1]Refers to treatments: Control diet (CO); Canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by marjoram)

[2]Refers to time: the experimental period (beginning and end)

[3]Means within a column not followed by the same letter differ (P> 0.05)

4.3.1.2. Characterization of the lipidic profile of milk

4.3.1.2.1. Fatty acid (FAs) composition of milk

The fatty acid profile of the milk fat of ewes fed with different diets (CO, CS and TM) was shown in the Table 4.9. As can be observed, there were significant differences in the composition of milk associated to the type of diet. No modifications of the fatty acid profile through the experimental period was observed for saturated, mono-unsaturated, poly-unsaturated and short chain fatty acids whereas significant effect of the time was reported for medium chain and long chain fatty acids.

The inclusion of canola seed in the diet produced a significant increase in the 18-C atom FA content, at the expense of a decrease in C10:0, C12:0, C14:0 and C16:0 saturated FA concentration. Similar results were reported in sheep in others studies where different plant oils were used (Gomez-Cortés *et al*., 2009; Gomez-Cortés *et al*., 2011a).

However, little information has been reported as regards dairy ewes fed canola seed. In dairy cows, a decrease in the saturated fatty acids (SFA; C8 to C16) with canola seed supplementation was as well reported (Delbecchi *et al*., 2001).

On the other hand, the marjoram diet induced an increase in the 18-C atom FA content, higher than that induced by the supplementation of canola seed ($P<0.001$). Significant decrease was observed for the total SFA and short chain fatty acids (SCFA) for TM diet in comparison with CS diet. This result was in agreement with the result found by Vázquez-Añón *et al*. (2008) in which a significant decrease in SCFA (4:0 to 12:0) was reported in milk fat content of cows fed with dietary antioxidant (Agrado Plus). The reasons for the decrease observed with CS diet and with TM diet might be associated to the fact that long-chain fatty acids (18 or more carbon atoms), from the feed or the mobilization of body reserves, inhibited the synthesis of acetyl-CoA carboxylase and thus decreasing de novo synthesis in mammary gland cells (Chilliard *et al*., 2003).

The reported results were advantageous for human health because the intake of these SFA is related with increments in the cardiovascular disease markers in plasma as LDL-cholesterol (Fontecha *et al*., 2011). Moreover, C16:0 and C14:0 are implicated as hypercholesterolemic agents (Bell *et al*., 2006) and C4:0 was described as an anticarcinogenic agent with ability to inhibit growth in a wide range of human cancer cell lines at lower concentrations (Blank-Porat *et al*., 2007).

The increased C18:0 levels in ewe milk with CS or TM diets could be the result of the total rumen biohydrogenation of part of the unsaturated dietary C18. According to Grummer (1991) it is easier to increase the C18:0 content of the fat in milk with supplements rich in mono and polyunsaturated fatty acids than with supplements that provide stearic acid *per se*, which was in agreement with the results reported in the present work.

Table 4. 9. Effect of diets on milk fatty acid profile (g/100g of fatty acids)

Item [1]	Treatments [2]			SEM [3]	P< [4]		
	CO	CS	TM		D	T	D×T
C4	4.50 [a]	4.40 [a]	4.08 [b]	0.054	0.001	0.613	0.238
C6	3.85 [a]	3.89 [a]	3.70 [b]	0.028	0.002	0.340	0.028
C8	2.90	2.87	2.72	0.029	0.023	0.642	0.049
C10	6.42 [a]	5.89 [b]	5.89 [b]	0.079	0.001	0.608	0.016
C10:1+11	0.33 [b]	0.35 [b]	0.41 [a]	0.013	0.002	0.014	<0.001
C12	4.86 [a]	4.52 [b]	4.57 [b]	0.062	0.021	0.019	0.141
C14 iso	0.06 [b]	0.07 [a]	0.07 [a]	0.003	0.007	0.046	0.111
C14	11.64 [a]	10.67 [b]	10.44 [b]	0.138	<0.001	0.004	0.013
C15 anteiso	0.26	0.29	0.28	0.007	0.235	0.045	0.472
C15 iso	0.14	0.13	0.13	0.003	0.789	<0.001	<0.001
C14:1	0.11 [a]	0.07 [b]	0.07 [b]	0.004	0.003	0.386	0.700
C15	0.65 [b]	0.66 [b]	0.67 [a]	0.008	0.007	<0.001	<0.001
C16 iso	0.21	0.21	0.20	0.005	0.373	0.273	<0.001
C16	24.28 [a]	23.96 [b]	23.20 [b]	0.120	<0.001	0.058	0.319
C17 anteiso	0.40	0.41	0.41	0.009	0.731	0.197	0.069
C16:1	0.71 [a]	0.62 [b]	0.56 [b]	0.024	0.009	0.068	0.036
C17	0.44	0.43	0.41	0.008	0.467	0.009	0.305
C18	8.96 [c]	10.48 [b]	10.95 [a]	0.179	<0.001	0.189	0.027
trans 6 to 8 C18:1	0.53 [a]	0.35 [b]	0.35 [b]	0.021	<0.001	0.045	0.181
trans-9 C18:1	0.44	0.32	0.34	0.025	0.189	0.586	0.500
trans- 10 C18:1	1.62	1.59	1.56	0.067	0.699	<0.001	0.662
trans-11 C18:1 (VA)	1.61 [c]	2.02 [b]	2.15 [a]	0.066	<0.001	<0.001	0.051
trans-12 C18:1	0.95 [b]	1.09 [a]	1.11 [a]	0.044	0.009	0.038	0.022
cis -9 C18:1	16.48 [c]	17.03 [b]	17.74 [a]	0.163	<0.001	0.026	<0.001
trans-15 C18:1	0.65	0.78	0.71	0.055	0.442	0.177	0.226
cis -11 C18:1	0.47	0.42	0.44	0.021	0.743	0.117	0.429
cis -12 C18:1	0.36 [b]	0.49 [a]	0.54 [a]	0.026	0.001	<0.001	0.020
trans 16 + *cis* -14 C18:1	0.23 [b]	0.37 [a]	0.34 [a]	0.020	0.007	0.206	0.185
Others *trans*- *trans* C18:2	0.31	0.29	0.28	0.014	0.852	0.083	0.654
cis 9, *cis* 12 C18:2	4.12 [a]	3.60 [c]	3.83 [b]	0.044	<0.001	0.429	0.648
C20	0.45	0.43	0.44	0.022	0.953	0.605	0.546
α C18:3 n3	0.27	0.27	0.25	0.008	0.356	0.888	0.983
cis 9, *trans* 11 C18:2 (RA)	0.52 [c]	0.59 [b]	0.66 [a]	0.012	<0.001	0.744	0.992
C22	0.22	0.26	0.31	0.020	0.372	0.784	0.991
AA	0.24	0.31	0.34	0.018	0.063	0.954	0.858
SFA	70.18 [a]	69.58 [b]	68.49 [c]	0.216	0.001	0.013	0.139
MUFA	24.05 [c]	25.03 [b]	25.76 [a]	0.209	<0.001	0.016	0.075
PUFA	5.43 [a]	5.06 [b]	5.35 [a]	0.039	<0.001	0.208	0.759
SCFA	22.83 [a]	21.90 [b]	21.37 [b]	0.073	<0.001	0.732	0.022
MCFA	38.85 [a]	37.52 [b]	36.46 [c]	0.236	<0.001	0.001	<0.001
LCFA	38.32 [c]	40.58 [b]	42.17 [a]	0.363	<0.001	0.001	<0.001
AI [5]	2.57 [a]	2.37 [b]	2.24 [c]	0.038	<0.001	0.004	0.056

[1] Abbreviations: VA, vaccenic acid; RA, rumenic acid; SFA, saturated fatty acids; MUFA, mono-unsaturated fatty acids; PUFA, poly-unsaturated fatty acids; SCFA, short chain fatty acids (C4:0-C12:0); MCFA, medium chain fatty acids (C14:0iso-C17:0); LCFA, long chain fatty acids (>C18:0)

[2] Refers to treatments: Control diet (CO); Canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by marjoram)

[3] SEM: standard error of least square means

[4] Probability of significant effects due to experimental diets (D), time (T) and their interaction (D×T)

[5] AI= Atherogenicity index: (12:0 + (4 × (14:0) + 16:0)/ (MUFA+PUFA)

The increase observed in the oleic acid (*cis*-9 C18:1) content in milk fat ($P < 0.05$) with CS and TM diet in comparison with the control diet could be due to the higher presence of unsaturated dietary C18 in the modified diets and to the action of the Δ^9-desaturase enzyme on the mammary gland on the portion of C18:0 generated in the rumen. Furthermore, the increase reported with TM diet was significantly higher than the reported for CS diet ($P<0.05$). The higher concentration of oleic acid in milk with TM diet might be related to the fact that antioxidants may preserve unsaturated fatty acids from oxidation before its absorption as reported by Vázquez-Añón *et al*. (2008).

Regardless of CS and TM diets in comparison with the control diet and except for *trans*-6+8 C18:1 where a significant decrease ($P>0.05$) was observed, no change or an increase was reported for the rest of the isomers of 18-C monounsaturated FA. Concerning *trans*-10 C18:1, no significant variation was observed and it was in agreement with that found by Gomez-Cortés *et al*. (2009) with extruded linseed included at 6 and 12% in the diet. However, it was in contrast with others where an increase was reported by using vegetal oils (Bodas *et al*., 2010; Gomez-Cortés *et al*., 2008, 2011; Hervas *et al*., 2008; Mele *et al*., 2006). The lack of decrease of *trans*-10 C18:1 with TM diet in comparison with CS diet was in agreement with the results reported by He and Armentano (2011) where Agrado Plus (commercial antioxidant) by oils interaction (palm oil; high-oleic safflower oil; linseed oil corn oil and high-linoleic safflower oil) did not inhibit the secretion of *trans*-10 C18:1. However, this result was in contrast with that of Pottier *et al*. (2006) where a lower concentration of *trans*-10 C18:1 was reported in dairy cows fed linseed diet supplemented with vitamin E.

The result observed for *trans*-10 C18:1 might be associated to the undetectable presence of *trans*-10, *cis*-12 C18:2 in the milk fat content, which is the precursor of *trans*-10 C18:1 in the rumen, under certain rumen conditions (Bauman and Griinari, 2001). Furthermore, Gomez-Cortés *et al*. (2009) reported that the lack of increase in *trans*-10 C18:1 might also be linked to the source of lipid supplementation.

In the other hand, the *trans*-11 C18:1 (VA) which is well-known as the major trans isomer produced during PUFA biohydrogenation (Chillard and Ferlay, 2004) increased substantially with CS and MP diet ($P<0.001$). According to Gaynor *et al*. (1994), diets containing high percentage of concentrate and unsaturated fats would likely increase the production of *trans*-11 C18:1. Moreover, the formation of *trans*-11 C18:1 in the rumen is a function of the overall accumulation of *trans* C18:1 with minimal shifts towards

trans-10 C18:1 (Bell *et al*., 2006). Consequently, the increase of VA content for CS and for TM diet ($P<0.01$) might be partly associated to the lack of increase of the *trans*-10 C18:1 content. Similar results, concerning VA content, was reported by others authors using different plant oils (Bodas *et al*., 2010; Gomez-Cortés *et al*., 2011). Moreover, the increase achieved with marjoram diet was significantly higher than that with CS diet. This result was in agreement with other studies in which an increase in VA was reported as well (Bell *et al*., 2006). However, this finding was in contrast with the results reported by Vázquez-Añón *et al*. (2008) in which a decrease of VA content was found and was associated to a lower ruminal hydrogenation and *trans*-isomerization of C18:1. Furthermore, a positive relationship was reported between milk fat depression and the increase of *trans*-11 C18:1 concentration (Bauman and Griinari, 2001; Gaynor *et al*. (1994) which was in agreement with the present results.

The decrease in linoleic acid level in the milk fat content of ewes fed canola seed might be partly justified by the lower content of linoleic acid in the CS-supplemented ration (Table 3.1). In the other hand, the increase induced by the inclusion of TM might be related to the fact that antioxidants may preserve unsaturated fatty acids from oxidation before its absorption as suggested for the oleic acid. The rumenic acid (RA) content followed the same pattern as that observed for VA, which was expected due to the significant correlation usually reported between VA and RA in ewe milk fat (Gomez-Cortés *et al*., 2011a). The reasons for the increase in RA content should be associated to the same reasons as for the increase in VA percentage because most of RA is produced in the mammary gland due to the activity of the Δ^9-desaturase enzyme on the VA generated in the rumen. Those results were in agreement with several studies in which supplemental fat increase substantially the RA content (Gomez-Cortés *et al*., 2008; Gomez-Cortés *et al*., 2009). Furthermore, the enhancement observed for RA content, as result of the inclusion of marjoram was in agreement with values reported by Bell *et al*. (2006) in which monensin in combination with safflower oil induced greater *trans*-11 C18:1 accumulation in the rumen and consequently an enhancement of *cis*-9, *trans*-11 CLA levels suggesting an apparent synergistic effect of monensin with safflower, which could be, as well, occurring in the present study. According to Hervás *et al*. (2008), from a nutritional point of view, those changes were in the direction of a healthier FA profile.

The level of PUFA in CS milk was lower than in CO diet which might be related to the high level of linoleic acid in the CO diet in comparison with the CS diet. Similarly, the highest content of PUFA in TM milk might be associated to its high level of linoleic acid since it was preserved from oxidation before its absorption as suggested above. These results were in agreement with values reported by Boutoial *et al*. (2013) in the milk of goats fed with rosemary (*Rosmarinus officinalis* spp.).

As a result of the higher levels of unsaturated FA and lower levels of saturated ones, a significant effect was observed on the atherogenicity index value (AI) in milk from ewes fed diets supplemented with canola seed alone or in combination with marjoram plant (Table 4.9). However, further studies need to be carried out for a better understanding of the effect of dietary antioxidant on the biohydrogenation process in the rumen.

4.3.1.2.2. CLA isomers profile of milk

The CLA isomers profile were identified by using the Silver ion (Ag^+)-HPLC. The Ag^+-HPLC profiles exhibited a first chromatographic zone where the different *trans-trans* compounds separated, followed by a zone in which the *cis/trans* compounds eluted (Figure 4.4). Under the Ag^+-HPLC conditions, the two groups of isomers eluted during 54 min of run. The *trans-trans* and the *cis-trans* groups eluted between 20 and 32 min and 46 and 54 min, respectively as shown in the Figure 4.7.

The CLA isomers identified corresponded to the *trans-trans* isomers (6-8, 7-9, 8-10, 9-11, 10-12, 11-13 and 12-14) (Figure 4.4 and Table 4.10). The effect of diets (CO, CS, and TM) on CLA isomers profile through the experimental period was showed in the Table 4.10. No modifications were observed during the experimental period for the CLA isomers profile (Table 4.10).

Most of those isomers decreased or remained constant with both modified diets; CS and TM in comparison with the control diet (CO), except the *trans-trans* 9-11 which level increased substantially. Moreover, a significant decrease was observed for the *cis*-7, *trans*-9 CLA. Those decreases might be associated to the level of linoleic acid in the diets, which was lower in CS and TM diets in comparison with CO diet, due to the fact that significant positive correlations were reported between diets containing high levels of linoleic acid and the concentrations of the CLA isomers (Collomb *et al*., 2004; Gómez-Cortés *et al*., 2011; Hervas *et al*., 2008).

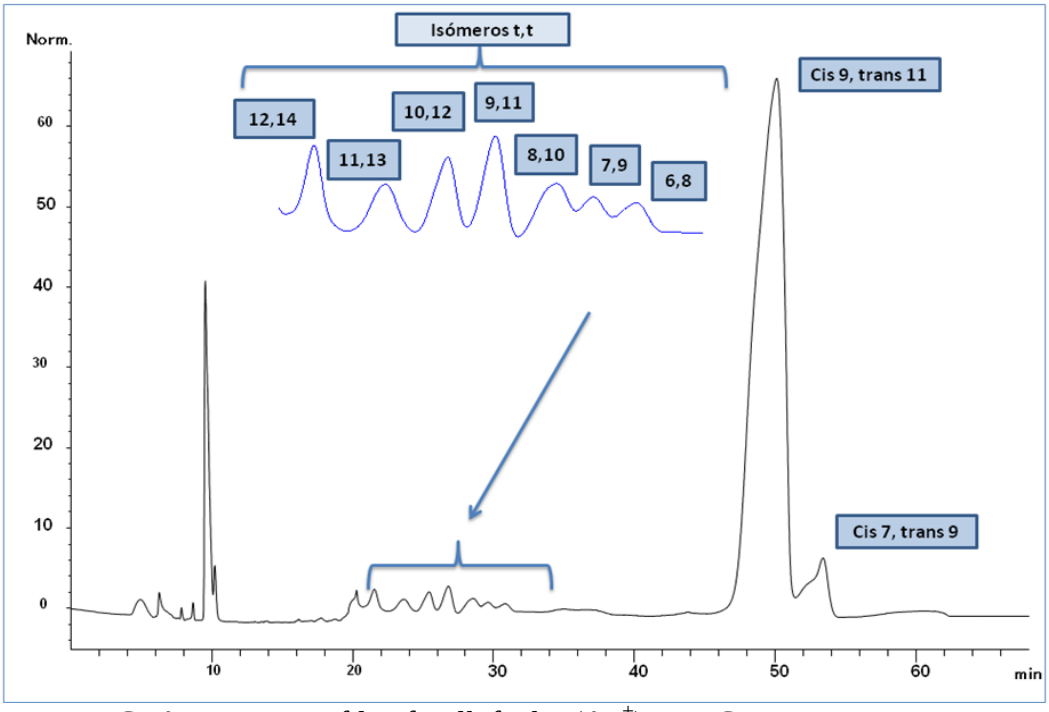


Figure 4. 7. CLA isomers profile of milk fat by (Ag^{+})-HPLC

In the other hand, the most important increase concerned *cis*-9, *trans*-11 CLA. As discussed above, the enhancement of *cis*-9, *trans*-11 CLA was directly correlated with the increase of VA. Furthermore, the undetectable level of *trans*-10, *cis*-12 C18:2 (Figure 4.4) which is the precursor of *trans*-10 C18:1 might confirm the fact that in absence of alteration of the biohydrogenation pathways, oleic acid generates more VA and RA.

Table 4. 10. Effect of diets on the CLA isomers profile

CLA isomer composition, % of total CLA	**Treatments[1]**			**SEM[2]**	***P*<[3]**		
	CO	**CS**	**TM**		**D**	**T**	**D×T**
trans,*trans*							
12,14	0.92 [a]	0.81 [b]	0.69 [c]	0.028	0.001	0.073	0.466
11,13	0.87 [a]	0.63 [b]	0.58 [b]	0.035	<0.001	0.142	0.406
10,12	2.14 [a]	1.75 [b]	1.35 [c]	0.081	<0.001	0.638	0.980
9,11	0.51 [b]	0.82 [a]	0.78 [a]	0.056	0.038	0.541	0.190
8,10	1.23	1.19	1.06	0.035	0.103	0.145	0.361
7,9	0.38	0.44	0.36	0.021	0.286	0.991	0.175
6,8	0.38	0.35	0.27	0.021	0.110	0.409	0.400
*cis*9, *trans*11	85.11 [c]	87.39 [b]	88.80 [a]	0.345	<0.001	0.098	0.078
*cis*7,*trans*9	8.43 [a]	6.62 [b]	6.10 [b]	0.233	<0.001	0.020	0.170

[1]Refers to treatments: Control diet (CO); Canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by marjoram)

[2]SEM: standard error of least square means

[3]Probability of significant effects due to experimental diets (D), time (T) and their interaction (D×T)

4.3.1.2.3. Milk triglycerides profile

Sixteen peaks were identified in the chromatograms obtained from the samples of milk from ewes fed different diets (CO, CS and TM), as shown in the Table 4.11, corresponding to triglycerides (TAG) with CN from 24 to 54. The distribution of the TG in ewe's milk by chain length (short-, medium-, and long-chain: C24- C34, C36-C44, and C46- C54, respectively) and according to the diet received; control, CS or TM was similar to that found by Goudjil *et al*., (2003) in ewes' milk (14.9, 51.8, and 33.3%, respectively).

Diets affected the triglyceride structure of milk fat (Table 4. 11). The modification of the triglyceride profile of the milk fat through the experimental period was not significant (P>0.05).

Table 4. 11. Effect of diets on triglyceride profile of milk fat

Item [1]	Treatments[2]			SEM[3]	P<[4]		
	CO	CS	TM		D	T	D×T
C24	0.10	0.11	0.09	0.004	0.073	0.767	0.132
C26	0.76	0.79	0.68	0.027	0.183	0.422	0.124
C28	1.39	1.40	1.28	0.042	0.420	0.315	0.720
C30	2.81	2.84	2.51	0.087	0.191	0.099	0.206
C32	4.42	4.35	3.96	0.121	0.193	0.141	0.165
C34	6.90 [a]	6.75 [b]	6.16 [c]	0.141	0.040	0.019	0.432
C36	9.68 [a]	9.78 [a]	8.68 [b]	0.177	0.004	0.005	0.623
C38	13.74 [a]	13.11 [b]	12.27 [c]	0.225	0.017	0.044	0.823
C40	12.15 [a]	12.43 [a]	11.18 [b]	0.178	0.002	0.011	0.648
C42	9.09 [a]	8.85 [b]	8.33 [c]	0.121	0.003	<0.001	0.799
C44	7.73 [a]	7.42 [b]	7.29 [c]	0.084	0.026	0.011	0.080
C46	6.74	6.24	6.46	0.154	0.455	0.511	0.557
C48	6.51	6.63	7.31	0.203	0.237	0.489	0.386
C50	7.95 [b]	8.24 [b]	9.77 [a]	0.277	0.001	0.001	0.143
C52	5.06 [b]	5.63 [b]	7.13 [a]	0.277	0.001	0.037	0.099
C54	4.88 [c]	5.71 [b]	6.70 [a]	0.276	<0.001	<0.001	0.051
SCT [a]	16.38	16.23	14.69	0.407	0.123	0.080	0.208
MCT [b]	52.39 [a]	51.59 [a]	47.75 [b]	0.721	0.004	0.004	0.719
LCT [c]	31.14 [b]	32.45 [b]	37.37 [a]	0.901	0.001	0.003	0.559

[1]Abbreviations: SCT, short chain triglycerides (C24-C34); MCT, medium chain triglycerides (C36-C44); LCT, long chain triglycerides (C46-C54)
[2] Refers to treatments: Control diet (CO); Canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by marjoram)
[3] SEM: standard error of least square means
[4] Probability of significant effects due to experimental diets (D), time (T) and their interaction (D×T)

No changes in the proportion of triglycerides associated with C24, C26, C28, C30, C32, C46 and C48 occurred. Canola seed supplementation decreased triglycerides in the C34, C38, C42 and C44 groups but did not affect triglycerides from C46 to C52 groups. A decrease in short chain and medium chain triglycerides was observed but was not significant and might be associated to the lower content of C12:0, C14:0 and C16:0. Similar results were reported by Depeters *et al*., (2001) in dairy cows with different canola oil additions (added to the diet, infused ruminally or infused abomasally).

The TG profile of milk with marjoram diet showed the same content of short-chain TG as that of CS diet. However, a lower content of medium-chain TG was observed because of the decrease in the C36, C38, C40, C42 and C40 groups. On the other hand, a significant increase was reported in the C50, C52 and C54 groups ($P<0.05$). This could be due to the high content of PUFA in the TM milk, since most of mono- and di-unsaturated C18 fatty acids are esterified in C50-C54 in ewe milk fat (Fontecha *et al*., 2005).

4.3.1.2.4. Milk phospholipids profile

The chromatographic profile of the lipid classes (neutral and polar) was showed in the Figure 4.8. The chromatographic profile of the polar lipids (phospholipid fraction) was showed in the Figure 4.9.

The peak appearance and the elution position upon HPLC-ELSD were highly reproducible for most neutral and polar lipid classes of the different milk samples. The separation of the phospholipids (phosphatidylethanolamine (PE), phosphatidyl-inositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC) and sphingomyelin (SM)) was resolved in less than 12 min.

Phospholipids are associated to the milk fat globule membrane (MFGM) and account for 0.5-1% of total milk lipids (Rodriguez-Alcala et al., 2009). PLs species are expressed as % of total phospholipids. The PLs identified in the Figure 4.9 were the most abundant found in all the milk samples (control, CS and MP). This ranging was in agreement with other author's results for milk, from buffalo (Andreotti *et al*., 2006) human, cow, camel and mare species (Garcia *et al*., 2013).

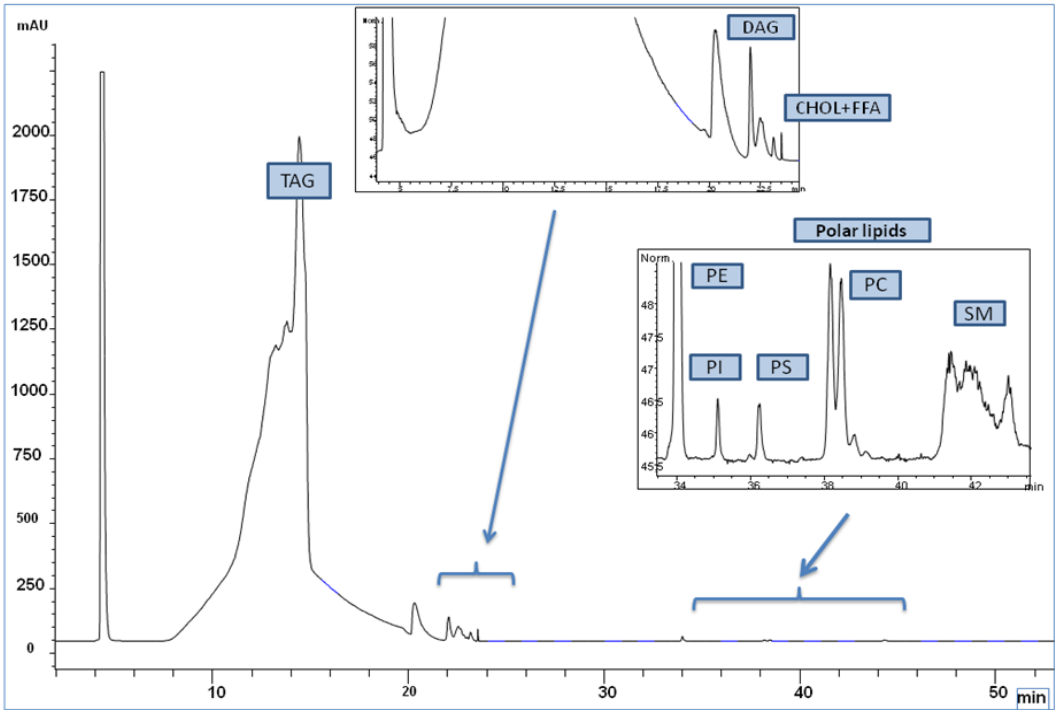


Figure 4. 8. Separation of the lipid classes by HPLC-ELSD. TAG: triacylglycerides; DAG: diacylglycerides; CH: cholesterol; FFAA: free fatty acids and Polar lipids (phospholipids)

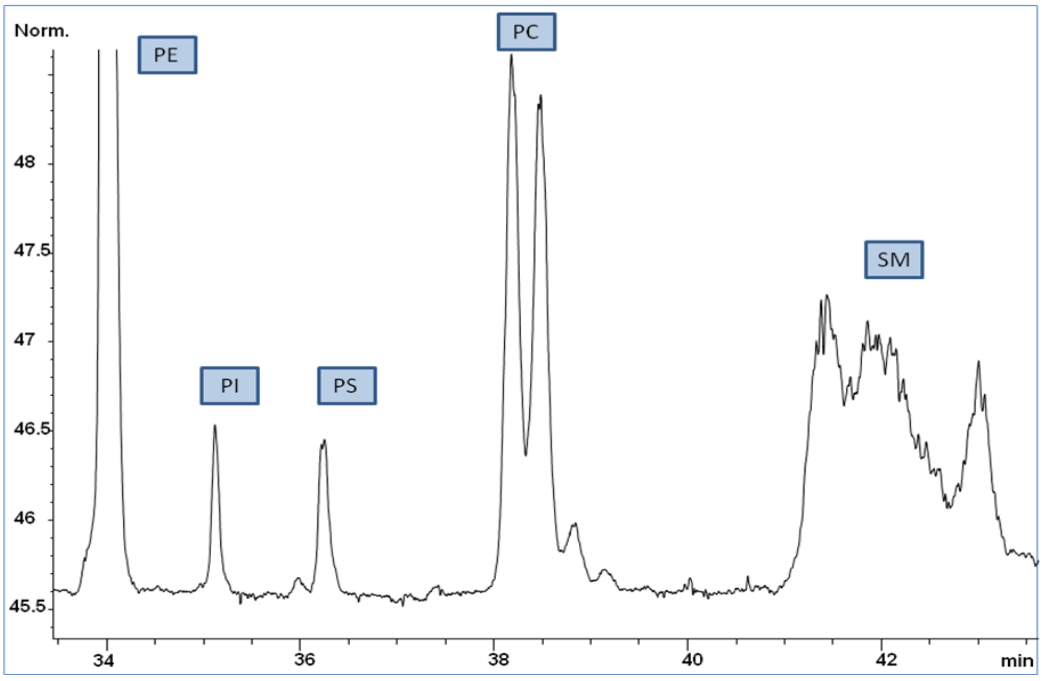


Figure 4. 9. Separation of the phospholipid fraction by HPLC-ELSD. PE: phosphatidylethanolamine; PI: phosphatidylinositol; PS: phosphatidylserine; PC: phosphatidylcholine and SM: sphingomyelin

Rombaut and Dewettinck (2006), based on bibliography, reported the different contents that could range the PL classes; PE (19.8–42.0%, w/w), PC (19.2–37.3%, w/w), PS (1.9–10.5%, w/w), PI (0.6–11.8%, w/w) and SM (18.0–34.1%, w/w). In the present study, the results observed for the different classes of PLs were agreement with the values advanced above. However, the SM content raised a level higher than that reported by Rodriguez-Alcalà and Fontecha (2010) for ewe's milk but comparable to that reported by Bitman and wood (1991) for bovine milk. According to Andreotti *et al*. (2006), factors such the age, breed, diet, and stage of lactation of the animal can affect the PLs content.

Table 4. 12. Effect of diets on lipid classes and phospholipid profile of milk fat

Lipid classes (%)[1]	**Treatments**[2]			**SEM**[3]	***P*<**[4]		
	CO	**CS**	**TM**		**D**	**T**	**D×T**
Neutral lipid fraction	(% of total lipids)						
Triacyglycerol	99.94	99.95	99.95	0.002	0.051	0.694	0.074
1,2_ Diacylglycerol	0.054	0.042	0.048	0.002	0.060	0.662	0.078
Cholesterol+Free fatty acids	0.006	0.006	0.007	0.001	0.478	0.547	0.429
Phospholipid fraction	(% of polar lipids)						
PE	38.44 [b]	39.80 [b]	42.69 [a]	0.964	0.002	0.006	0.000
PI	1.13	1.05	0.86	0.078	0.082	0.006	0.028
PS	1.47	1.37	1.39	0.113	0.861	0.265	0.014
PC	21.40	21.83	22.94	0.461	0.171	0.893	0.013
SM	37.55 [a]	35.94 [a]	32.12 [b]	1.280	0.003	0.023	0.001

[1]Abbreviations: PE, phosphatidylethanolamine; PI, phosphatidyl-inositol; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin

[2] Refers to treatments: Control diet (CO); Canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by marjoram)

[3] SEM: standard error of least square means

[4] Probability of significant effects due to experimental diets (D), time (T) and their interaction (D×T)

As presented in the Table 4.12, no significant differences were observed between the diets as regards the total proportion of triacyglycerol, 1,2_diacylglycerol and cholesterol+free fatty acids. However, significant differences were observed regardless of several phospholipid classes (PLs).

The supplementation with canola seed did not affect the PLs. In the other hand, the inclusion of marjoram increased the PE proportion in TM milk comparison with the CS milk. One explanation could be the fact that PE is highly unsaturated (Contarini and

Povolo, 2013) and consequently more prone to oxidation which render the effect of the marjoram as antioxidant more efficient. The enhancement observed is interesting from a nutritional point of view since it was demonstrated that PE exerts a hypocholesterolemic effects in rats (Imaizumi *et al*., 1991).

However a significant decrease was observed in the SM proportion which is as well reported as reducer of the cholesterol absorption (Eckhardt *et al*., 2002). Nonetheless, little literature is available on this subject and more researches need to be carried out to understand the observed differences.

4.3.1.3. Total antioxidant capacity (TAC) of milk

The total antioxidant capacity of milk samples (TAC) determined by measuring the $ABTS^{\cdot-}$ scavenging capacity, was showed in the Table 4.13. Significant differences were observed between the different milks (P<0.001). In decreasing order TM milk, CO milk and CS milk showed the highest antioxidant activity. No significant changes were found through the experimental period. The effect of the extractant solvent on TAC values was not significant (P>0.05).

Table 4. 13. Effect of diets on the antioxidant capacity of milk

Item	Treatments[1]			SEM[2]	*P*<[3]			
	CO	CS	TM		D	T	S	D×T×S
Antioxidant capacity (mg Trolox/ 100g of DWE)	26.00 [b]	24.13 [c]	28.12 [a]	1.396	<0.001	<0.001	0.165	0.546

[1]Refers to treatments: Control diet (CO); Canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by marjoram)

[2]SEM: standard error of least square means

[3]Probability of significant effects due to experimental diets (D), time (T), solvent (S) and their interaction (D×T×S)

According to Pulido *et al*. (2003), hydrophilic fraction normally contributes more to the total antioxidant than the lipophilic fraction. In the current study, antioxidant activity was measured in the supernatant in order to evaluate the hydrophilic fraction. Therefore, results can only be correlated to the non-fat fraction of the milk components.

Many factors related to the sample preparation might affect the antioxidant activity of food matrices and one of those factors is the extractant solvent, since an efficient extraction of antioxidant requires the use of solvents with different polarities (Pèrez-

Jimènez *et al*., 2008). However, in the present study, the effect of the extractant solvent was not significant.

The increase of the antioxidant capacity of TM milk might be associated to the phenolic compounds transmitted to the milk through ewe's diet. The present study did not report evidence that phenolic compounds were present in milk after feeding ewes *T. mastichina*. However, De Gordoa *et al*. (2008) reported that large differences were observed in TAC values of milk samples from various flocks of ewes after grazing and suggested that those differences might be associated to the botanical species of pastures.

4.3.2. Evaluation of the properties of the dairy products

4.3.2.1. Cheese properties

4.3.2.1.1. Physicochemical composition of cheese

The physicochemical composition of the cheeses (CO, CS and TM) was showed in the Table 4.14. The modification of the physicochemical parameters during the experimental period and during 120 days of ripening (2 and 4 months) was studied and showed in the Table 4.15 and 4.16. Significant differences between diets (CO, CS and TM) and significant changes throughout the experimental period and during ripening were observed.

The moisture content and the pH values in the present study were in agreements with the results observed by Blanco *et al*. (1999) who reported the intervals in which the physicochemical parameters of Castellano cheese could vary; moisture (18.8-35.7% DM), fat (45.2-62.0% DM), protein (30.1-43.7% DM) and pH (4.5-5.5). However the present values of fat and protein content were slightly lower (Table 4.14).

TM cheeses in comparison with CO and CS cheeses had the lowest fat content and the highest moisture and protein content, which was in agreement with the results observed in raw milk. No significant differences were found between CO and CS cheeses, except for protein content. The difference between CO and CS diet for fat content was significant in milk and not significant in cheese, which might suggest that a loss of fat in whey occurred during the manufacture of CO cheeses.

Table 4. 14. Effect of diets on the physicochemical composition of cheese

Item	Treatments[1]			SEM[2]	*P*<[3]			
	CO	CS	TM		D	T	R	D×T×R
Fat (%)	36.31[a]	36.78 [a]	33.46 [c]	0.328	<0.001	<0.001	<0.001	<0.001
Protein (%)	23.86 [b]	23.06 [c]	24.40 [a]	0.465	<0.001	<0.001	<0.001	0.056
Moisture (%)	30.80 [b]	30.80 [b]	33.51 [a]	0.376	<0.001	<0.001	<0.001	<0.001
Sodium chloride	1.14 [a]	1.14 [a]	1.07 [b]	0.019	<0.001	<0.001	<0.001	<0.001
S/M	3.74 [a]	3.69 [a]	3.19 [b]	0.026	<0.001	<0.001	<0.001	<0.001
pH	5.21 [a]	5.20 [a]	5.06 [b]	0.081	0.031	0.635	0.432	0.343

[1]Refers to treatments: Control diet (CO); Canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by marjoram)

[2] Means within a column not followed by the same letter differ (*P*> 0.05)

[3]Probability of significant effects due to experimental diets (D), time (T), ripening and their interaction (D×T×R)

The inclusion of canola seed in the control diet did not affect the major part of the physicochemical parameters of cheese (fat and moisture content), which was in agreement with the results reported by Coakley *et al*. (2007), in which diet supplemented by sunflower oil affected significantly milk composition but not cheese composition. However, it was in contrast with the results found by Mughetti *et al*. (2012), where the inclusion of extruded linseed affected cheese composition in the same way as it affected milk composition.

Cheeses produced from TM milk showed the lowest fat content and the highest protein and moisture content. These values were similar to the results found in milk. The present results were in contrast with those reported by Boutoial *et al*. (2013), in which the inclusion of 10 and 20% of rosemary (*Rosmarinus officinalis* spp.) in dairy goat diet did not affect the dry matter, the fat and the protein content of cheese. TM cheeses exhibited the lowest pH value (*P*<0.05) in comparison with CO and CS cheeses. The significant differences observed in pH were in agreement with the findings of Misttry and Kasperson (1998), who advanced that pH was always higher in cheeses with higher level of sodium chloride. And, this result might be associated to the partial inhibition of lactic acid bacterial growth by the sodium chloride. However, the results were in contrast with others published by Boutoial *et al*. (2013) and Hala *et al*. (2010), in which phenolic compounds produced an increase on pH as a result of the antimicrobial activity of the phenolic compounds.

On the other hand, cheeses with high sodium chloride had lower moisture content and this correlation was observed in other studies, in which adding sodium chloride

promotes synerisis and decreases the moisture content of cheese (Pastorino *et al*., 2003; Misttry and Kasperson, 1998).

Throughout the experimental period, significant changes were observed for fat, moisture and sodium chloride (Table 4.15). The moisture content in all cheeses increased significantly during the experimental period in agreement with the pattern observed in milk. The protein content remained steady and the fat content decreased significantly in CO and CS cheeses but not in TM cheeses.

Table 4. 15. Effect of time on the physicochemical composition of cheese

Diets [1]	**Time** [2]	**Fat (%)**	**Moisture (%)**	**Sodium chloride**	**S/M (%)**
CO	Beginning	36.55 ± 0.46 [a]	29.35 ± 0.40 [b]	1.21 ± 0.11 [a]	4.12 ± 0.34 [a]
	End	36.07 ± 0.34 [b]	32.26 ± 1.10 [a]	1.08 ± 0.07 [b]	3.35 ± 0.10 [a]
CS	Beginning	37.09 ± 0.53 [a]	30.64 ± 0.95 [a]	1.14 ± 0.04 [a]	3.74 ± 0.23 [a]
	End	36,47 ± 0.53 [b]	30.95 ± 1.33 [a]	1.13 ± 0.14 [a]	3.64± 0.31 [a]
TM	Beginning	33.82 ± 0.61 [a]	32.68 ± 0.45 [b]	1.09 ± 0.11 [a]	3.35 ± 0.05 [a]
	End	33.11 ± 0.85 [a]	34.35 ± 1.08 [a]	1.05 ± 0.01 [a]	3.06 ± 0.22 [b]

[1]Refers to treatments: Control diet (CO); Canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by marjoram)

[2]Refers to time: the experimental period (beginning and end)

[3]Means within a column not followed by the same letter differ (P> 0.05)

The ripening modify significantly the physicochemical parameters of CO, CS and TM cheeses (P<0.001, Table 4.7). In the present study the sodium chloride increased during ripening. However, no variation was reported in all cheeses for the other components, fat, protein, moisture content and pH value, which means that the proteolysis was not significant during ripening. Those results were in agreement with values reported by Bonanno *et al*. (2013), in which no differences were observed for fat and protein content between 60 and 120 days of ripening. Coppa *et al*. (2011) also found no difference in fat content during 12 weeks of ripening, independently of the diet.

Table 4. 16. Effect of ripening on the physicochemical composition of cheese

Diets [1]	Cheese age [2]	Fat (%)	Protein (%)	Moisture (%)	Sodium chloride	S/M (%)
CO	2 month	35.96 ± 0.22 [a]	22.47 ± 1.95 [a]	31.45 ± 2.03 [a]	1.07 ± 0.05 [b]	3.91 ± 0.56 [a]
	4 month	36.66 ± 0.34 [a]	25.26 ± 2.08 [a]	30.16 ± 1.33 [a]	1.22 ± 0.10[a]	3.55 ± 0.32 [a]
CS	2 month	36.35 ± 0.33 [a]	21.54 ± 1.66 [a]	30.97 ± 1.32 [a]	1.06 ± 0.06 [b]	3.92 ± 0.03[a]
	4 month	37.21 ± 0.40 [a]	24.58 ± 1.65 [a]	30.63 ± 0.96 [a]	1.21 ± 0.05 [a]	3.46 ± 0.11 [b]
TM	2 month	32.83 ± 0.54 [a]	22.99 ± 2.12 [a]	34.18 ± 1.28 [a]	1.02 ± 0.03 [a]	3.26 ± 0.12 [a]
	4 month	34.09 ± 0.29 [a]	25.81 ± 1.61 [a]	32.85 ± 0.65 [a]	1.11 ± 0.08 [a]	3.12 ± 0.15 [a]

[1]Refers to treatments: Control diet (CO); Canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by marjoram)

[2]Refers to ripening (2 and 4 months)

[3]Means within a column not followed by the same letter differ ($P > 0.05$)

4.3.2.1.2. Lipidic profile of cheese

The fatty acid composition and the triglyceride profile of cheeses according to the diets were showed in the Table 17 and 4.18, respectively. The results showed that significant differences were observed in fatty acids and triglycerides profile between cheeses regardless of diets. However, no significant modifications were found during the experimental period or during ripening for the major part of fatty acids (vaccenic acid, rumenic acid, unsaturated fatty acids, mono-unsaturated and polyunsaturated fatty acids) and triglycerides (short chain, medium chain and long chain triglycerides).

There was a complete transfer of fatty acid profile from milk to cheese, which means that the cheesemaking did not change the lipidic profile of milk and that the differences observed between control, CS and TM cheeses were associated to the diets. Results in the present study were in agreement with other studies carried out on dairy sheep, in which cheesemaking did not affect the FA profile of cheese (Buccioni *et al*., 2010; Coakley *et al*., 2007; Luna *et al*., 2008; Mele *et al*., 2011).

As expected, the content of polyunsaturated fatty acids (PUFA) and saturated fatty acids (SFA) in CS cheeses was lower than in CO cheeses. The current results were in agreement with values reported by Gómez-Cortés *et al*. (2009) and Mele *et al*. (2011), in which including extruded linseed in dairy sheep diet decreased the saturated fatty acid content in cheese.

The TM cheeses showed the lowest content of saturated fatty acids and the highest content of polyunsaturated fatty acids in comparison with the other cheeses. However, no difference was observed between TM and CO cheeses as regards the PUFA content and those differences were completely transferred from milk.

For ripening, the present results were in agreement with values reported by Mele *et al*. (2011), in which ripening did not affect FA composition and triglyceride profile of cheese. The *cis*-9, *trans*-10 CLA was stable during ripening in all the groups (CO, CS and TM), which was in agreement with other studies, where ripening does not substantially modify the total CLA content of cheese (Luna *et al*., 2007; Jiang *et al*., 1997). However, it was in contrast with others, in which changes in *cis*-9, *trans*-10 CLA content were reported during aging (Kumar *et al*., 2006; Laskaridis *et al*., 2012). Laskaridis *et al*. (2012) observed an increase in *cis*-9, *trans*-10 CLA content and this enhancement was associated to the proteolytic activity of the microbial flora enzymes present in cheese. In the present study, based on the physicochemical data, no proteolysis was found in all cheeses, which may explain the lack of changes in CLA content during ripening.

Table 4. 17. Effect of diets on the fatty acid profile of cheese (g/100g of fatty acids)

Item[1]	Treatments (mean values)[2]				*P*-value[4]	
	CO	CS	TM	SEM[3]	D	D×T×R
C4	4.49[a]	4.43[a]	4.13[b]	0.034	<0.001	0.498
C6	3.87	3.83	3.75	0.015	0.015	0.992
C8	2.82[a]	2.81[a]	2.77[b]	0.012	0.004	0.050
C10	6.63[a]	6 .10[b]	6.07[b]	0.056	<0.001	0.340
C10:1+11	0.39	0.35	0.42	0.019	0.245	0.489
C12	4.78[b]	4.68[a]	4.74[b]	0.013	0.003	0.182
C14 iso	0.07	0.07	0.07	0.001	0.178	0.016
C14	11.33[a]	10.21[b]	10.21[b]	0.111	<0.001	0.727
C15 anteiso	0.28	0.29	0.27	0.003	0.203	0.244
C15 iso	0.15	0.15	0.14	0.003	0.085	0.159
C14:1	0.09	0.08	0.08	0.002	0.139	0.954
C15	0.67[a]	0.64[b]	0.67[a]	0.004	0.008	0.295
C16 iso	0.23[a]	0.24[a]	0.17[b]	0.011	0.014	0.317
C16	24.28[a]	23.40[b]	22.81[c]	0.129	<0.001	0.001
C17 anteiso	0.42	0.45	0.42	0.008	0.069	0.158
C16:1	0.74[a]	0.65[b]	0.60[c]	0.014	<0.001	0.238
C17	0.46[a]	0.46[a]	0.44[b]	0.005	0.001	0.006
C18	8.78[c]	10.75[b]	10.91[a]	0.203	<0.001	0.253
trans 6 to 8 C18:1	0.51[a]	0.35[c]	0.37[b]	0.016	<0.001	0.084
trans-9 C18:1	0.38[a]	0.37[b]	0.36[b]	0.004	0.016	0.036
trans-10 C18:1	1.52[a]	1.44[b]	1.46[b]	0.047	0.033	0.063
trans-11 C18:1 (VA)	1.63[c]	2.14[b]	2.29[a]	0.061	<0.001	0.480
trans-12 C18:1	1.01	1.14	1.15	0.029	0.094	0.243
cis-9 C18:1	16.51[c]	17.01[b]	17.53[a]	0.125	<0.001	0.515
trans-15 C18:1	0.60[c]	0.84[a]	0.74[b]	0.021	<0.001	0.179
cis-11 C18:1	0.44	0.44	0.44	0.003	0.828	0.877
cis-12 C18:1	0.40[b]	0.51[a]	0.51[a]	0.011	<0.001	0.004
trans 16 + cis-14 C18:1	0.27[b]	0.37[a]	0.37[a]	0.012	<0.001	0.016
Others trans-trans C18:2	0.32	0.32	0.31	0.003	0.188	0.845
cis 9, cis 12 C18:2	4.16[a]	3.60[c]	3.84[b]	0.048	<0.001	0.985
C20	0.44	0.43	0.41	0.004	0.008	0.130
α C18:3 n3	0.27[a]	0.26[c]	0.26[b]	0.003	<0.001	<0.001
cis 9, trans 11 C18:2 (RA)	0.52[c]	0.58[b]	0.65[a]	0.012	<0.001	0.185
C22	0.26[b]	0.24[c]	0.30[a]	0.006	<0.001	0.546
AA	0.24	0.31	0.34	0.009	<0.001	0.112
SFA	69.98[a]	69.20[b]	68.295[c]	0.049	<0.001	0.122
MUFA	24.11[c]	25.39[b]	25.90[a]	0.160	<0.001	0.123
PUFA	5.52[a]	5.07[b]	5.41[a]	0.0451	<0.001	0.324
SCFA	22.98[a]	22.27[b]	21.89[c]	0.105	<0.001	0.178
MCFA	38.75[a]	36.65[b]	35.90[c]	0.254	<0.001	0.197
LCFA	38.27[c]	41.14[b]	42.24[a]	0.350	<0.001	0.399
cis-9 C18:1 / C16	0.68[c]	0.74[b]	0.78[a]	0.009	<0.001	0.110

[1] Abbreviations: VA, vaccenic acid; RA, rumenic acid; SFA, saturated fatty acids; MUFA, mono-unsaturated fatty acids; PUFA, poly-unsaturated fatty acids; SCFA, short chain fatty acids (C4:0-C12:0); MCFA, medium chain fatty acids (C14:0iso-C17:0); LCFA, long chain fatty acids (>C18:0)

[2] Refers to treatments: Control diet (CO); Canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by marjoram)

[3] SEM: standard error of least square means

[4] Probability of significant effects due to experimental diets (D), time (T) and their interaction (D×T)

For the triglycerides profile, the differences observed in the fatty acid composition between CO, CS and TM cheeses were also found in the triglyceride (TG) profile (Table 4.18). The TG profile of CS cheese showed a lower content of short-chain TG as a consequence of the lower content of fatty acids from C8:0 to C14:0. The content of TG with 40 carbon atoms did not vary, which might be related to the lack of change in C4:0. Most of the increments in LCT (C46-C52) was observed in the TG with 52 and 54 carbon atoms, which might explain the lack of significant differences between CO and CS for LCT. Similar results were reported by Mel *et* al. (2011) as regards short-chain TG.

Table 4. 18. Effect of diets on the triglyceride profile of cheese

Item [1]	Treatments (mean values) [2]			SEM[3]	*P*-value[4]	
	CO	CS	TM		D	D×T×R
C24	0.13	0.13	0.11	0.008	<0.001	0.061
C26	0.42 [a]	0.44 [a]	0.38 [b]	0.008	0.001	0.835
C28	2.18 [a]	2.08 [b]	1.68[c]	0.047	<0.001	0.054
C30	3.19 [a]	3.07 [b]	2.51 [c]	0.311	<0.001	0.071
C32	5.00 [a]	4.52 [b]	3.82 [c]	0.502	<0.001	0.012
C34	7.13 [a]	6.27 [b]	5.65 [c]	0.128	<0.001	0.043
C36	9.79 [a]	9.25 [b]	8.68 [c]	0.100	<0.001	0.405
C38	12.99 [b]	13.32 [a]	12.33[c]	0.091	<0.001	0.828
C40	12.83 [a]	12.78 [a]	11.71 [b]	0.117	<0.001	0.043
C42	9.44 [a]	8.88 [b]	8.42 [c]	0.104	<0.001	0.438
C44	7.32 [a]	6.96 [b]	7.08 [b]	0.048	0.017	0.868
C46	5.39 [b]	4.81 [c]	5.64 [a]	0.081	<0.001	0.504
C48	5.78 [b]	5.68 [b]	7.90 [a]	0.216	<0.001	0.085
C50	7.29 [b]	7.40 [b]	8.67 [a]	0.135	<0.001	0.211
C52	5.91 [c]	7.01 [b]	7.19 [a]	0.122	<0.001	0.335
C54	6.19 [c]	7.61 [b]	8.24 [a]	0.278	<0.001	0.081
SCT [a]	17.05 [a]	16.52 [b]	14.16 [b]	0.337	<0.001	0.281
MCT [b]	52.38 [a]	51.20 [a]	48.21 [b]	0.370	<0.001	0.975
LCT [c]	30.57 [b]	32.50 [b]	37.62 [a]	0.698	<0.001	0.064

[1]Abbreviations: SCT, short chain triglycerides (C24-C34); MCT, medium chain triglycerides (C36-C44); LCT, long chain triglycerides (C46-C54)
[2] Refers to treatments: Control diet (CO); Canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by marjoram)
[3] SEM: standard error of least square means
[4] Probability of significant effects due to experimental diets (D), time (T) and their interaction (D×T)

The triglyceride profile of TM cheese followed the same pattern observed for milk. TM cheese in comparison with CS cheese showed lower content of MCT (C36-C44), higher

content of LCT (C46-C54) and no significant differences as regards SCT (C28-C34). As regards CS cheese in comparison with CO cheese, the similarity with the milk profile was related to the content of MCT, since a lack of modification was reported in cheeses.

4.3.2.1.3. Organoleptic aspects of cheese

4.3.2.1.3.1. Colour parameters of cheese

The effect of the diet on the colour parameters was reported in the Table 4.19. The results showed that there was no modification of the colour attributes of cheeses (CO, CS and TM) during the experimental period and during ripening.

Table 4. 19. Effect of diets on the colour parameters of cheese

Item	Treatments[1]			SEM[2]	*P*<[3]			
	CO	CS	TM		D	T	R	D×T×R
L*	81.22 [b]	80.99 [b]	81.91 [a]	1.27	<0.001	0.475	0.080	0.068
a*	-0.85 [b]	-0.57 [c]	-1.19 [a]	0.44	<0.001	0.535	0.216	0.625
b*	22.07 [a]	22.49 [a]	20.79 [b]	1.35	<0.001	0.995	0.060	0.912

[1]Refers to treatments: Control diet (CO); Canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by marjoram)

[2]SEM: standard error of least square means

[3]Probability of significant effects due to experimental diets (D), time (T), ripening (R) and their interaction (D×T× R)

L*: light/dark chromaticity (changing from 0% dark to 100% light)

a*: green/red chromaticity (changing from -60% green to 60% red)

b*: yellow/blue chromaticity (changing from -60% yellow to 60% blue)

Significant differences were observed on L*, a* and b* according to the diet (Table 4.19). No significant differences were reported between CO and CS cheeses as regards L* and b*, which might be associated to their similar structure (fat and moisture content). CO cheese had the lowest parameter a*, which presented low values (green colour). TM cheeses had the highest values of L* and the lowest values of b* in comparison with CO and CS cheeses which means that CO and CS cheeses have more intense yellow colour but less lightness than MP cheeses. The present results were in agreement with the statement of Coppa *et al*. (2011) in which it suggest that fat content and fat composition variations found among treatments account for most of the differences in colour. Furthermore, indirect correlation was reported between moisture and colour by Tejada *et al*. (2007). Therefore differences in colour parameters might be mostly related to changes in the physicochemical parameters.

On the other hand, ripening did not significantly affect the colour parameters of all cheeses. According to Tejado *et al*. (2006), colour intensity could be related to higher proteolysis, which may explain the absence of differences in colour between the cheeses as no proteolysis was observed during ripening.

4.3.2.1.3.2. Texture attributes of cheese

The effect of the diets on the texture attributes was showed in the Table 4.20. The texture attributes of the different cheeses did not changed during the experimental period and during the ripening period. The texture of cheeses depended principally on diets (CO, CS and TM).

Table 4. 20. Effect of diets on the texture attributes of cheese

Item	Treatments[1]			SEM[2]	*P*<[3]			
	CO	CS	TM		D	T	R	D×T×R
Hardness (g)	7508.82 [a]	7506.08 [a]	6872.74 [b]	200.3	0.04	0.716	0.068	0.771
Adhesiveness (g.s)	-5.97 [c]	-6.80 [b]	-7.75 [a]	0.331	0.06	0.654	0.880	0.938
Cohesiveness	0.48	0.42	0.40	0.010	0.10	0.100	0.060	0.717
Springiness (s)	0.83	0.80	0.80	0.005	0.12	0.928	0.711	0.986
Gumminess (g)	3642.67 [a]	3227.26 [b]	2778.90 [c]	144.3	0.001	0.100	0.300	0.426
Chewiness (g.s)	3024.86 [a]	2650.73 [b]	222.23 [c]	122.0	0.001	0.089	0.210	0.389

[1]Refers to treatments: Control diet (CO); Canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by marjoram)

[2]SEM: standard error of least square means

[3]Probability of significant effects due to experimental diets (D), time (T), ripening (R) and their interaction (D×T× R)

The texture profile analysis revealed significant changes in firmness, adhesiveness, gumminess and chewiness according to the feeding treatments. However, no changes were observed for cohesiveness and springiness (P>0.05).

According to Coppa *et al*. (2011), differences in texture are linked to the combined effects of fat in DM since fat globules reinforce the gel strength, PUFA content and cis-9-C18:1/C16:0 ratio. Moreover, Liu *et al*. (2008) advanced that the higher water content breaks up the protein matrix and plays the role of lubricant to provide smoothness and a softer texture. Additionally, a positive correlation was reported between pH and firmness in cheese because rennet coagulation is influenced by the pH (Han *et al*., 2011; Pastorino *et al*., 2003).

Low fat content, high PUFA content and high cis-9-C18:1/C16:0 ratio has been positively correlated to creamy and less firm cheese (Coppa *et al*., 2011; Hurtaud *et al*.,

2009; Liu *et al*., 2008; Martin *et al*., 2005), which was in agreement with the results observed for TM cheeses. However, in spite of the difference observed between CO and CS as regards fat and PUFA content, no significant difference was reported respect to the firmness. On the other hand, phenolic compounds have been reported to exert an indirect action on the curd firmness making the cheese less firm through modifications of pH (Han *et al*., 2011) as occurred in TM cheeses.

Significant differences were observed for chewiness between cheeses (CO, CS and TM), which was the parameter that combines the overall texture attributes because it involved compressing, shearing, piercing, grinding, tearing and cutting, along with adequate lubrication by saliva at body temperatures (Liu *et al*., 2008). TM cheese was the cheese with the less chewiness value which means the one that needs less mastication in order to breaks up.

4.3.2.1.3.3. Sensory evaluation of cheese

A sensory analysis, based on a discriminative test by using a multiple comparisons, was carried out in order to detect differences between cheeses, manufactured from the milk of ewes that fed different diets (CO, CS and TM), throughout the experimental period (beginning and end) and during ripening (2 and 4 months).

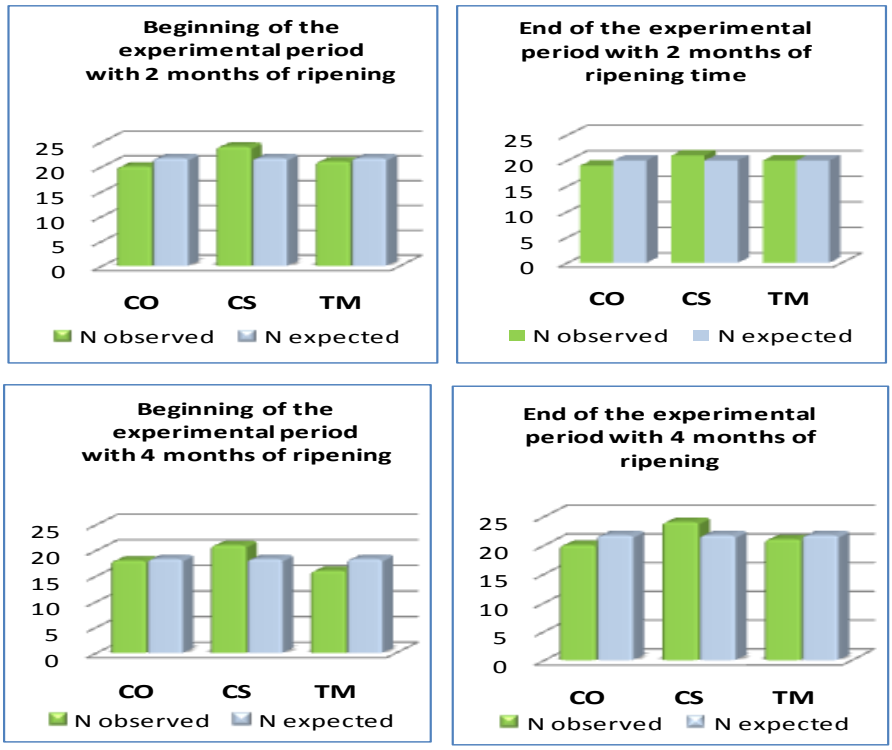


Figure 4. 10. Observed and expected frequencies of cheese throughout the experimental period and during ripening

As can be seen in the Figure 4.9, the panelists did not differentiate between the tested samples and the reference samples (P>0.05), of the different cheeses manufactured during the experimental period independently of the ripening time.

No significant difference was reported between the different cheeses (CO, CS and TM). This result means that including canola seed or marjoram in ewe's diet did not affect significantly the sensorial characteristics of cheese, as judged by the trained panelist (P>0.05).

4.3.2.1.4. Total antioxidant capacity (TAC) of cheese

The total antioxidant capacity (TAC) of cheeses was determined through measuring the $ABTS^{\cdot-}$ scavenging capacity. Significant differences were observed between cheeses (CO, CS and TM) according to the diets. However, no modification of the antioxidant capacity was found during ripening (2 and 4 months). Moreover, the effect of the solvent used for the extraction was not significant (water and methanol), which means that differences between cheeses were principally associated to the diets (Table 4.21).

Table 4. 21. Effect of diets on the antioxidant capacity of cheese

Item	**Treatments**[1]			**SEM**[2]	***P*<**[3]			
	CO	**CS**	**TM**		**D**	**R**	**S**	**D×S×R**
Antioxidant capacity (mg Trolox/ 100g of DWE)	31.34 [b]	29.18 [c]	33.24 [a]	0.871	0.001	0.00	0.316	0.830

[1]Refers to treatments: Control diet (CO); Canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by marjoram)

[2]SEM: standard error of least square means

[3]Probability of significant effects due to experimental diets (D), Solvent (S), ripening (R), and their interaction (D×E×R)

The highest antioxidant capacity was observed in TM cheeses meanwhile the lowest was found in CS cheeses, which was in agreement with the milk TAC results. Those results confirmed once again that cheesemaking did not affect the differences between treatments (CO, CS and TM). However the antioxidant capacity in cheeses was higher than that observed in milk, independently of the feeding treatments. This enhancement might be associated to several factors.

The heat treatment was reported to enhance the antioxidant properties of milk through affecting antioxidant enzymes of milk, such as superoxide dismutase (SOD), which catalyse the dismutation of superoxide anion to H_2O_2 (Jiménez *et al*., 2008). However, several authors found that only severe heating (>100ºC) was associated to the formation

of brown melanoidins and therefore permits the recovery and even a possible increase in milk antioxidant properties (Zulueta *et al*., 2009; Jiménez *et al*., 2008). In the present study, during cheesemaking milk was heated to not more than 30ºC, which discard the effect that could have this factor on TAC values.

Another reason could be the bioactive peptides produced during proteolysis. Enzymes present in milk (especially plasmin), from rennet, or released by microorganisms, hydrolyze casein (α_{s1}-, α_{s2}-, β- and κ- caseins) and may enrich cheeses with bioactive peptides (Pihlanto, 2006). The hydrolysis of casein by pepsin and trypsin produce bioactive peptides with low molecular weight (Pinto *et al*., 2013). And, the presence of those peptides in the supernatant might explain the high TAC values in cheese samples in comparison with milk samples. Furthermore, the complex formed by the phenolic compounds present in milk and the bioactive peptides released during proteolysis could be responble of the changes observed on the antioxidant activity of cheeses.

In the other hand, no significant difference was reported between the different cheeses during ripening, which mean that the proteolysis was weak between 2 and 4 months of ripening.

4.3.2.2. Fermented milk: Yogurt

4.3.2.2.1. Physicochemical properties of yogurt

The physicochemical composition of yogurts according to the diets (CO, CS and TM) was showed in the Table 4.22. Significant modifications in the physicochemical parameters of the different yogurts were observed during the experimental period (Table 4.23).

Haenlein (1998) advanced that sheep milk is especially suitable for yoghurt production because of its high protein and total solids content. The overall properties of yoghurt such as its level of acidity and physicochemical composition are affected by the physicochemical composition of the milk base, the processing conditions employed and the activity of the starter culture during the incubation period (Vivar-Quintana *et al*., 2006).

Serafeimidou *et al*. (2013) reported the intervals in which the physicochemical parameters of yogurt manufactured from sheep milk, at day 1 of storage, could vary; fat (6.08 ± 1.35), protein (4.71 ± 0.67) and total solids (20.27 ± 0.60). The results observed

for CO, CS and TM yogurts were in agreement with those reported above, except for total solids content which was slightly lower (Table 4.22).

The physicochemical parameters of yogurts were significantly affected by the diets (4.22). The yogurts manufactured from the milk of ewes fed CS diet had significantly lower fat, protein and total solids content than the yogurts manufactured from the milk of ewes fed CO diet. The fat content in TM yogurts was significantly lower than those observed in CS and CO yogurts. However, TM yogurts showed higher protein content (P<0.001) in comparison with CS yogurts and no significant change in comparison with CO yogurts. The difference between yogurts according to the diets was similar to that reported for milks. Therefore the modifications observed for the physicochemical parameters were mainly associated to the diets through changes in milk composition. However, the manufacturing process was responsible of minor changes (fat content) without affecting the differences between yogurts (CO, CS and TM). As can be seen in the Table 4.22, the physicochemical parameters of yogurt (protein and total solids content) increased during the transformation of milk in yogurts, except the fat content in TM yogurts, which decrease from 5.31% to 5.22%. This reduction may be related to the fact that lactic acid bacteria may consume some fat to produce flavour compounds during the fermentation process (Ye *et* al., 2013).

Table 4. 22. Effect of diets on the physicochemical composition of yogurt

Item	**Treatments**[1]			**SEM**[2]	***P*<**[3]		
	CO	**CS**	**TM**		**D**	**T**	**D×T**
Fat (%)	5.73 [a]	5.64 [b]	5.22 [c]	0.029	<0.001	<0.001	<0.001
Protein (%)	5.23 [a]	5.09 [b]	5.30 [a]	0.025	0.001	0.422	0.039
Total solids (%)	16.84 [a]	16.53 [b]	16.32 [c]	0.033	<0.001	<0.001	<0.001
pH	4.59	4.60	4.60	0.006	0.530	<0.001	<0.001
Acidity (%)	0.91 [a]	0.88 [b]	0.88 [b]	0.004	<0.001	<0.001	<0.001

[1]Refers to treatments: Control diet (CO); Canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by marjoram)

[2]SEM: standard error of least square means

[3]Probability of significant effects due to experimental diets (D), time (T) and their interaction (D×T)

No significant differences were observed between the different yogurts (CO, CS and TM) for pH and the values ranged from 4.59 to 4.6. As regards the titratable acidity, significant differences were reported between yogurts and the values ranged from 0.88 to 0.9.

The CO yogurts had the highest titratable acidity in comparison with CS and TM yogurts. A correlation between the total solids content and the titrable acidity was reported by Estévez *et al*. (2008), which was in agreement with the present results since the CO yogurts that had the highest total solids content had as well the highest titrable acidity. This relationship might be associated to the fact that higher solid content may have produced a higher buffer capacity and consequently a good acid production (Ankeman and Morr, 1996). However, no significant differences in acidity were observed between TM and CS yogurts in spite of the differences observed in the total solids content between both groups of yogurts. This lack of change in acidity between CS and TM yogurts may be associated to the acid production in the TM yogurts, which depends on the growth of the microorganisms and their ability to ferment the available carbohydrates (Favaro *et al*. 2001), rather than to the total solids content. Indeed, at day 1 of storage, as showed in the Figure 4.7, the *L. bulgaricus* count in TM yogurts was higher than that in CS yogurts.

On the other hand, it was reported by Vivar-Quintana *et al*. (2006) that yogurt manufactured from milk with high somatic cell count had low level of acidity, since an inhibition of the starter cultures occurs as a result of the antimicrobial action of the somatic cells. However, this relationship was not suitable for the observed results since there was no inhibition of the starter cultures in TM and CS yogurts, at day 1 of storage, as a result of higher somatic cell count in TM and CS milks, in comparison with CO yogurts as shown in the Figure 4.7.

The physicochemical composition of yogurts (CO, CS and TM,) during the experimental period was showed in the Table 4.23. Significant modifications during the experimental period were found for all the physicochemical parameters, except for the protein content. The fat and total solids content decreased substantially ($P<0.05$). This reduction was in agreement with that described for milk.

Table 4. 23. Effect of time on the physicochemical composition of yogurt

Diets [1]	Time [2]	Fat (%)	Total solids (%)
CO	Beginning	5.87 ± 0.07 [a]	16.94 ± 0.13 [a]
	Middle	5.76 ± 0.09 [b]	17.01 ± 0.22 [a]
	End	5.56 ± 0.05 [c]	16.57 ± 0.09 [b]
CS	Beginning	5.86 ± 0.12 [a]	16.87 ± 0.11 [a]
	Middle	5.60 ± 0.10 [b]	16.55 ± 0.19 [b]
	End	5.47 ± 0.10 [c]	16.17 ± 0.16 [c]
TM	Beginning	5.57 ± 0.10 [a]	16.56 ± 0.07 [a]
	Middle	5.01± 0.05 [b]	16.24 ± 0.09 [b]
	End	5.08 ± 0.11 [b]	16.15 ± 0.15 [b]

[1]Refers to treatments: Control diet (CO); Canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by marjoram)

[2]Refers to time: experimental period (beginning, middle and end)

[3]Means within a column not followed by the same letter differ (P> 0.05)

4.3.2.2.2. Microbiological analysis of yogurt

The Table 4.24 showed the *Streptococcus thermophilus* (ST) and the *Lactobacillus bulgaricus* (LB) counts in the different yogurts (CO, CS and TM) and their viabilities during 28 days of storage. No significant differences were reported between yogurts regardless of diets. For the storage period, no significant differences were observed for *S. thermophilus* whereas significant differences were found for *L. bulgaricus* (Figure 4.11 and 4.12).

Table 4. 24. Effect of diets on the yogurt culture bacteria (ST and LB)

Item	Treatments[1]			SEM[2]	P<[3]		
	CO	CS	TM		D	S	D×S
ST (Log UFC/g)	9.08	9.09	9.03	0.127	0.160	0.934	0.131
LB (Log UFC/g)	6.29	6.44	6.44	0.035	0.145	<0.001	0.001

[1]Refers to treatments: Control diet (CO); Canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by marjoram)

[2]SEM: standard error of least square means

[3]Probability of significant effects due to experimental diets (D), storage (S) and their interaction (D×S)

The counts were higher in all the cases to 10^7 CFU which was in accordance with the food regulations of yogurt in which yogurt should contain at least 10^7 CFU of viable

bacteria (S. *thermophilus* and *L. bulgaricus*) per grams o millilitres of yogurt. Moreover, the yogurt microflora was found to be present at sufficiently high levels both at the beginning and the end of the 28 days of storage. The *S. thermophilus* counts were higher than *Lb. bulgaricus* counts, which is the normal behavior of these bacteria as it has been previously reported by Tamime and Robinson (1999) and Wang *et al*. (2008).

The effect of diets (CO, CS and TM) on *S. thermophilus* and *L. bulgaricus* counts was not significant (P>0.05) (Table 4.24). These findings indicated that changes in milk properties through the modification of ewe's diets did not affect the lactic acid bacteria count in the yogurt manufactured from this milk. These results were in agreement with values found by Jaziri *et al*. (2009) and Najgebauer-Lejko *et al*. (2011) in which phenolic compounds did not inhibit lactic acid bacteria in opposition to other pathogenic bacteria.

However, the study of the effect of diets throughout the shelf life of yogurts showed that *S. thermophilus* and *L. bulgaricus* have a different behavior (Figure 4.10 and 4.11). The viability of *S. thermophilus* was not affected during storage and remained stable during the shelf life of all the yogurts (Figure 4.11). However, the viability of *L. bulgaricus* decreased throughout the shelf life in all the yogurts (Figure 4.12).

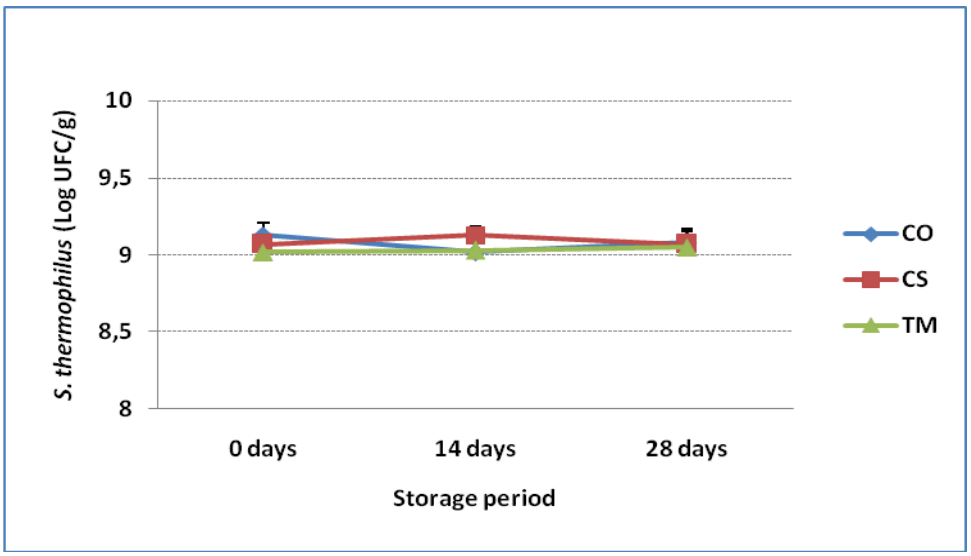
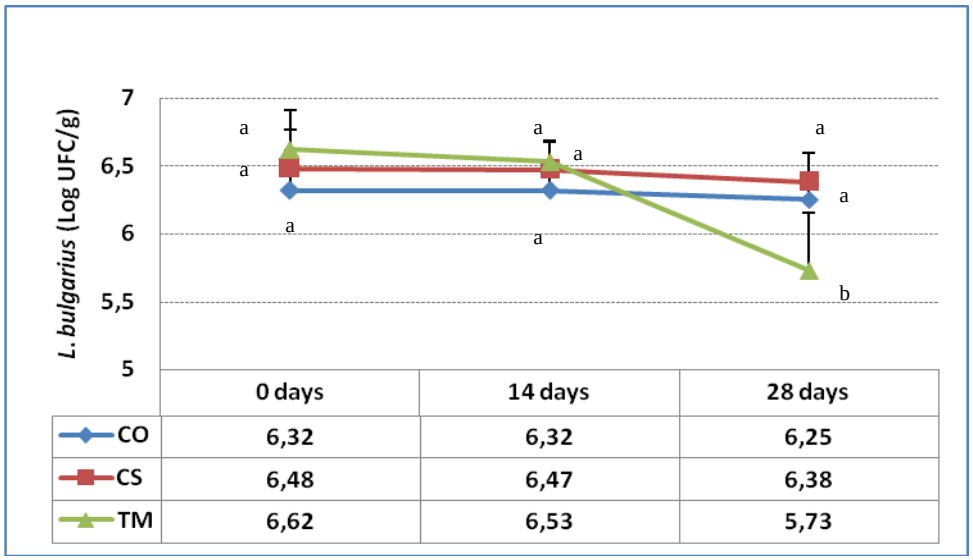


Figure 4. 11. Yogurt *S. thermophilus* survival during 28 days of storage



	0 days	14 days	28 days
CO	6,32	6,32	6,25
CS	6,48	6,47	6,38
TM	6,62	6,53	5,73

Figure 4. 12. Yogurt *L. bulgaricus* survival during 28 days of storage

The decrease in *L. bulgaricus* was in agreement with previously reported observations indicating a negative effect of the storage on *L. bulgaricus* viability in yogurt during shelf life due to their high sensitivity to low temperatures (Birollo *et al.*, 2000). Although the decrease was only significant for TM yogurts, CO and CS showed the same pattern. After 28 days of storage, the number of *L. bulgaricus* in TM yogurts decreased significantly in comparison with day 1 and 14 of storage, which suggest that the viability of *L. bulgaricus* was affected by the presence of *T. mastichina* in CS diet at day 28 of storage. However, as can be seen in the Figure 4.11, the *L. bulgaricus* counts in TM yogurts were higher than CO and CS yogurts at day 1 and 14 of storage.

These results were in contrast with those reported by Jaziri *et al*. (2009), who showed that the addition of green or black tea at 2.0% to 4.0% (w/v) in the milk used for making yogurts, did not influence the fermentation of yogurts and the survival of characteristic microorganisms in yogurts during a six-week storage period at 4 ºC.

4.3.2.2.3. Lipidic profile of yogurt

The fatty acids composition and the triglycerides profile of the yogurts manufactured from the milks of ewes fed different diets (CO, CS and Tm) were showed in the Tables 4.25 and 4.26. The modifications of the fatty acids and triglycerides composition during the experimental period were as well reported in the Tables 4.25 and 4.26. Significant differences were observed regardless of diets. However, throughout the experimental period, no significant changes were observed for the triglycerides profile and for the unsaturated, mono-unsaturated, short chain, medium chain and long chain fatty acids.

The differences observed between yogurts were associated to the differences found in milks (CO, CS and TM). The effect of canola seed and marjoram on yogurt FA composition was similar to that of milk. No significant differences were observed between CS and CO yogurts for saturated and mono-unsaturated and short chain fatty acids whereas polyunsaturated and medium chain fatty acids decreased and only the long chain fatty acids increased ($P<0.05$). Including marjoram in CS diet decreased saturated, short chain and medium chain fatty acids but increased polyunsaturated, mono-unsaturated and long chain fatty acids. The differences between yogurts were mainly associated to the diets since the FA composition of yogurts showed a profile similar to that found in milk.

The only difference observed between the profile of yogurts and milks was related to the *cis*-9 *trans*-11 CLA content, which increased in all the groups of yogurts (CO, CS and TM). The results showed that the *cis*-9 *trans*-11 CLA content of yogurts ranged between 0.57-0.76 g/100g of fatty acids which was in agreement with the values reported by Serafeimidou *et al*. (2012) for sheep yogurt (0.405-1.250 g/100g fat) and which corresponds to 0.18-1.09 of total fatty acids. The increase observed in the CLA content of yogurts in comparison with milks might be related to the manufacturing process. Actually, according to Serafeimidou *et al*. (2012), the effect of manufacturing process on CLA content in dairy products is subject of controversy. Several studies reported that manufacturing process affected significantly CLA content (Kim and Liu, 2002; Sieber *et al*., 2004). However, Boylston and Beitz (2002) observed that processing of milk into yogurt did not have a significant effect on the content of CLA.

According to Jiang *et al*. (1998) and Aneja and Murthi, (1990), microbial fermentation increases CLA concentration. Additionally, Sieber *et al*. (2004), suggests that strains of various traditional dairy cultures may possess different abilities to produce CLA during fermentation of milk and other dairy substrates. On the other hand, various studies reported that CLA content can be affected by numerous factors such as bacterial strain, cell number, optimal substrate concentration and the period of incubation at neutral pH (Kim and Liu, 2002) or processing parameters such as heat treatment, composition of starter culture, storage and aging (Serafeimidou *et al*., 2012). Based on the studies mentioned above, the increase observed in the CLA content in CO, CS and TM yogurts might be associated to the starter culture used during the manufacturing process.

Table 4. 25. Effect of diets on the fatty acid profile of yogurt (g/100g of fatty acids)

Item [1]	Treatments (mean values) [2]			SEM [3]	*P*-value [4]	
	CO	CS	TM		D	D×T
C4	4.43	4.34	4.20	0.050	0.163	0.133
C6	3.81^{a}	3.82^{a}	3.63^{b}	0.030	0.009	0.174
C8	2.57^{a}	2.57^{a}	2.45^{b}	0.031	0.066	0.04
C10	6.40^{a}	5.11^{b}	5.90^{b}	0.075	0.007	0.400
C10:1+11	0.87^{b}	0.94^{b}	1.13^{a}	0.041	0.006	0.012
C12	4.62	4.50	4.40	0.061	0.357	0.348
C14 iso	0.07	0.07	0.07	0.002	0.301	<0.001
C14	11.78^{a}	11.02^{b}	10.48^{c}	0.132	0.004	0.811
C15 anteiso	0.25^{b}	0.30^{a}	0.30^{a}	0.009	<0.001	<0.001
C15 iso	0.14	0.15	0.15	0.003	0.369	<0.001
C14:1	0.12^{a}	0.09^{b}	0.09^{b}	0.004	0.004	0.082
C15	0.68^{b}	0.71^{a}	0.71^{a}	0.009	0.023	<0.001
C16 iso	0.21	0.20	0.21	0.004	0.004	<0.001
C16	23.97^{a}	23.79^{a}	22.87^{b}	0.124	<0.001	0.424
C17 anteiso	0.39	0.36	0.41	0.019	0.286	0.083
C16:1	0.76	0.72	0.68	0.010	0.00	0.001
C17	0.44	0.46	0.45	0.007	0.107	<0.001
C18	9.45^{b}	10.76^{a}	11.00^{a}	0.203	0.002	0.541
trans 6 to 8 C18:1	0.47^{a}	0.41^{b}	0.41^{b}	0.010	0.002	0.525
trans-9 C18:1	0.35	0.34	0.34	0.007	0.601	0.004
trans-10 C18:1	1.65^{a}	1.58^{b}	1.56^{b}	0.071	0.054	0.063
trans-11 C18:1 (VA)	1.66^{c}	2.07^{a}	2.31^{a}	0.095	<0.001	<0.001
trans-12 C18:1	0.71	0.77	0.80	0.019	0.146	0.164
cis-9 C18:1	16.66^{c}	17.28^{b}	18.02^{a}	0.137	<0.001	0.975
trans-15 C18:1	0.62	0.47	0.54	0.032	0.173	0.580
cis-11 C18:1	0.46	0.43	0.48	0.010	0.076	0.043
cis-12 C18:1	0.36^{c}	0.51^{b}	0.56^{a}	0.021	<0.001	<0.001
trans 16 + cis-14 C18:1	0.23^{b}	0.37^{a}	0.37^{a}	0.016	<0.001	0.277
Others trans- trans C18:2	0.29	0.31	0.31	0.006	0.131	0.001
cis 9, cis 12 C18:2	4.13^{a}	3.62^{b}	3.85^{c}	0.043	<0.001	0.750
C20	0.35	0.36	0.36	0.010	0.829	0.001
α C18:3 n3	0.28	0.28	0.26	0.007	0.468	0.002
cis 9, trans 11 C18:2 (RA)	0.57^{c}	0.65^{b}	0.76^{a}	0.020	<0.001	0.001
C22	0.17	0.16	0.19	0.006	0.083	0.035
AA	0.25	0.23	0.28	0.012	0.99	<0.001
SFA	69.55^{a}	69.48^{a}	67.79^{b}	0.207	<0.001	0.246
MUFA	24.07^{b}	24.50^{b}	25.63^{a}	0.198	<0.001	0.040
PUFA	5.52^{a}	5.08^{b}	5.46^{a}	0.049	<0.001	0.007
SCFA	22.69^{a}	22.08^{a}	21.72^{b}	0.163	0.048	0.340
MCFA	38.63^{a}	37.87^{b}	36.44^{c}	0.223	<0.001	0.171
LCFA	38.68^{c}	40.04^{b}	41.85^{a}	0.357	<0.001	0.325

[1] Abbreviations: VA, vaccenic acid; RA, rumenic acid; SFA, saturated fatty acids; MUFA, mono-unsaturated fatty acids; PUFA, poly-unsaturated fatty acids; SCFA, short chain fatty acids (C4:0-C12:0); MCFA, medium chain fatty acids (C14:0iso-C17:0); LCFA, long chain fatty acids (>C18:0)

[2] Refers to treatments: Control diet (CO); Canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by marjoram)

[3] SEM: standard error of least square means

[4] Probability of significant effects due to experimental diets (D), time (T) and their interaction (D×T)

The effect of diet on the triglyceride profile (TG) was significant since significant differences were observed between the different yogurts (Table 4.26). The triglyceride profile of yogurts followed the same pattern as that observed for milks. The conclusions drawn for milks were suitable for yogurts; TM yogurts had the lowest medium chain triglycerides and the highest long chain triglycerides and CS yogurts had higher long chain triglycerides in comparison with CO yogurts.

Table 4. 26. Effect of diets on the triglyceride profile of yogurt

Item [1]	Treatments (mean values) [2]			SEM[3]	*P*-value[4]	
	CO	CS	TM		D	D×T
C24	0.12 [a]	0.11 [a]	0.08 [b]	0.004	<0.001	0.278
C26	0.83	1.04	0.76	0.067	0.192	0.356
C28	1.88 [a]	1.59 [b]	1.42 [b]	0.063	0.009	0.534
C30	2.84 [a]	2.63 [b]	2.30 [c]	0.053	<0.001	0.044
C32	4.33 [a]	4.11 [b]	3.67 [c]	0.069	<0.001	0.062
C34	6.67 [a]	6.49	5.97 [b]	0.080	<0.001	0.668
C36	9.67 [a]	9.14 [b]	8.70 [c]	0.096	<0.001	0.780
C38	13.70 [a]	13.23 [b]	12.07 [b]	0.174	<0.001	0.373
C40	12.86 [a]	12.50 [b]	10.95 [c]	0.172	<0.001	0.253
C42	9.11 [a]	8.31 [b]	7.96 [c]	0.115	<0.001	0.576
C44	7.70 [a]	7.28 [b]	6.99 [c]	0.079	<0.001	0.530
C46	5.71 [b]	6.63	6.56 [a]	0.101	<0.001	0.774
C48	6.66 [b]	6.76 [b]	7.29 [a]	0.072	<0.001	0.650
C50	6.73 [c]	8.63 [b]	9.90 [a]	0.289	<0.001	0.125
C52	5.69 [c]	6.80 [b]	7.46 [a]	0.170	<0.001	0.791
C54	5.67 [c]	6.76 [b]	7.90 [a]	0.212	<0.001	0.358
SCT [a]	16.67 [a]	15.97 [b]	14.22 [c]	0.254	<0.001	0.093
MCT [b]	52.86 [a]	50.44 [b]	46.67 [c]	0.586	<0.001	0.402
LCT [c]	30.47 [c]	35.59 [b]	39.11 [a]	0.788	<0.001	0.493

[1] Abbreviations: SCT, short chain triglycerides (C24-C34); MCT, medium chain triglycerides (C36-C44); LCT, long chain triglycerides (C46-C54)
[2] Refers to treatments: Control diet (CO); Canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by marjoram)
[3] SEM: standard error of least square means
[4] Probability of significant effects due to experimental diets (D), time (T) and their interaction (D×T)

4.3.2.2.4. Organoleptic aspects of yogurt

In order to evaluate the organoleptic aspects of yogurts, colour, texture and sensory characteristics were assayed.

4.3.2.2.4.1. Colour parameters of yogurt

The use of different diets (CO, CS and TM) affected significantly the colour parameters (L*, a* and b*) of yogurts as shown in the Table 4.27. Significant changes were observed throughout the experimental period in the different groups of yogurts (Table 4.28).

Table 4. 27. Effect of diets on the colour parameters of yogurt

Item	Treatments[1]			SEM[2]	*P*<[3]		
	CO	CS	TM		D	T	D×T
L*	91.67 [b]	91.63 [c]	91.72 [a]	0.008	<0.001	<0.001	<0.001
a*	-3.34 [a]	-3.28 [b]	-3.28[b]	0.006	<0.001	<0.001	<0.001
b*	11.63[a]	11.45 [b]	11.41[c]	0.016	<0.001	<0.001	<0.001

[1]Refers to treatments: Control diet (CO); Canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by marjoram)

[2]SEM: standard error of least square means

[3]Probability of significant effects due to experimental diets (D), time (T) and their interaction (D×T)

L*: light/dark chromaticity (changing from 0% dark to 100% light)

a*: green/red chromaticity (changing from -60% green to 60% red)

b*: yellow/blue chromaticity (changing from -60% yellow to 60% blue)

Significant differences were observed for the colour parameter between the different yogurts as regards the diet (Table 4.25). The TM yogurts manufactured from the milk of ewes fed TM diet, was lighter in colour (L*) and less yellowish (b*) than the rest of yogurts (CO and CS). The CO yogurt was more yellowish (b*) than TM and CS yogurts. Actually, the yellow colour (b*) was mostly correlated to the level of fat in milk (Coppa *et al*., 2011), which was in agreement with the present results since TM yogurt had the lowest fat content and CO yogurt the highest.

The parameter a* presented low values, regardless of the diet used (Table 4.27). Significant differences were reported between CO and the rest of yogurts (CS and TM) as regards the parameter a*. According to Hilali *et al*. (2011), the parameter a* was negatively correlated with milk protein content and positively correlated with lactose content but the present results did not showed relationship between protein or lactose content and the parameter a*.

Significant changes in all the colour parameters were observed in the different yogurts throughout the experimental period (Table 4.28). Significant decreases were found in the L*, a* and b* parameters. The parameter b* decreased significantly during the

experimental period (P<0.01), indicating that the yogurts became less yellowish, probably due to the decrease in fat content.

Table 4. 28. Effect of time on the colour parameters of yogurt

Diets [1]	Time [2]	L*	a*	b*
CO	Beginning	91.74 ± 0.05 [a]	-3.38 ± 0.14 [a]	11.82 ± 0.07 [a]
	Middle	91.66 ± 0.06 [b]	-3.42 ± 0.03 [a]	11.59 ± 0.011 [b]
	End	91.59 ± 0.11 [c]	-3.21 ± 0.03 [b]	11.29 ± 0.07 [c]
CS	Beginning	91.77 ± 0.12 [a]	-3.37 ± 0.04 [a]	11.71 ± 0.14 [a]
	Middle	91.64 ± 0.09 [b]	-3.21 ± 0.03 [b]	11.32 ± 0.12 [b]
	End	91.45 ± 0.05 [c]	-3.21 ± 0.06 [b]	11.26 ± 0.10 [c]
TM	Beginning	91.87 ± 0.04 [a]	-3.33 ± 0.04 [a]	11.53 ± 0.12 [a]
	Middle	91.69 ± 0.06 [b]	-3.26 ± 0.07 [b]	11.18 ± 0.13 [b]
	End	91.61 ± 0.06 [c]	-3.21 ± 0.04 [c]	11.13 ± 0.11 [b]

[1]Refers to treatments: Control diet (CO); Canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by marjoram)

[2]Refers to time: experimental period (beginning, middle and end)

[3]Means within a column not followed by the same letter differ (P> 0.05)

L*: light/dark chromaticity (changing from 0% dark to 100% light)

a*: green/red chromaticity (changing from -60% green to 60% red)

b*: yellow/blue chromaticity (changing from -60% yellow to 60% blue)

4.3.2.2.4.2. Texture attributes of yogurt

Significant differences in texture attributes (firmness, apparent viscosity and adhesiveness) were reported between the different yogurts according to the diets (CO, CS and TM) and throughout the experimental period (Table 4.29 and 4.30).

Table 4. 29. Effect of diets on the texture attributes of yogurt

Item	Treatments[1]			SEM[2]	P<[3]		
	CO	CS	TM		D	T	D×T
Firmness (g)	158.56 [a]	151.48 [b]	163.09 [a]	1.715	0.03	<0.001	<0.001
Adhesiveness (g.s)	-161.40	-158.21	-171.35	3.163	0.054	<0.001	0.002
Apparent viscosity (cps)	84370 [a]	63127 [b]	95522 [a]	4045	0.002	0.341	0.387

[1]Refers to treatments: Control diet (CO); Canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by marjoram)

[2]SEM: standard error of least square means

[3]Probability of significant effects due to experimental diets (D), time (T) and their interaction (D×T)

Significant differences were observed between TM and CS yogurts ($P<0.05$). The yogurts manufactured from milk of ewes fed with TM diet, had higher firmness and apparent viscosity than the yogurts manufactured from milk of ewes fed with CS diet. On the other hand, TM and CO yogurts had similar gel structure, which was more firm and viscous than CS yogurts. The textural differences between the yogurts were associated to the milk used and its physicochemical composition (Vivar-Quintana *et al*., 2006). TM and CO yogurts that displayed the highest firmness and viscosity had also the highest protein content. Those results were in agreements with values reported by Estévez *et al*. (2008) and Magenis *et al*. (2006), in which a positive correlation was reported between protein content and firmness. Actually, milk protein content affect firmness through the formation of aggregates, by interactions with casein micelles, making the gel structure of yogurt more rigid (Lucey *et al*., 1998). Furthermore, the milk low in fat and high in protein would theoretically promote the curd structure and consequently increase its firmness (Abeddou *et al*., 2011).

The viscosity showed similar results to the results reported by Abu-Jdayil (2003) in yogurts of the type labneh, in which higher viscosity was correlated with higher protein content. Moreover, Martín-Diana *et al*. (2004) reported that protein content affected significantly yogurt viscosity.

No significant differences between the yogurts were found respect to the adhesiveness. Although a not significant difference was observed, TM yogurts were more adhesive than CO and CS yogurts, which was in agreement with the results reported by Herrero and Requena (2006) in which the increase in protein content enhanced the adhesiveness of yogurt.

The modifications of the texture attributes of yogurts (CO, CS and TM) during the experimental period were significant with the exception of the apparent viscosity, which remained steady. The firmness and the adhesiveness decreased in all the yogurts during the experimental (Table 4.30).

Table 4. 30. Effect of time on the texture attributes of yogurt

Diets [1]	Time [2]	Firmness (g)	Adhesiveness (g.s)
CO	Beginning	170.93 ± 13.9 [a]	-191.18 ± 35.7 [a]
	Middle	161.80 ± 8.59 [a]	-153.96 ± 30.4 [b]
	End	144.73 ± 4.61 [b]	-133.72 ± 12.3 [b]
CS	Beginning	166.37 ± 12.8 [a]	-179.93 ± 28.3 [a]
	Middle	138.90 ± 8.52 [b]	-152.60 ± 28.3 [b]
	End	144.30 ± 3.09 [b]	-133.12 ± 17.4 [b]
TM	Beginning	173.30 ± 16.0 [a]	-186.27 ± 27.9 [a]
	Middle	159.41 ± 20.6 [b]	-169.87 ± 35.6 [b]
	End	142.13 ± 7.52 [b]	-159.40 ± 15.5 [b]

[1]Refers to treatments: Control diet (CO); Canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by marjoram)

[2]Refers to time: experimental period (beginning, middle and end)

[3]Means within a column not followed by the same letter differ (P> 0.05)

4.3.2.2.4.3. Sensory evaluation of yogurt

A sensory analysis was carried out in order to evaluate the differences between the yogurts, manufactured from the milk of ewes fed with different diets (CO, CS and TM), throughout the experimental period (beginning, middle and end).

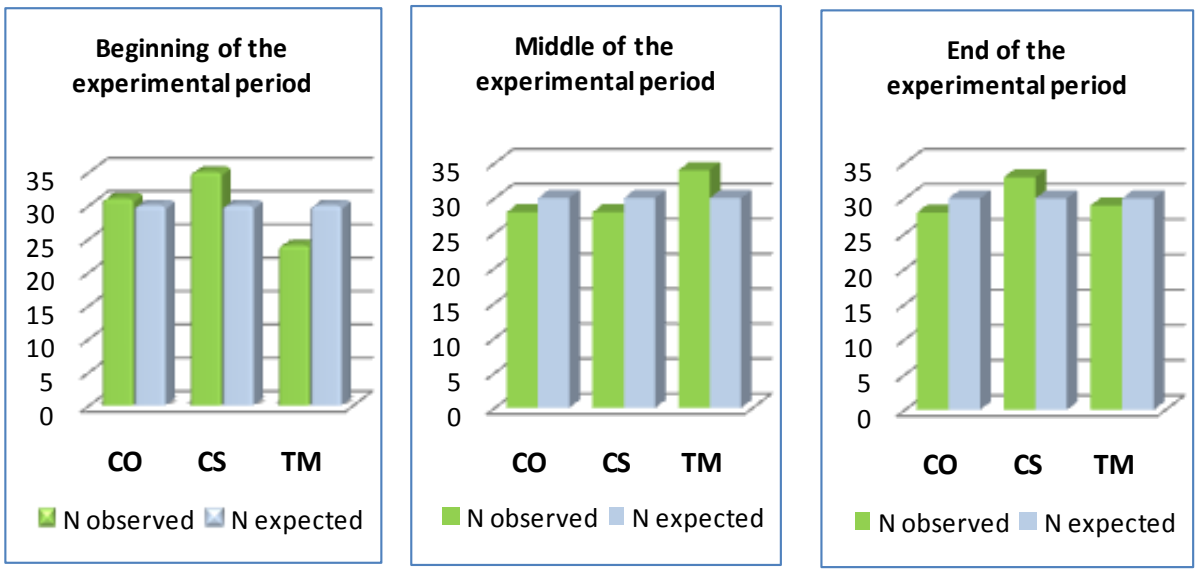


Figure 4. 13. Observed and expected frequencies of yogurts (CO, CS and TM) during the experimental period

The sensory evaluation of yogurts was performed using a multiple comparisons test in order to allow the comparison between the observed and the expected frequencies in each group of yogurt (CO, CS and TM). No significant differences between the different yogurts (CO, CS and TM) were observed ($P>0.05$). The results showed that the panelists did not differentiate between the manufactured yogurts independently of the diet used to feed the ewes (Figure 4.13).

The sensory evaluation of the yogurts revealed that the different yogurts had the same acceptability. This result means that including canola seed or marjoram plant in dairy sheep diet did not affect milk characteristics in a way that panelists were able to note changes in the organoleptic aspects of the manufactured yogurts.

4.3.2.2.5. Characterization of phenolic compounds in yogurt

Separation and detection of phenolic compounds in milk-based food products have been reported in literature by Redeuil *et al*. (2009). However the number of studies is limited in comparison with the studies published on the analysis of polyphenols in plants.

The characterization of the phenolic compounds was performed by using LC-MS, the same method used for the characterization of polyphenols in marjoram. The aim of this characterization was to identify the phenolic compounds present in yogurt and to observe if there is any transfer of phenolic compounds from the plant to the yogurt manufactured from the milk of ewes fed with marjoram diet.

The phenolic characterization of yogurt was performed on the hydrophilic fraction of yogurt extracted with methanol. The fat layer was removed and only the supernatant was used in order to avoid any interference with the lipidic fraction.

The tentative of identification was based on the use of the same reference standards used for the marjoram plant and also based on the data available in the library. The compounds found did not correspond to any of the compounds identified in marjoram or existing in the library. However, based on the charge:mass ratio observed in the chromatograms, it was concluded that these peaks corresponded to bioactive peptides or to complexes peptides-polyphenols.

The Figure 4.14 showed the analysis of the phenolic compounds of the yogurt extract by LC-MS. A total of seventeen peaks eluted during 18 min were found.

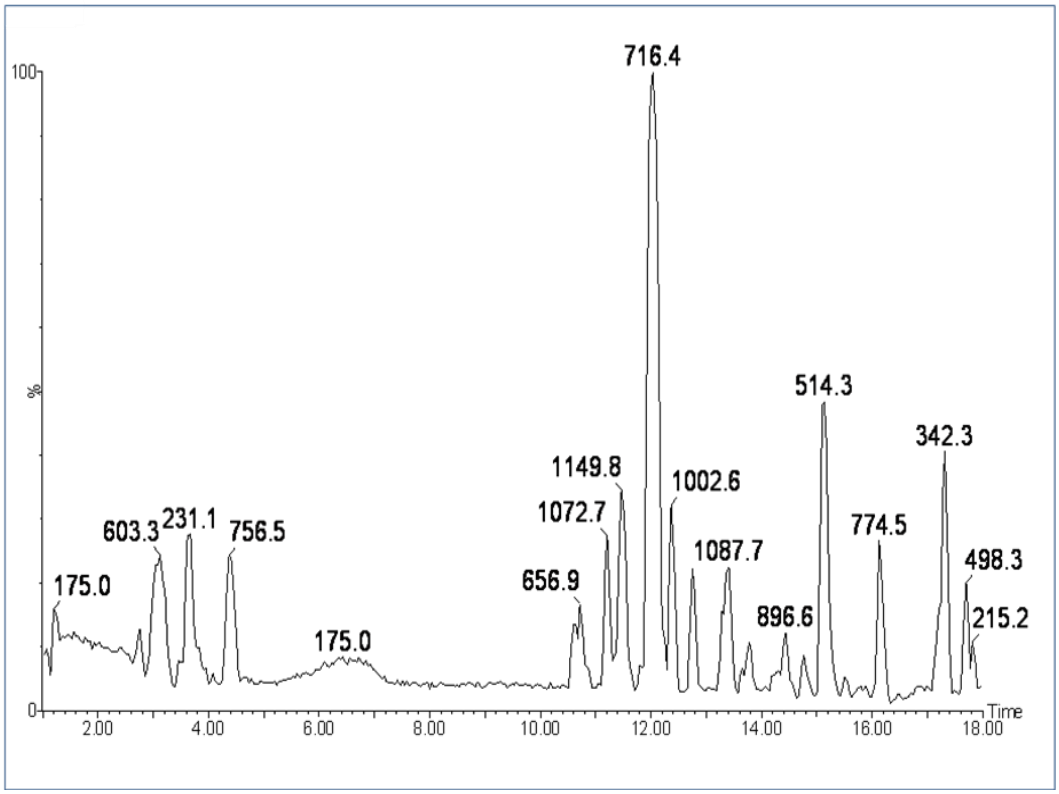


Figure 4. 14. Phenolic compounds identified in yogurt by LC-ESI-MS/MS

Actually, as observed in the marjoram, polyphenols was mostly eluted bounded to glycosylated compounds and the use of the accurate mass measurements allowed the identification of each compounds due to its fragmentation. In dairy products, other type of interactions is possible such the interaction between protein and phenolic compounds, which received a considerable attention in recent years (Gallo *et al*., 2013). However, there is a lack of information on the protein-polyphenol interactions in fermented milk (Najgebauer-Lejko *et al*., 2011).

4.3.2.2.6. Bioactive properties of yogurt

Due to the high antioxidant capacity of yogurts in comparison with cheeses, further studies were carried out in order to study extensively the bioactive properties of yogurts and to identify the components associated to the increase of the antioxidant capacity.

Yogurt extract showed better $ABTS^{\cdot-}$ scavenging capacity than cheese extract, as observed in the Table 4.21 and 4.32. The lower antioxidant capacity of cheese in comparison with yogurt might be principally associated to the type of bacterial strain used during the manufacturing process and also to the nature of bioactive peptides

released upon proteolysis or fermentation, depending on the starter cultures and the proteolytic enzymes (Pinto *et al*., 2013).

4.3.2.2.6.1.1. Total antioxidant capacity (TAC)

In order to study the antioxidant capacity of yogurt extracts, several factors of variation were taken into account. The first one was the extraction procedure, which could significantly affect the results of the antioxidant activity.

Milk and milk-based products contain hydrophilic and lipophilic phases which have different contribution to the antioxidant activity (Jiménez *et al*., 2008). For this reason, two extraction procedures were carried out, with methanol as extraction solvent, in order to measure the antioxidant capacity in the whole matrix (hydrophilic and lipophilic phase) and in the supernatant (hydrophilic phase) of yogurts.

The difference observed in the antioxidant capacity between both extractions was significant ($P<0.05$). The antioxidant capacity of yogurts was significantly higher in the supernatant in comparison with the whole matrix (56.85 ± 13.60 and 31.01 ± 4.41, respectively). These differences were probably due to the fact that beyond the capacity of phenolic compounds to scavenge free radicals, other biochemical mechanisms can explain their antioxidant effects. And, these mechanisms are related to polyphenols-lipid and polyphenols-protein interactions (Abu-Reidah. 2013). Moreover, Serafini *et al*. (2009) suggested a possible novel involvement of lipids in the interaction between, protein and antioxidants ingredients.

The effect of the diets (CO, CS and TM), received by ewes, on the antioxidant capacity of the whole matrix of yogurts, throughout the experimental period, was showed in the Table 4.31. No significant differences were observed between CO and TM yogurts. However, TM yogurts had significantly higher antioxidant capacity than CS yogurts. No modifications in the antioxidant activity of yogurts were observed throughout the experimental period ($P>0.05$).

Table 4. 31. Effect of diets on the DPPH scavenging activity of the whole matrix of yogurt

Item	Treatments[1]			SEM[2]	*P*<[3]		
	CO	CS	TM		D	T	D×T
Antioxidant capacity (mg Trolox/ 100g of DWE)	31.32 [a]	28.36 [b]	33.12 [a]	0.636	0.006	0.783	0.454

[1]Refers to treatments: Control diet (CO); Canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by marjoram)

[2]SEM: standard error of least square means

[3]Probability of significant effects due to experimental diets (D), time (T), and their interaction (D×T)

According to Zulueta *et al*. (2009), the antioxidant activity increased in parallel with milk fat content, which was in contrast with the observed results. Actually, TM yogurts had the lowest fat content in comparison with CO yogurts but both have similar antioxidant capacity (P>0.05). These results were in agreement with those of Ye et *al*. (2013), in which no correlation was reported between the fat content of yogurt and its antioxidant capacity. However, a positive correlation between the polyunsaturated fatty acids content (PUFA) and the antioxidant activity was observed in all the yogurts (CO, CS and TM). Indeed, no significant differences were reported between TM and CO yogurts for PUFA content and also for the antioxidant capacity. Similar results were reported by Vitas *et al*. (2012) for fermented milk products. However, no significant relationship between PUFA and antioxidant capacity was found by Tijerina-Sáenz *et al*. (2009) for human milk.

On the other hand, a positive correlation was also observed between the antioxidant activity and the protein content in all yogurts (CO, CS, and TM). Further results in the hydrophilic phase will be show latter for a better discussion and understanding of the interaction between phenolic compounds and peptides. More extensive research is needed in order to clarify the role of lipids and its interaction with proteins and phenolic compounds on the antioxidant capacity of milk and dairy products.

From this point forward, the results will not be related to the whole matrix but will refer only to the supernatant, which means that the antioxidant capacity will be mainly associated to the antioxidant compounds present in the hydrophilic phase.

The effect of the dietary treatments on the bioactive properties of yogurts occurred through changes on the milk properties. However, the antioxidant capacity of yogurts was higher than that of milk, both assayed in the supernatant with the ABTS method, as observed in the Table 4.13 and 4.32. This difference could be mainly associated to the bioactive peptides hydrolyzed from milk through the fermentation process (Pinto *et al*., 2013). Indeed, in yogurt, enzymes could give rise to the liberation of a particularly high number of bioactive peptides (Pinto *et al*., 2012). Few studies related the production of antioxidant peptides in fermented milk to the lactic bacteria indicating that radical scavengers were related to proteolysis (Hernández-Ledesma *et al*., 2005; Aloğlu and Öner, 2011). In fact, Lin and Yen (1999), demonstrated that lactic acid bacteria (L. *acidophilus,* L. *bulgaricus,* S. *thermophilus, Bifido-bacterium longum)* scavenge reactive oxygen species ($OH^{\cdot}$, H_2O_2) and show reducing activity. Therefore, changes in the profile of nitrogenous compounds in yogurt, compared with milk, were due to the proteolytic activity of *S. thermophilus* and *L. bulgaricus*, both during fermentation. And this change basically induced an increase in the level of soluble nitrogenous compounds, which included the liberation of amino acids and low molecular peptides from the milk proteins (Tamine and Robinson, 1997).

In order to study the effect of the diets (CO, CS and TM) on the antioxidant capacity of yogurt throughout the experimental period, the supernatant of yogurt was extracted by two different solvent (methanol and water). Antioxidant capacity was assayed by using three different methods (ABTS, DPPH and ORAC) as shown in the Table 4.32.

Table 4. 32. Effect of diets on the antioxidant capacity of yogurt

Antioxidant capacity	**Treatments[1]**			**SEM[2]**	***P*<[3]**			
	CO	**CS**	**TM**		**D**	**T**	**S**	**D×T×S**
ABTS (mg Trolox/ 100 g of DWE)	54.64 ^{b}	52.00 ^{c}	62.11 ^{a}	1.126	<0.001	<0.001	<0.001	<0.001
DPPH (mg Trolox/ 100g of DWE)	54.08 ^{b}	54.06 ^{b}	62.41 ^{a}	1.109	<0.001	<0.001	<0.001	0.028
ORAC (µM)	46.48 ^{b}	40.50 ^{C}	52.47 ^{a}	1.661	<0.001	0.145	0.052	<0.001

[1]Refers to treatments: Control diet (CO); Canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by marjoram)

[2]SEM: standard error of least square means

[3]Probability of significant effects due to experimental diets (D), Solvent (S), time (T), and their interaction (D×T×S)

As previously reported by Pérez-Jiménez *et al*. (2008), the solvent in which the reaction takes place is a key factor in the results, since the polarity of the solvent affects the mechanisms of the reaction. Moreover, there is no solvent that would be entirely satisfactory for the extraction of all the antioxidants present in food, especially those associated with complex carbohydrates and proteins (Bravo *et al*., 1994).

As observed in the Table 4.32, the effect of the solvent, on the antioxidant activity, assayed with three different methods (ABTS, DPPH and ORAC), was significant (P<0.05). Methanol was the best solvent for the extraction of the antioxidant compounds in the different yogurts as showed in the Table 4.33.

Table 4. 33. Effect of the extractant solvent on the antioxidant capacity of yogurt

Diets [1]	**Solvent** [2]	**ABTS**	**DPPH**	**ORAC**
CO	Water	46.24 ± 2.22 [b]	44.22 ± 4.13 [b]	47.53 ± 3.75 [a]
	Methanol	62.75 ± 2.83 [a]	63.93 ± 0.18 [a]	45.43 ± 13.62 [a]
CS	Water	42.57 ± 4.25[b]	44.54 ± 3.19 [b]	40.60 ± 5.81 [a]
	Methanol	61.67 ± 1.56 [a]	63.59 ± 1.38 [a]	40.38 ± 5.87 [a]
TM	Water	50.74 ± 8.14 [b]	51.72 ± 8.93 [b]	47.53 ± 6.91 [b]
	Methanol	72.80 ± 8.24 [a]	73.09 ± 7.38 [a]	57.41 ± 9.07 [a]

[1]Refers to treatments: Control diet (CO); Canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by marjoram)

[2]Refers to solvent: water and methanol

[3]Means within a column not followed by the same letter differ (P> 0.05)

The solvent affected more ABTS and DPPH assays than ORAC assay for CO and CS yogurts. And, the difference between methanol and water was highly significant regardless of the method used for TM yogurts.

The effect of the diets was significant since significant differences were found between the different yogurts as showed in the Table 4.32 (P<0.001). Independently, of the method used, TM yogurts had always the highest antioxidant capacity. However, the antioxidant capacity of CO and CS yogurts varied according to the method used. CS yogurts had the lowest antioxidant capacity if assayed with ABTS and ORAC methods whereas no differences were reported between CS and CO if assayed with DPPH method. This might be explained by the fact that ABTS and ORAC are more specific to measure the antioxidant capacity of hydrophilic compounds whereas DPPH has been

routinely applied in both hydrophilic and lipophilic extracts (Pèrez-Jimènez *et al*., 2008). These differences between assays were excepted because the antioxidant activity, measured by an individual assay reflects only the chemical reactivity under the specific conditions applied in that assay (Huang *et al*., 2005). And, this observation was in agreement with many others that recommend the use of different methods of measurement to evaluate the antioxidant capacity in food matrices (Frankel and Meyer, 2000; Pèrez-Jimènez *et al*., 2008; Zulueta *et al*., 2009).

Throughout the experimental period, the antioxidant activity of TM yogurts increased significantly meanwhile the antioxidant capacity of CO and CS yogurts increased or did not change (Table 4.34). The increase showed in TM yogurts might suggest that the time that ewes spent under feeding TM diet was associated to the increase found in the antioxidant capacity.

Table 4. 34. Effect of time on the antioxidant capacity of yogurt

Diets [1]	**Time** [2]	**ABTS**	**DPPH**	**ORAC**
CO	Beginning	52.74 ± 10.27 [b]	51.49 ± 13.78 [b]	45.28 ± 6.10 [b]
	Middle	55.73 ± 8.87 [a]	55.46 ± 9.18 [a]	53.41 ± 10.99 [a]
	End	55.46 ± 8.22 [a]	55.28 ± 9.53 [a]	40.76 ± 8.17 [b]
CS	Beginning	51.52 ± 10.94 [b]	51.65 ± 11.23 [c]	40.98 ± 7.25 [a]
	Middle	55.11 ± 7.81 [a]	53.71 ± 11.06 [b]	38.29 ± 4.17 [a]
	End	49.37± 12.84 [c]	56.82 ± 9.02 [a]	39.96 ± 8.33 [a]
TM	Beginning	52.57 ± 10.24 [c]	47.90 ± 17.20 [c]	49.94 ± 10.92 [b]
	Middle	62.97 ± 16.45 [b]	63.76 ±13.61 [b]	44.44 ± 10.52 [b]
	End	70.80 ± 9.95 [a]	75.57 ±11.30 [a]	63.04 ± 17.25 [a]

[1]Refers to treatments: Control diet (CO); Canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by marjoram)

[2]Refers to time: experimental period (beginning, middle and end)

[3]Means within a column not followed by the same letter differ ($P > 0.05$)

Significant correlations were observed between the different assays used to measure the antioxidant activity of yogurts. DPPH values were highly correlated with the ABTS values (r = 0.744) as shown in the Figure 4.14, which might suggest that the antioxidant capacity measured with ABTS assay could be predicted on the basis of DPPH results. However, it was not possible to correlate the values measured by the DPPH and ABTS

assays with those determined through using the ORAC assay because the units of measure were completely different.

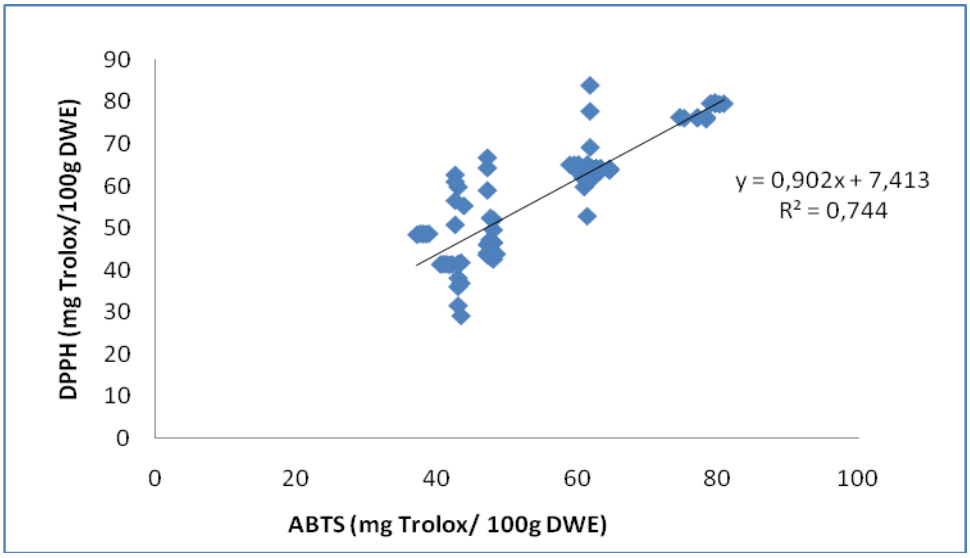


Figure 4. 15. Relationship between the antioxidant capacity as measured by ABTS and DPPH assay

As reported above, the antioxidant capacity of CO and CS yogurts varied according to the method used. Moreover, the correlation between DPPH or ABTS results and ORAC values were not possible due to differences in the unit of measurements. For those reasons, it was necessary to use the relative antioxidant capacity index (RACI) in order to take into account the results of all the assays performed. This index combined the three methods used in this study (DPPH, ABTS and ORAC) with no limitation unit and no variance among methods.

As shown in the Figures 4.15, TM yogurts showed the highest antioxidant activity followed by CO and CS yogurts. The trend of the RACI value matched with the standard score of the 3 three methods. These differences between yogurts might be associated to the antioxidant compounds present in the hydrophilic phase.

The increase of the antioxidant capacity in CO yogurts in comparison with CS yogurts might be related to its high protein content and then higher concentration of bioactive peptides which are widely reported to have an important antioxidant activity. On the other hand, the increase observed in TM yogurts in comparison with CO yogurts may be principally related to the polyphenols present in yogurts, through the inclusion of marjoram in the diet, or to the bioactive peptides present in the yogurt extracts.

However, as can be seen in the Table 4.22 and 4.32, TM yogurts in comparison with CO yogurts, showed the highest antioxidant capacity but similar protein content. This high antioxidant capacity in TM yogurts was probably due to the phenolic compounds present in marjoram. However, the characterization of the phenolic compounds of yogurt extracts showed that the peaks identified corresponded to bioactive peptides or to complexes of polyphenols-peptides.

These observations may suggest that the antioxidant capacity in TM yogurts may be associated to a complex peptide-phenol formed in the yogurt extracts. Similar results were reported by Ye *et al*., (2013), in which the increase of the antioxidant capacity in yogurt, supplemented with hickory and black soybean, was associated to small peptides derived in the fermentation process of yogurt, phenols in hickory and flavonoids in the black soybean.

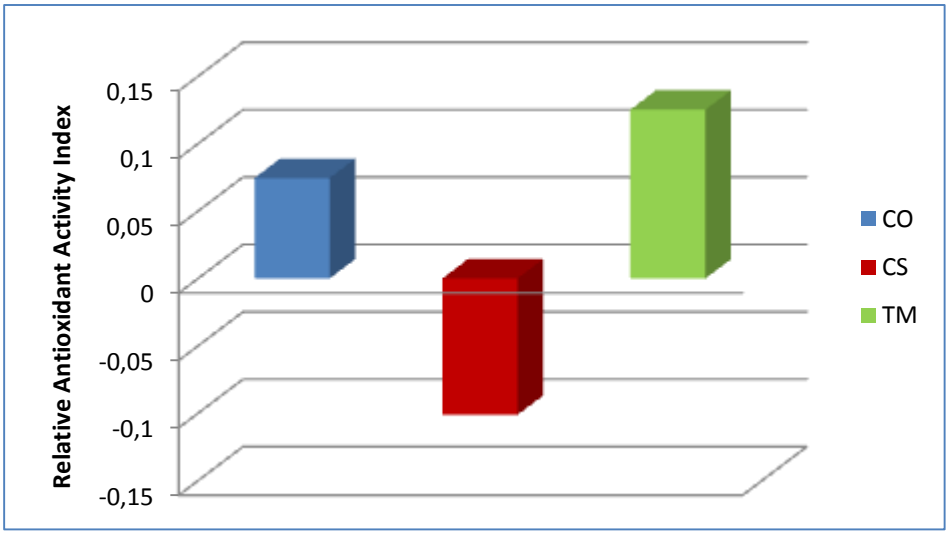


Figure 4. 16. The relative antioxidant capacity index (RACI) according to the feeding treatments

The effect of the extractant solvent was already reported as one of the most important factor that affect the antioxidant capacity in yogurt and the RACI value confirmed this observation as showed in the Figure 4.16.

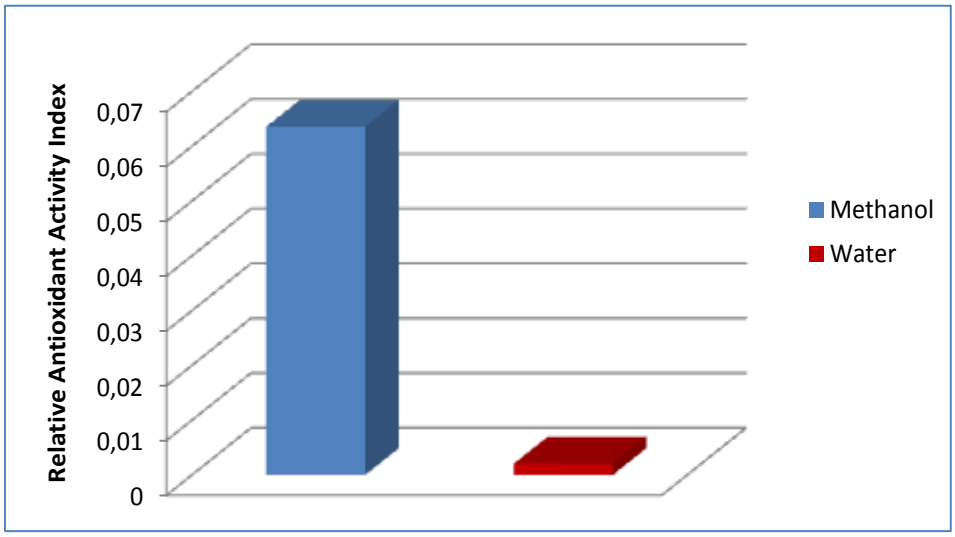


Figure 4. 17. The relative antioxidant capacity index (RACI) according to the extractant solvent

The Figure 4.17 showed the effect of the time on the antioxidant capacity of yogurt, and as can be seen throughout the experimental period, the highest values of antioxidant capacity were observed at the end of period. Similar observation was observed with the three methods reported above (ABTS, DPPH and ORAC).

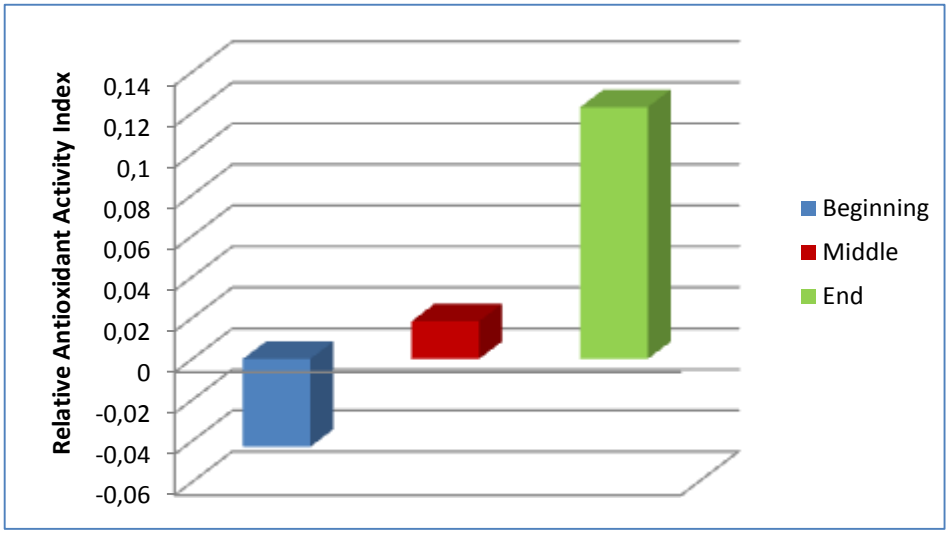


Figure 4. 18. The relative antioxidant capacity index (RACI) throughout the experimental period

4.3.2.2.6.2. Anti-inflammatory capacity of yogurt

The anti-inflammatory capacity in yogurt extracts was measured with the semicarbazide-sensitive amine oxidase (SSAO) inhibitory assay. The extraction was performed by using methanol as extractant solvent, since it was observed above that it was the best for extracting antioxidant compounds. Moreover, the anti-inflammatory capacity was measured in yogurt manufactured at the end of the experimental period because it was shown that the antioxidant capacity was the highest at the end of the period.

As presented in the Table 4.35, significant differences were observed between the different yogurts (CO, CS and TM). The highest anti-inflammatory capacity was observed in TM yogurts in comparison with CO and CS yogurts. No significant difference was reported between CO and CS yogurts.

Table 4. 35. Effects of diets on the anti-inflammatory activity of yogurt (SSAO)

Anti-inflammatory activity capacity	**Treatments[1]**			**SEM[2]**	***P*<[3]**
	CO	**CS**	**TM**		**D**
SSAO (µM of semicarbazide)	34.69 [b]	34.13 [b]	60.53 [a]	2.58	<0.001

[1]Refers to treatments: Control diet (CO); Canola seed (CS); *T. mastichina* (TM) (a substitution of 7.5% of the CS by marjoram)

[2]SEM: standard error of least square means

[3]Probability of significant effects due to experimental diets (D)

The results showed that TM yogurts had significant higher anti-inflammatory activity than CO and CS yogurts, which might suggest that bioactivas compounds in TM yogurts acted as anti-inflammatory compounds and inhibited the SSAO. It is known that SSAO plays a key role in inflammation through its catalytic products, hydrogen peroxide, and reactive aldehydes. Therefore, the inhibition of SSAO activity might represent a therapeutic strategy for controlling inflammation (Lin *et al*., 2008).

There was a positive correlation between SSOA inhibitory activity and ABTS, DPPH and ORAC values (r= 0.942, 0.929, 0.734, respectively) as shown in the Table 4.36. Those results were in agreements with others, in which positive correlation was reported between anti-inflammatory activity and antioxidant capacity (Hossain *et al*., 2010; Trouillas *et al*., 2003).

Table 4. 36. Correlation matrix of antioxidant and anti-inflammatory methods

	SSAO	ABTS	DPPH	ORAC
SSAO	1	_	_	_
ABTS	0.942	1	_	_
DPPH	0.929	0.744	1	_
ORAC	0.734	_	_	1

Chapter 5: Conclusions

5. Conclusions

- The whole plant, *Thymus mastichina,* from wild origin, showed a high antioxidant and anti-inflammatory activity, associated to its high content in phenolic compounds, especially flavonoids, mainly luteolin and quercetin.
- The inclusion of canola seed (2.85%) in the control diet of sheep did not modify the intake, the nutrients digestibility and the nitrogen balance. The substitution of 7.5% of canola seed diet by the whole marjoram plant increased the digestibility of fat.
- The use of the canola seed diet or the marjoram diet did not affect the milk production of the lactating ewes. The dry matter intake decreased significantly with the canola seed diet. And, this effect was more accurate in the case of marjoram diet.
- The milk produced from ewes fed with canola seed diet or marjoram diet showed lower milk fat, total solids and saturated fatty acids content. Both modified diets increased the CLA content in milk in comparison with the control diet, and this increase was higher with marjoram diet. Ewes fed with marjoram diet showed higher level of protein, casein, polyunsaturated fatty acids and phosphatidylethanolamine in milk.
- The milk produced from ewes fed with marjoram diet showed high antioxidant capacity compared with canola seed diet and control diet.
- The cheeses manufactured with the milk of ewes fed with canola seed diet showed lower protein content in comparison with the other cheeses. Meanwhile, the use of marjoram diet increased the protein and decreased the fat and total solids. The lipidic profile of the cheeses manufactured from the milk of ewes fed with both diets was similar to the lipidic profile observed in milk.
- The canola seed diet increased the adhesiveness in cheeses. The marjoram diet reduced hardness in cheeses, which showed less intense yellow colour. Both diets did not affect the sensorial characteristics of cheeses.
- The antioxidant capacity in cheeses was higher than in milk. The cheeses manufactured from the milk of ewes fed with marjoram diet showed higher antioxidant capacity than the other cheeses.

- The yogurts manufactured from the milk of ewes fed with canola seed diet or marjoram diet showed low fat and total solids content. The use of marjoram diet increased the protein content in yogurts. The lipidic profile of yogurts was similar to the lipidic profile observed in milk and cheeses. Furthermore, the CLA content increased during the fermentation in the manufacturing process.
- The firmness and viscosity of yogurts decreased with canola seed diet while increased with marjoram diet, but no changes were showed in the sensorial characteristics. The marjoram diet fed to the ewes increased the lightness in yogurts.

- The antioxidant capacity of yogurt was 2 times higher to the antioxidant capacity of milk. The use of marjoram diet increased the antioxidant and anti-inflammatory capacity in yogurts in comparison with the canola seed and the control diet.

As main conclusion, the present thesis demonstrates that the combination of canola seed and marjoram plant in sheep diet doesn't affect milk yield and induces the production of milk and dairy products with a healthy lipidic profile and high antioxidant and anti-inflammatory activity, which might help to reduce the risk of cardiovascular diseases.

Chapter 6: Resumen

6.1. Introducción

6.1.1. Alimentos funcionales

La principal función de la dieta es aportar los nutrientes necesarios para satisfacer las necesidades nutricionales de las personas. Sin embargo, hoy en día, la ciencia de la nutrición ha evolucionado a partir de conceptos clásicos, como evitar las deficiencias de nutrientes y la suficiencia nutricional básica, a los conceptos de nutrición "positiva" u "optima".

El concepto de alimentos funcionales nació en Japón. En los años 80, las autoridades sanitarias japonesas se dieron cuenta que para controlar los gastos sanitarios, generados por la mayor esperanza de vida de la población anciana, había que garantizar también una mejor calidad de vida (Nagashima *et al*., 2013; Szakály *et al*., 2012). Se introdujo un nuevo concepto de alimentos, que se desarrollaron especialmente para mejorar la salud y reducir el riesgo de contraer enfermedades.

Los alimentos funcionales no han sido definidos hasta el momento por la legislación Europea. Sin embargo, esto no parece ser un obstáculo directo para el desarrollo de este mercado, lo que significa que los consumidores se sienten más atraídas por un mensaje de salud en lugar de la utilización de un término técnico particular. Y como respuesta al creciente interés sobre este tipo de alimentos, las autoridades se centraron en la necesidad de establecer normas y directrices que regulen el desarrollo y la publicidad de dichos alimentos.

Muchos académicos, científicos y organismos reguladores están trabajando para encontrar maneras de establecer una base científica que apoye las alegaciones beneficiosas que se asocian a los componentes funcionales o los alimentos que los contienen. Sería necesario que un marco regulador protegiera a los consumidores de las atribuciones de propiedades falsas o confusas, y que además pudiera responder a las necesidades de la industria en cuanto a innovación en el desarrollo de productos, su comercialización y su promoción. Para que los alimentos funcionales puedan aportar todos los benéficos posibles para la salud pública, los consumidores tienen que comprender bien y confiar en los criterios científicos utilizados para documentar sus efectos y atribuciones beneficiosas.

En la Unión Europea, se han adoptado directivas y procedimientos prácticos para facilitar el uso de las alegaciones de salud. La directiva 1924/2006/EC establece disposiciones específicas relativas al uso de las alegaciones nutricionales y de salud en alimentos que se vayan a proporcionar como tales a los consumidores, y esta directiva complementa los principios generales de la Directiva 2000/13/CE.

Entre los distintos grupos de alimentos funcionales, los productos lácteos tienen un papel creciente y representan el 43% del mercado (Özer and Kirmaci, 2010), debido a que los productos lácteos gozan en general de una imagen "saludable" ante los consumidores.

6.1.2. Antioxidantes

Durante las últimas décadas, la utilización de los antioxidantes en la industria aumentó significativamente (Franz. 2011). La importancia de los antioxidantes se debe a que contribuyen eficientemente en la preservación de los alimentos de la deterioración oxidativa (Souza Cruz *et al*., 2012). Muchos alimentos son muy sensibles al oxígeno, que es responsable de sus deterioros de una manera directa o indirecta. De todas las grasas, los ácidos grasos poli-instaurados son los más susceptibles al deterioro (Boroski *et al*., 2012), lo que afecta las características físico-químicas, nutricionales y sensoriales de los productos (Boroski *et al*., 2012; Petit, 2009). Además, los radicales libres implicados en la oxidación de los poli-insaturados están involucrados en varias enfermedades, incluyendo aterosclerosis y cáncer (Yin and Porter, 2005).

Halliwell and Gutteridge (2007) definen los antioxidantes como “cualquiera substancia que retrasa, previene o remueve los daños oxidativos de las moléculas objetivo”. Además, Alamed *et al*. (2009), define los antioxidantes como compuestos sintéticos o naturales que tienen la habilidad de disminuir la oxidación lipídica. Otros autores definen los antioxidantes como atrapadores de radicales libres que previenen los daños debidos a las especias reactivas de oxigeno (ROS) (Niki *et al*., 2011; Gordon, 2012).

El mecanismo de defensa contra los radicales libres involucra muchos mecanismos; atrapar oxidantes, reparar/ remover daños oxidativos o encapsular daños irreparables. La primera línea de defensa consiste en interferir en la reacción inicial que genera los ROS, la segunda en atrapar los radicales libres para romper la cadena de propagación de las reacciones y la ultima en reparar los daños oxidativos generados (Sgorlon *et al*., 2006; Lobo *et al*., 2010; Lykkesfeldt and Svendsen, 2007).

Los antioxidantes pueden ser clasificados según muchos criterios; naturaleza, propiedades físico-químicas, estructura y mecanismo de acción (Vertuani *et al*., 2004). Sin embargo, el criterio más utilizado es el del origen (natural o sintético).

Los antioxidantes tienen además de la capacidad antioxidante, unas propiedades anti-inflamatorias al poder interferir en los procesos anti-inflamatorios.

Durante el proceso oxidativo, son los lípidos, proteínas y carbohidratos de los alimentos las moléculas que son atacadas por los diferentes radicales libres y por ello están involucrados diferentes mecanismos. La evaluación de las propiedades bioactivas de los alimentos, capacidad antioxidante y anti-inflamatoria, se obtiene utilizando varios métodos, desarrollados, fundamentalmente, durante la última década. La evaluación de la capacidad antioxidante se determina o bien utilizando ensayos basados en la transferencia de electrones o en otros basados en la transferencia de átomos de hidrogeno. Los métodos basados en la transferencia de electrones incluyen FCR (Folin-Ciocalteau), ABTS (2,2' ácido azinobis-3-etil- benzotiazolin-6- sulfónico), FRAP (Ferric Reducing Antioxidant Power) y DPPH (1,1-difenil-2-picrilhidrazil) y otros aunque los más utilizados en alimentos son los tres primeros. Existen varios métodos basados en la transferencia de átomos de hidrógenos pero el más utilizado es el ORAC por la utilización del radical peroxilo, que es el más común en las matrices biológicas. Por otro lado, dado que los métodos utilizados son varios y basados en diferentes reacciones y también pueden estar expresadas en diferentes unidades, la comparación entre ellos se encuentra difícil y de allí la necesidad de calcular el índice de capacidad antioxidante relativa (RACI), que combina los datos de los diferentes ensayaos.

La evaluación de la capacidad anti-inflamatoria se puede evaluar mediante diferentes métodos y uno de ellos es el SSAO (amino oxidasa sensible al semicarbazide), que es un método enzimático cuyos resultados presentan una buena correlación con los datos de la capacidad antioxidante.

6.1.3. La mejorana y la colza

La mejorana *(Thymus mastichina* L.*)* pertenece al género *Thymus* que es uno de los géneros más importantes de la familia Lamiaceae. Es una planta endémica de la península ibérica y está ampliamente distribuida con una excelente adaptación a múltiples ecosistemas (Blanco. 2007). Es una planta aromática y medicinal,

caracterizada por su alto contenido en aceites esenciales y compuestos fenólicos. Los principales componentes de los aceites esenciales son el 1,8-cineol y el linalol, conocidos por sus propiedades terapéuticas (Aggarwal and Kunnumakkara, 2009). Los compuestos fenólicos están presentes en las diferentes partes de la planta. Dentro de los compuestos fenólicos, la clase más amplia es el grupo de los flavonoides, que son conocidos por actividad antioxidante. También, la capacidad antioxidante de los compuestos fenólicos depende de sus estructuras químicas (solubles e insolubles). Estos compuestos actúan dando un átomo de nitrógeno y un electrón que convierte los radicales libres en compuestos inofensivos. Los polifenoles tienen altas propiedades bioactivas y dentro de ellos, los flavonoides son los más potentes y juegan un papel importante en la reducción del desarrollo de enfermedades degenerativas (Pandey and Rizvi, 2009; Dashwood. 2007; Vauzour *et al*., 2010; Mink *et al*., 2007).

La colza (*B. napus* L.) pertenece a la familia Cruciferae y procede de una hibridación natural de la colza, tiene un perfil nutricional diferente caracterizado por un bajo contenido en glucosinolates y ácido erúcico. Durante los últimos diez años, el principal énfasis en la alimentación fue la reducción de la grasa total en las dietas y reemplazar los ácidos grasos saturados y los *trans* por ácidos grasos poli-instaurados para disminuir los riesgos asociados a las enfermedades degenerativas (Christen *et al*., 2013; Erkkilä*et al*., 2008; Guesnet *et al*., 2005; Zampatti. 2013).

6.1.4. Efecto de la dieta sobre los rendimientos animales y la composición de la leche

La modificación de la dieta de los animales con el objetivo de modificar el perfil nutricional y saludable de la leche y de los productos lácteos derivados ha sido el objetivo de varios estudios.

La inclusión de antioxidantes en la dieta se puede hacer de diferentes maneras, como la incorporación de aceites esenciales, extractos de plantas, etc.. Otra manera seria incluir las plantas con alto contenido en antioxidantes en las raciones de los animales con la finalidad de obtener unos productos enriquecidos en estos compuestos. Pocos estudios han investigado el efecto de incluir compuestos fenólicos mediante la utilización de las plantas enteras en la dieta de los animales. Se han alcanzado avances y uno de ellos ha sido el aumento del contenido en proteína de la leche. Este incremento ha sido asociado principalmente a la formación de un complejo proteína-fenol, que permite proteger la

proteína inhibiendo su utilización por los microorganismos del rumen y subsecuentemente mejorar su disponibilidad en la glándula mamaria para producir más leche o una leche con un mayor contenido en proteína.

En cuanto a la inclusión de grasa insaturada en la dieta con el fin de modificar el perfil lipídico hacia una leche más saludable, baja en grasa saturada y menor contenido de ácidos grasos *trans* y con un mayor contenido en grasa poli-insaturada, han sido llevados a cabo varios estudios pero son pocos los realizados en ovejas recibiendo semillas de plantas oleaginosas (Gómez-Cortés *et al*., 2009; Mughetti *et al*., 2012; Zhang *et al*., 2006a; Zhang *et al*., 2006b).

6.1.5. Sector ovino lácteo y características de la leche

Durante las últimas décadas se ha producido un crecimiento importante en el censo ovino mundial, que ha venido a coincidir con un aumento en la producción láctea, principalmente asociado al aumento de la producción individual de leche por oveja, debido a una mejora de los sistemas de producción. En España, el sector ovino juega un papel importante asociado a aspectos culturales y ecológicos y también por su contribución a la vida socio-económica del país. Durante las últimas décadas, el censo ovino ha experimentado unos cambios importantes asociados a la incorporación de España a la unión europea, a la última reforma de la PAC y también a los cambios en los gustos del consumidor y a las subidas de precios de las materias primas y de los tratamientos zoosanitarios (MARM, 2011).

El control de la composición físico-química y microbiológica de la leche es esencial para un buen desarrollo de los productos lácteos. Las leches de las diferentes especies tienen los mismos componentes básicos (carbohidratos, proteína, grasa y minerales). Sin embargo, se han observado importantes diferencias entre las especies (vaca, oveja y cabra). La leche de oveja tiene el más alto contenido en grasa, proteína y solidos totales y también en minerales en comparación con la de vaca y la de cabra (Tamime *et al*., 2011; Barlowska *et al*., 2011; Park *et al*., 2007).

La producción y la calidad de la leche depende de varios factores; unos inherentes al animal como la genética, el estado de lactación o fisiológico y otros que dependen de los factores externos como el sistema de ordeño, la dieta y la época de lactación.

6.1.6. Productos lácteos

Los productos lácteos, quesos y leches fermentadas tienen una elevada aceptabilidad por parte del consumidor ya que han sido generalmente asociados a productos saludables. Por otro lado, los productos lácteos fabricados a partir de leche de oveja juegan un papel importante dado que son fabricados, en parte, de forma tradicional y ayudan a mantener una población y una economía perdurable en las zonas rurales.

Los quesos y los yogures son fuentes de componentes nutritivos con unas propiedades funcionales que van más allá de sus beneficios nutritivos. Algunos de estos componentes pueden reducir los riesgos de enfermedades degenerativas como los péptidos bioactivos que se liberan en el queso durante la maduración y en el yogurt durante la fermentación. En relación con las propiedades organolépticas de estos productos, lo mas importantes son la textura, el aroma y la aceptabilidad, y todas ellas influyen en la decisión de compra por parte del consumidor.

6.2. Materiales y métodos

6.2.1. Evaluación de la mejorana (*T. mastichina* L.) como fuente de antioxidantes naturales

Material vegetal: mejorana

En este estudio se ha utilizado principalmente mejorana cultivada, cuya procedencia eran semillas silvestres y también en una pequeña proporción, mejorana silvestre recolectada directamente del campo. La mejorana cultivada provenía de dos campos de agricultores en la provincia de Valladolid, España (Villabrágima en Ctra. Castromonte y San Pedro Latarce), que han sido pioneros en este cultivo. La mejorana silvestre se recolectó en los alrededores de la Escuela de la Santa Espina (Castromonte, Valladolid). Toda la mejorana utilizada en este estudio se recogió en Junio de 2010, con una altura de planta de unos 40 cm, procediéndose directamente a su secado, a una temperatura de 19.5 ºC en una nave de unos 80 m^2, bien aireada y protegida de la exposición solar. Una vez seca se picó finamente (tamaño ≈ 1 cm) para su envasado, almacenamiento y posterior incorporación en la dieta.

Evaluación de las propiedades bioactivas de la mejorana

Para evaluar las propiedades bioactivas de la mejorana, se procedió a la determinación de su capacidad antioxidante y anti-inflamatoria, mediante la extracción previa de los

compuestos fenólicos de la planta y utilizando varios solventes (agua, etanol y metanol) a través de un proceso de homogenización, agitación, centrifugación, filtración y evaporación.

Para la evaluación de la capacidad antioxidante, se utilizaron dos métodos:

- Determinación del contenido en fenoles totales según el método descrito por Singelton et al. (1999).
- Determinación de la capacidad atrapadora del radical libre DPPH según el método descrito por Sánchez-Moreno (2002).

La evaluación de la capacidad anti-inflamatoria fue llevada a cabo midiendo la actividad inhibidora de SSAO de los extractos de mejorana.

Caracterización de los compuestos fenólicos del *T. mastichina* L.

La caracterización de los polifenoles de *T. mastichina* fue llevada a cabo utilizando la cromatografía líquida-electrospray-espectrometría de masas (LC-ESI-MS/MS). Se realizó previamente una extracción en fase-sólida según el método descrito por García et al. (2004), para una limpieza de muestras antes de la separación en el LC- MS.

6.2.2. Evaluación de los parámetros productivos de las ovejas

Diseño experimental

El experimento se llevo a cabo entre enero y marzo 2011. Todas las prácticas de manejo de los animales siguieron la Directiva del Consejo General Europeo 86/6097EEC y las recomendaciones de la Comisión Europea (2007/526/EC) para la protección de los animales utilizados para propósitos experimentales y otros científicos. Los animales fueron divididos en tres grupos experimentales que recibieron diferentes dietas, que consistieron en una ración completa (TMR), incluyendo melaza para evitar la selección de los componentes y basada en un heno de alfalfa deshidratada y concentrado.

El grupo control (CO) recibió una TMR sin ningún suplemento, el segundo grupo recibió una TMR suplementada con 2.85% de semilla de colza (CS), ambas dietas formuladas para ser iso-energéticas y iso-proteicas. El tercer grupo recibió una ración modificada, substituyendo 7.5% de la ración de CS con mejorana (TM).

Caracterización de la dieta

La composición química de las dietas y sus contenidos en materia seca, materia orgánica, nitrógeno y extracto etéreo fue evaluada siguiendo el método descrito en el AOAC (2006). La fibra neutro detergente y el acido neutro detergente fueron determinados según el método descrito por Goering and Van Soest (1970) y Van Soest et al. (1991), añadiendo el sulfato sódico. La fibra neutro detergente fue analizada con α-amilasa. El contenido medio de las dietas en proteína, fibra neutro detergente, acido neutro detergente y extracto etéreo fue 17.79 ± 0.80, 30.82 ± 1.10, 20.41 ± 1.20 y 4.75 ± 0.42, respectivamente.

Evaluación de los parámetros productivos de las ovejas secas

La evaluación de los rendimientos de las ovejas incluyó dos ensayos; el control de la ingestión individual y del peso vivo, y el control de la digestibilidad de los nutrientes y del balance de nitrógeno. En estos dos ensayos se utilizaron ovejas de raza Assaf no-gestantes y secas, pero el número de animales y las instalaciones utilizadas difieren de un ensayo a otro.

Para el control de la ingestión y del peso vivo se utilizaron treinta ovejas (10 animales en cada grupo). El control de las cantidades ofrecidas y rechazadas se efectuó diariamente durante 5 días consecutivos. Las muestras de alimentos y de los rechazos se secaron a 60º C hasta peso constante y luego se guardaron. El peso vivo se midió al principio y al final del periodo experimental (5dias de intervalo).

Para el control de la digestibilidad de los nutrientes y del balance de nitrógeno, se utilizaron veinticuatro ovejas (8 animales en cada grupo). Durante 5 días consecutivos, en las jaulas de digestibilidad, se controlaron las cantidades consumidas de alimentos y las cantidades generadas de heces. También se procedió a la recolección de las heces, orina y residuos de alimentos, y se guardó una muestra representativa para su posterior análisis.

Evaluación de los parámetros productivos de las ovejas en lactación

Para el control de los rendimientos animales de las ovejas en lactación, se utilizaron treinta ovejas multíparas de raza Assaf en mitad de lactación, divididas en tres grupos homogéneos (12 animales en cada grupo) en función de la producción de leche, el peso vivo y el grado de condición corporal. El experimento duró 4 semanas.

Antes del control de la ingestión, hubo un periodo de adaptación de 10 días, durante el cual las ovejas recibieron la dieta control. Las dietas fueron ofrecidas diariamente *ad libitum* con disponibilidad permanente del agua. La ingestión fue registrada diariamente para cada grupo de ovejas calculando la diferencia entre lo ofrecido y lo rechazado. El peso vivo de cada oveja fue registrado semanalmente.

Las ovejas se ordeñaron diariamente a las 8: 30 y 18: 30 h y la producción individual de cada oveja se registró diariamente en cada ordeño, mañana y tarde.

6.2.3. Caracterización de la leche y de los productos derivados

Diseño experimental

La leche que se utilizó para la fabricación de los productos lácteos fue producida por las ovejas en lactación descritas en el ensayo anterior. La mezcla de leche de cada grupo de ovejas (CO, CS y TM) fue colectada en tres tiempos (principio, mitad y final) de un periodo experimental que duró 4 semanas, y que fue utilizada para fabricar yogures y quesos.

Para la fabricación de yogures se colectaron tres lotes de leche, de cada grupo de ovejas (CO, CS y TM), al principio, mitad y final del periodo experimental con 15 días de intervalo. La leche utilizada provenía del ordeño de la mañana y se necesitaron 5 L de leche para cada fabricación tradicional de yogures.

Se fabricaron 12 quesos del tipo “queso castellano”, dos quesos de cada tipo de leche (CO, CS y TM), al principio y al final del periodo experimental y con un intervalo entre fabricaciones de 20 días. La leche utilizada para las fabricaciones provenía del ordeño de la tarde del día anterior que se guardó a 4ºC y luego se mezcló con la leche del ordeño de la mañana para obtener una cantidad de leche suficiente para las fabricaciones de quesos con cada tipo de leche (se necesitan unos 14 L para fabricar un queso de 2kg). El tiempo de maduración de los quesos fue también considerado (2 y 4 meses). Se analizó un queso a los 2 meses de maduración y otro diferente a los 4 meses. Los datos de los quesos al día 1 de fabricación fueron excluidos dado que el “queso Castellano”, se fabrica con leche cruda y esto hace que, por razones de seguridad, los quesos no se pueden consumir hasta después de 2 meses de maduración.

Caracterización de la leche

Las muestras de la leche de mezcla de cada grupo de ovejas fueron colectadas antes de cada fabricación de quesos o de yogures, y guardadas a -20ºC para su posterior análisis. Las muestras utilizadas en los ensayos de bioactividad fueron liofilizadas.

Se llevaron a cabo una serie de análisis para la evaluación de las propiedades de la leche de mezcla de las ovejas; análisis de la composición físico-química, perfil lipídico y capacidad antioxidante.

En el análisis de la composición físico-química, se determinó el contenido en grasa, proteína, lactosa y sólidos totales con el método de espectrometría infrarroja. El contenido en caseína se analizó según el procedimiento ISO 17997-1: 2004.

Para la caracterización del perfil lipídico de la leche, se llevaron a cabo diferentes extracciones y fueron utilizadas diferentes técnicas cromatográficas. Para el análisis del perfil de ácidos grasos y de los isómeros del ácido linoleico conjugado (CLA), la grasa fue extraída según el procedimiento ISO 14156: 2001 y la metilación se realizó según el procedimiento ISO 15884:2002 y para llevar a cabo el análisis se utilizó cromatografía gas-líquido (GLC) acoplada a espectrometría de masas (MS) y cromatografía líquida de alta eficacia (HPLC) acoplada a un detector UV para ácidos grasos e isómeros del CLA, respectivamente. Par el análisis de los triglicéridos y de los fosfolípidos, se hicieron otras extracciones de la grasa y se utilizó cromatografía de gases para analizar los triglicéridos y cromatografía líquida de alta eficacia (HPLC) acoplada a un detector ELSD para analizar los fosfolípidos.

Para la evaluación de la capacidad antioxidantes de la leche, se procedió a la extracción de los compuestos fenólicos según el método descrito por Redeuil et al. (2009) con algunas modificaciones con respecto al temperatura de extracción.

La capacidad antioxidante de las muestras de leche fue evaluada midiendo la capacidad atrapadora del radical libre ABTS según el método descrito por Chen et al. (2002).

Caracterización de los quesos and yogures

Después de cada fabricación las muestras de yogures y de quesos se guardaron a 4ºC para el análisis organoléptico, y a -20ºC para los análisis físico-químicos y la caracterización del perfil lipídico, y las destinadas a los ensayos de bioactividad fueron liofilizadas.

6.2.3.1.1. Caracterización de los quesos

Durante el proceso de elaboración del queso, se procedió al calentamiento de la leche, luego se añadieron el cloruro cálcico, los fermentos y el cuajo. Una vez producida la coagulación, la cuajada se cortó en cubos y siguió bajo calentamiento progresivo y agitación hasta que hubo sinéresis. Posteriormente, se eliminó el suero y la cuajada se introdujo en moldes para el prensado. El saldo se realizó con la introducción de los quesos en una salmuera. La maduración se llevó a cabo en una cámara frigorífica con unas características determinadas de temperatura y humedad.

Se llevaron a cabo varios análisis para la evaluación de las propiedades de los quesos; análisis de la composición físico-química, perfil lipídico, aspectos organolépticos y capacidad antioxidante. Para los análisis físico-químicos, el contenido en grasa, proteína y sólidos totales se determinaron según los procedimientos ISO 1735: 2004, 8968-1: 2001 y 5534: 2000, respectivamente. El pH fue medido a temperatura ambiente y el contenido en sal fue determinado según el método descrito por Cimiano (1999).

El perfil lipídico del queso incluyó la caracterización del perfil de ácidos grasos y de triglicéridos. La extracción de la grasa del queso se hizo según el procedimiento ISO 14156: 2001, pero la metilación de esta grasa se hizo igual que en la leche. Los métodos cromatografícos de análisis utilizados fueron los mismos descritos para la leche.

La evaluación de los aspectos organolépticos del queso incluyó un análisis del color, de la textura y un análisis sensorial. Los parámetros de color se evaluaron mediante el espacio de color CIELAB con las coordenadas de cromaticidad (a* y b*) y luminosidad (L*). El análisis del perfil de textura (TPA) mediante la determinación de diferentes atributos de manera instrumental (firmeza, adhesividad, elasticidad, cohesividad, pegajosidad y masticabilidad). Las pruebas sensoriales se realizaron con un panel de cata entrenado a través de un test discriminativo basado en pruebas de comparaciones múltiples.

Para la evaluación de la capacidad antioxidantes de los quesos, se realizó la extracción de los compuestos fenólicos según el método descrito por Meira et al. (2012) con algunas modificaciones. Esta capacidad antioxidante fue evaluada midiendo la capacidad atrapadora del radical libre ABTS, igual que para las muestras de leche.

6.2.3.1.2. Caracterización de los yogures

El proceso de elaboración de los yogures consistió en la filtración de la leche, pasteurización, adición del fermento, fermentación y posterior almacenamiento para conservación en cámara fría.

Se realizaron una serie de análisis para evaluar las propiedades de los yogures; análisis físico-químicos y microbiológicos, caracterización del perfil lipídico, análisis de los aspectos organolépticos y determinación de la capacidad antioxidante.

Los análisis físico-químicos para determinar el contenido en grasa y sólidos totales, y la acidez total, se determinaron según los procedimientos ISO 1211: 2010, 13580: 2005 y 11869: 1997, respectivamente. El contenido en proteína y el pH fueron medidos igual que en el queso. El análisis microbiológico de los yogures se realizó únicamente en la primera fabricación de yogures, para determinar su contenido en bacterias lácticas y verificar el cumplimiento de la Norma Española para el yogurt. El control de la viabilidad de la microflora láctica (*Streptococcus thermophilus y Lactobacillus bulgaricus*) se realizó a los días 1, 7 y 14 de fabricación.

El perfil lipídico de los yogures incluyó la caracterización del perfil de ácidos grasos y de triglicéridos. La extracción de la grasa de los yogures, la metilación de esta grasa y los métodos cromatografícos utilizados para analizarla fueron los mismos descritos para la leche.

Las pruebas sensoriales utilizadas para diferenciar ente los yogures fabricados con los tres tipos de leche fueron las mismas que las utilizadas para los quesos.

Para la caracterización de los compuestos fenólicos en los extractos de yogures se utilizó el mismo método descrito para la mejorana. La evaluación de la bioactividad de los yogures se realizó llevando a cabo varios ensayos de capacidad antioxidante y un ensayo de capacidad anti-inflamatoria.

El procedimiento de la extracción de los compuestos fenólicos de los yogures se realizó en dos fases; la primera utilizó el sobrenadante y fue igual al procedimiento descrito en la leche y la segunda se realizó según el método descrito por Serafini et al. (2009). Los ensayos de capacidad antioxidantes incluyeron; la capacidad atrapadora del radical libre DPPH descrita por Sánchez-Moreno (2002), la capacidad atrapadora del radical libre ABTS descrita por Chen et al. (2002) y la capacidad de absorción de radicales de

oxígeno (ORAC) descrita por Huang et al. (2005). La evaluación de la capacidad anti-inflamatoria fue llevada a cabo midiendo la actividad inhibidora de SSAO de los extractos de yogures, igual que en los extractos de mejorana.

6.3. Resultados y discusión

6.3.1. Evaluación de la mejorana (*T. mastichina* L.) como fuente de antioxidantes naturales

Evaluación de las propiedades bioactivas del *T. mastichina* L.

6.3.1.1.1. Contenido en fenoles totales de los extractos de mejorana

El contenido en fenoles totales de los extractos de la planta fue evaluado utilizando diferentes solventes (agua, etanol y metanol). Se observó que las extracciones con metanol tenían un contenido en fenoles totales significativamente más elevado con respecto a las demás extracciones (etanol y agua). Además, comparando estos resultados con valores en otros estudios, donde se utilizó el metanol para las extracciones, se apreció que los valores actuales fueron más elevados. Este incremento se asoció principalmente al origen silvestre de la planta, y también al hecho que las muestras procedían de la planta entera y no únicamente de partes de la planta, como flores, hojas o tallos.

6.3.1.1.2. Capacidad atrapadora del radical libre DPPH de los extractos de mejorana

La evaluación de la capacidad atrapadora del radical libre DPPH de los extractos de mejorana reveló que las extracciones con metanol tenían los valores más elevados con respecto a las extracciones con agua y con etanol ($P<0.05$). La capacidad antioxidante de los diferentes extractos sigue un patrón que depende de la concentración utilizada. Se observó que la concentración adecuada para comparar las diferentes extracciones fue 0,03 mg/mL.

Por otro lado, y similarmente a los resultados observados en el contenido de fenoles totales, la mejorana tenía una capacidad antioxidante más alta que otras plantas y esto fue asociado a diferencias entre estudios como el hecho de utilizar la planta entera y no partes de la planta para el análisis. De hecho una correlación significativa se observó entre los resultados del contenido en fenoles totales y los valores de la capacidad

antioxidantes debido a que los extractos con alto contenido en polifenoles suelen exhibir una alta capacidad antioxidante.

Además, la alta capacidad antioxidante en los extractos de mejorana se asoció a sus elevadas concentraciones en flavonoides (luteolina, quercetina, apigenina) y en otros quimiotipos como el 1,8cineol, dado sus elevadas propiedades antioxidantes.

De otro parte, se observó que el efecto del solvente fue significativo en la correlación entre el contenido en fenoles totales y la capacidad antioxidante cuando se incluyeron los datos de las extracciones con agua y metanol excluyendo los datos de las extracciones con etanol.

6.3.1.1.3. La actividad inhibidora de SSAO de los extractos de mejorana

La capacidad anti-inflamatorio de los extractos de mejorana se evaluó a través de la determinación de la actividad inhibidora de SSAO. La capacidad inhibidora de los extractos de mejorana, extraídos con agua, fue comparada a la actividad del semicarbazide para poder cuantificar sus actividades inhibidoras.

La actividad inhibidora de SSAO de los extractos de mejorana sigue un patrón que depende de la concentración utilizada. Se observó que la mejorana tenía una elevada capacidad anti-inflamatoria, dado su alto poder inhibidor de SSAO, que se asoció principalmente al hecho de que los análisis se efectuaron en la planta entera y no en compuestos aislados. Este resultado fue en acorde con un estudio que utilizó otro método para medir la capacidad anti-inflamatoria del *T. mastichina*, y que observó que la capacidad anti-inflamatoria del *T. mastichina* fue superior a la de otras especies pertenecientes a la misma familia (*Lamiaceae*).

Caracterización de los compuestos fenólicos del *T. mastichina* L.

La caracterización de los polifenoles en *T. mastichina* fue llevada a cabo utilizando la cromatografía líquida-electrospray-espectrometría de masas (LC-ESI-MS/MS). Para la caracterización, se utilizaron extractos de planta preparados con metanol, dado que los valores más elevados de contenido en fenoles totales y de capacidad antioxidante se obtuvieron en extractos de metanol.

Se identificaron dieciocho polifenoles durante el análisis cromatografíco y la mayoría fueron flavonoides (diecisiete), probablemente debido a que se utilizó una extracción en fase solida durante la preparación de las muestras. De hecho, el único acido fenólico

identificado fue el ácido rosmarínico. La mayoría de estos flavonoides se presentaron enlazados a compuestos glucósidos o glucurónidos.

La cantidad de cada compuesto fue expresada en µg kaempferol/mg de extracto seco. Los compuestos más abundantes identificados fueron en un orden decreciente; luteolina-7-O-glucosida, quercetina glucosida y luteolina. Además, apigenina, narginina y eriodictiol estuvieron presentes en elevadas cantidades. La presencia elevada de luteolina, quercetina y apigenina en los extractos de mejorana fue acorde a otros estudios realizados con varias plantas aromáticas. Sin embargo, el ácido rosmarínico que suele ser el compuesto más importante en plantas de la misma familia (*Lamiaceae*), estuvo presente en gran cantidad pero no como compuesto principal.

Optimización de la extracción y de la producción de mejorana

Se estudió la eficiencia de los diferentes solventes (agua, etanol y metanol) con respecto a la extracción de los polifenoles de la mejorana. La producción varió significativamente según el solvente utilizado para la extracción y se observó que el metanol fue el solvente más eficaz para la extracción de los compuestos fenólicos en comparación con extractos de agua y etanol

6.3.2. Evaluación de los rendimientos animales y de lactación de las ovejas

Evaluación de los rendimientos animales de las ovejas secas

6.3.2.1.1. Ingestión y peso vivo

Se observaron diferencias significativas entre los diferentes grupos de ovejas en relación con la dieta recibida (CO, CS y TM) con respecto a la materia seca ingerida y al peso medio. Las ovejas que recibieron la dieta con mejorana tenían el peso vivo medio más bajo mientras que no se observaron diferencias en el peso entre las ovejas que recibieron la dieta con colza y las que recibieron la dieta control. Por otro lado, el grupo que recibió una dieta con colza tenía una ingestión individual más alta que el grupo que recibió la dieta con mejorana y una ingestión no significativamente distinta que el grupo control. Sin embargo, cuando la ingestión se expresó en g/kg de peso vivo, las diferencias entre grupos no fueron significativas y esto supone que una parte de las diferencias observadas en la ingestión se puede asociar a las diferencias observadas en el peso de los animales.

6.3.2.1.2. Digestibilidad y balance de nitrógeno

El efecto de la dieta fue significativo con respecto a la digestibilidad de los nutrientes (materia seca, proteína bruta, fibra neutro detergente, fibra acido detergente y grasa) y balance de nitrógeno (nitrógeno retenido). No se observaron diferencias significativas entre las ovejas que recibieron la dieta control y las que recibieron la dieta con colza, y esto puede ser debido a la similar composición de nutrientes en ambas raciones por el porcentaje de colza incluido en la dieta (2.85%).

Por otro lado, la inclusión de la mejorana en la dieta con colza, afectó significativamente la digestibilidad de todos los nutrientes. No obstante, no se observaron diferencias significativas en el balance de nitrógeno. Se observó una reducción de la digestibilidad de la materia seca, proteína bruta, fibra neutro detergente y fibra acido detergente. Esta reducción puede ser asociada al mayor contenido en fibra de la dieta con mejorana y también a la inhibición de los protozoarios, bacterias celulolíticas y hongos en el rumen como resultado de la inclusión de compuestos antioxidantes en la dieta. Además, los poli-fenoles pueden unirse a macromoléculas como los carbohidratos, las proteínas alimentarias y las enzimas digestivas, lo que podría reducir su digestibilidad.

Sin embargo, la digestibilidad de la grasa del grupo alimentado con mejorana (TM) aumentó significativamente con respecto a los grupos alimentados con las otras dos dietas (CO y CS). Este incremento puede ser debido a los compuestos fenólicos que actuaron como atrapadores de radicales libres y quelantes de metales, responsables de catalizar la peroxidación lipídica, y de esta manera pudieron preservar los lípidos de la oxidación e hicieron que fueran más disponibles para los microorganismos del rumen. La digestibilidad de la grasa, que disminuyó debido a la inclusión de la colza en la dieta, se mejoró con la inclusión de la mejorana.

Evaluación de los rendimientos animales de las ovejas en lactación.

6.3.2.1.3. Ingestión y peso vivo

Las diferencias en la materia seca ingerida entre los grupos de ovejas fueron significativas. Se observó que el grupo control tuvo el valor de ingestión más alto (P>0.05). La inclusión de colza en la dieta control disminuyó la ingestión de materia seca de las ovejas, y esta reducción puede estar asociada a la cantidad de ácidos grasos en la dieta y también a su grado de instauración. No obstante, la materia seca ingerida

del grupo de ovejas que recibió la mejorana fue la más baja con respecto a los demás grupos y esto durante todo el periodo experimental. Esta disminución puede ser debida al alto contenido en fibra de la ración con mejorana.

El peso vivo de las ovejas en lactación que recibieron diferentes dietas (CO, CS y TM) no varió significativamente durante el periodo experimental.

6.3.2.1.4. Producción de leche

El efecto de la modificación de la dieta con la inclusión de la colza o de la mejorana no afectó significativamente la producción de leche de las ovejas durante todo el periodo experimental. Este resultado puede estar asociado al hecho de que un aumento de la producción de leche está más asociado a la cantidad de energía en la dieta y al estado energético de las ovejas que al cambio del perfil lipídico de la dieta. El incremento de la producción de leche estuvo también asociada a una mejor ingestión de materia seca y una mayor digestibilidad de la materia seca y de la fibra. No obstante, esto no fue el caso en este estudio dado que las ovejas con mejorana produjeron un cantidad de leche, similar a los demás grupos, ingiriendo menos materia seca y teniendo la más baja digestibilidad de materia seca y de fibra, y esto puede ser debido a que los antioxidantes presentes en la mejorana pudieron mejorar el estado antioxidativo de las ovejas. Además, la movilización de reservas corporales reflejadas en los cambios de peso vivo pueden ayudar a explicar la no diferencias en producción de leche a pesar de las diferencias en digestibilidad.

6.3.3. Caracterización de le leche y de los productos derivados

Caracterización de la leche

6.3.3.1.1. Composición físico-química de la leche

La composición fisicoquímica de la leche de cada grupo de ovejas (CO, CS y TM) fue evaluada durante el periodo experimental (inicio, mitad y final). Las diferencias entre leches (CO, CS y TM) fueron significativas para todos los parámetros fisicoquímicos a excepción del contenido en lactosa. La leche proveniente de las ovejas que recibieron colza en la dieta tuvo menor contenido en grasa, proteína y sólidos totales que la procedente del grupo de ovejas que recibieron la dieta control. La inclusión de la mejorana (TM) en la ración con colza (CS), causó una disminución significativa del contenido en grasa y sólidos totales en la leche. Sin embargo, el mayor contenido en proteína y caseína se observó en la leche procedente de las ovejas que recibieron la dieta

con mejorana (TM). El contenido en lactosa no varió significativamente entre dietas (P>0.05). El efecto del tiempo fue también significativo para todos los parámetros medidos en la leche a excepción de la lactosa.

La disminución del contenido en grasa en la leche de las ovejas que consumieron la ración con colza puede ser debido a varios factores; la composición de la dieta que era baja en fibra con alto contenido en carbohidratos rápidamente fermentables, el nivel de ácidos grasos insaturados en la dieta, y el uso de una semilla de colza extrusionada. Todos estos factores pueden inducir la inhibición de la síntesis *de novo* de la leche. En cuanto a la disminución del contenido en proteína, la razón puede ser la disponibilidad insuficiente de los aminos ácidos necesarios para la síntesis de la leche en la glándula mamaria o cambios en los metabolismos de glucosa e insulina debido a la inclusión de grasa en la dieta.

La disminución del contenido en grasa en la leche con mejorana fue superior a la reducción observada en la leche con colza y esto puede estar asociado al alto contenido en grasa poli-insaturada en la dieta con mejorana debido a que los antioxidantes naturales pueden proteger los ácidos grasos insaturados de la oxidación. Sin embargo, el contenido en proteína y caseína aumentó significativamente y este incremento puede estar relacionado con el hecho que los compuestos fenólicos pueden formar complejos con la proteína reduciendo entonces su degradabilidad en el rumen hasta llegar al abomaso donde este complejo se rompe para liberar la proteína que vuelve a estar de nuevo disponible para ser utilizada por el rumiante para la síntesis de la leche.

6.3.3.1.2. Perfil lipídico de la leche

Las diferencias en el perfil de ácidos grasos fueron significativas entre las diferentes leches (CO, CS and TM). La modificación de la dieta al incluir colza o mejorana modificó el perfil de ácidos grasos de la leche. Se observó una disminución del contenido en ácidos grasos saturados ¿en cual tratamiento?. Esta reducción puede estar asociada a la presencia de ácidos grasos de cadena larga (≥18 átomos de carbono), proveniente de la dieta o de la movilización de las reservas corporales, que inhibieron la síntesis de la acetil-CoA carboxilasa y entonces redujeron la síntesis *de novo* en la glándula mamaria. Igualmente, el nivel de los ácidos grasos poli-insaturados disminuyó significativamente en la leche con colza, probablemente debido a la composición de la dieta con colza (CS) que tiene un contenido bajo en ácido linoleico con respecto a la

dieta control (CO). Sin embargo, la inclusión de la mejorana en la dieta con colza aumentó significativamente el contenido en ácidos grasos poli-insaturados de la leche, un incremento probablemente debido al efecto de la mejorana como antioxidante natural, capaz de proteger los ácidos grasos insaturados de la oxidación antes de su absorción.

El contenido en acido vacénico de las leches con colza y con mejorana aumentó significativamente con respecto a la leche control. Este incremento fue debido a un nivel alto de concentrado y de grasas insaturadas en la dietas. Además, la formación del *trans*-11 C18:1 depende de la acumulación total de los *trans* C18:1 en el rumen, con mínimas desviaciones hacia *trans*-10 C18:1. Se apreció siempre una correlación positiva entre el contenido en acido vacénico y la disminución en el contenido de grasa en la leche. El ácido ruménico *cis*-9, *trans*-11 CLA, el isómero más importante del ácido linoleico conjugado, siguió el patrón observado por el ácido vacénico, debido a que este ácido es el precursor del ácido ruménico en el rumen. Así se observó un incremento significativo en las leches con mejorana y con colza con respecto al control.

La inclusión de la colza en la dieta control no afectó significativamente el perfil de triglicéridos de la leche. No obstante, el hecho de introducir mejorana en la dieta aumentó el contenido en triglicéridos de cadenas medias y largas ($P<0.05$), debido a que la mayoría de los ácidos grasos C18 mono y di-insaturados se esterifican en triglicéridos con un numero de carbonos de C50 a C54.

El perfil de fosfolípidos en la leche no cambió con la dieta modificada por la inclusión de la colza pero se modificó significativamente con la dieta con mejorana. El contenido en esfingomielina (SM) de la leche disminuyó mientras que el contenido en fosfatidiletanolamina (PE) se incrementó. Este aumento puede ser debido a que la fosfatidiletanolamina, siendo altamente insaturada, es propensa a la oxidación y por tanto puede beneficiar más el efecto antioxidante de la mejorana.

6.3.3.1.3. Capacidad antioxidante de la leche

La capacidad antioxidante de la leche producida por las ovejas que consumieron diferentes raciones (CO, CS y TM) fue determinada en la fracción hidrofílica mediante el método TEAC. Las dietas que recibieron las ovejas afectaron significativamente la capacidad antioxidante de las leches. El solvente utilizado para la extracción de los compuestos fenólicos no modificó los resultados de la capacidad antioxidante.

El hecho de incluir colza en la dieta de ovejas disminuyó significativamente la capacidad antioxidante de la leche. Sin embargo, la inclusión de mejorana incrementó la capacidad antioxidante. El aumento que se observó en la leche con mejorana se puede asociar a los compuestos fenólicos que están presentes en la dieta.

Caracterización de los quesos

6.3.3.1.4. Composición físico-química de los quesos

La composición físico-química de los quesos varió significativamente según las dietas que recibieron las ovejas (CO, CS y TM). Durante el periodo experimental (inicio y final) las modificaciones en los parámetros fisicoquímicos de los quesos siguieron el mismo patrón que el observado en la composición de la leche.

Los parámetros físico-químicos de los quesos, fabricados a partir de la leche que proviene de las ovejas que recibieron colza en la dieta, eran similares a los valores observados en los quesos control a excepción de su contenido en proteína. Sin embargo, la ración con mejorana modificó significativamente la composición físico-química de los quesos siguiendo el mismo patrón que se observó en la leche; un bajo contenido en grasa y sólidos totales y un alto contenido en proteína. Además, los quesos elaborados con la leche de las ovejas que consumieron mejorana tenían un menor valor de pH, debido a la inhibición del crecimiento de las bacterias lácticas en el queso por el cloruro de sodio.

El tiempo de maduración no cambió los parámetros físico-químicos de los quesos (grasa, proteína, humedad), y esto puede implicar que no hubo proteólisis entre los 2 y 4 meses de maduración.

6.3.3.1.5. Perfil lipídico de los quesos

El efecto de las dietas sobre el perfil de los ácidos grasos y de los triglicéridos en los quesos fue similar al efecto observado en la leche. La inclusión de la colza en la ración de las ovejas disminuyó el contenido en ácidos grasos saturados y poli-insaturados en los quesos elaborados con la leche producida. No obstante, el efecto de la mejorana fue disminuir los ácidos grasos saturados y aumentar los poli-insaturados. No se encontraron diferencias entre leches y quesos con respecto al contenido en ácido ruménico. Se observaron modificaciones en el perfil de los triglicéridos, el contenido en triglicéridos de cadena corta disminuyó significativamente en los quesos elaborados con leche de ovejas que recibieron colza, mientras que el contenido en triglicéridos de

cadena media y larga aumentó en los quesos elaborados con la leche procedente de las ovejas que tenían mejorana en la ración. Esto permite concluir que el proceso de fabricación no afectó al perfil lipídico de las leches.

Por otro lado, el tiempo de maduración no afectó significativamente el perfil de ácidos grasos y de triglicéridos en los quesos.

6.3.3.1.6. Aspectos organolépticos de los quesos

Los aspectos organolépticos de los quesos se vieron afectados significativamente por las dietas que recibieron las ovejas. Los parámetros de color en los quesos se vieron significativamente afectados por las dietas (CO, CS y TM) recibidas por las ovejas. Los valores de L* y b* no estuvieron influidos por la inclusión de la colza en la dieta, lo que coincide con una estructura (contenido en grasa y en solidos totales) similar entre los quesos elaborados con la leche procedente de las ovejas que tenián colza en su ración y los de los procedente de ovejas que recibieron la dieta control. Sin embargo, se observó que los quesos elaborados con la leche de ovejas que recibieron una ración con mejorana tuvieron los valores más altos de L* y los valores más bajos de b*, lo que supone que estos quesos fueron más luminosos y menos amarillos con respecto a los demás quesos (CO y CS). Esta diferencia en el parámetro b* se puede asociar principalmente al menor contenido en grasa de los quesos con mejorana. El tiempo de maduración no modificó los parámetros de color en los quesos dado que las variaciones en color son principalmente debidas a variaciones en la composición físico-químicas y que estas últimas no fueron significativas durante la maduración.

La textura de los quesos se encontró afectada por las modificaciones en la dieta de las ovejas. Se observaron cambios significativos en la firmeza, adhesividad, pegajosidad y masticabilidad según la dieta recibida por las ovejas. Los quesos procedentes de la ovejas con la ración control y los de la ración con colza tenían una firmeza similar. No obstante, la textura menos firme se observó en los quesos procedentes de las ovejas que recibieron mejorana y esto se puede asociar a varios factores como el contenido en grasa, el contenido en grasa insaturada y el índice *cis*-9 C18:1/C16:0. Además, existe una correlación positiva entre el pH y la firmeza del queso lo que está en concordancia con los resultados observados en este estudio. La masticabilidad es el parámetro que combina los diferentes atributos de textura para caracterizar un queso. Los quesos procedentes de las ovejas que recibieron mejorana en su dieta presentaron el valor más

bajo de masticabilidad con respecto a los quesos procedentes de la ovejas de los tratamientos control y con colza, lo que indica que estos quesos necesitan menos fuerza de masticación para romperse.

El análisis sensorial reveló que la modificación de la dieta de las ovejas al incluir colza o mejorana no afectó las características organolépticas de los quesos de manera que un panel de cata entrenado no fue capaz de diferenciar los quesos elaborados a partir de la leche de cada tratamiento alimenticio de las ovejas.

La capacidad antioxidante de los quesos

La capacidad antioxidante de los quesos elaborados a partir de la leche obtenida de los distintos tratamientos alimenticios (CO, CS y TM) fue determinada en la fracción hidrofílica mediante el método TEAC. Las dietas de las ovejas afectaron significativamente la capacidad antioxidante de los quesos (P<0.05). El solvente utilizado para la extracción y el tiempo de maduración no modificaron los resultados de la capacidad antioxidante.

Por otro lado, la capacidad antioxidante en los quesos fue superior a la capacidad antioxidante observada en las leches. Este incremento puede estar asociado a los péptidos bioactivos producidos, durante el proceso de fabricación a través de la hidrólisis de la caseína de la leche (α_{s1}-, α_{s2}-, β- and κ- caseínas) por las enzimas en cuajo (especialmente plasmina) o durante la proteólisis por las enzimas que liberan los microorganismos.

La capacidad antioxidante de los quesos siguió el mismo patrón que la capacidad antioxidante en las leches. Con la inclusión de la colza en la dieta, se obtuvo un queso con una capacidad antioxidante inferior a la del queso fabricado con la leche control. No obstante, el queso fabricado a partir de la leche que provenía de las ovejas que comieron mejorana tenía la capacidad antioxidante más alta. Esta diferencia entre quesos puede estar asociada al contenido en proteína y caseína de los quesos, a su contenido en compuestos fenólicos o los dos factores combinados, al igual que en la leche.

Caracterización de los yogures

6.3.3.1.7. Composición físico-química de los yogures

La composición físico-química de la leche estuvo significativamente afectada por las modificaciones efectuadas en la dieta de las ovejas. Las modificaciones observadas en

los yogures durante el periodo experimental (inicio, medio y final) siguieron el mismo patrón que el ya comentado para la leche.

Los yogures fabricados a partir de la leche de ovejas alimentadas con colza tenían una composición diferente a los yogures control, con un contenido más bajo en grasa, proteína y sólidos totales. Los yogures fabricados a partir de una leche con mejorana se caracterizaron por tener un contenido bajo en grasa y sólidos totales pero un alto contenido en proteína. Las diferencias que se observaron entre las leches se mantuvieron en los yogures. Los cambios en los parámetros fisicoquímicos que se observaron entre yogures, se asociaron a las dietas a través de las modificaciones producidas en las leches. El proceso de transformación de la leche en yogur aumentó el contenido de todos los parámetros físico-químicos en el yogur a excepción de la grasa en los yogures con mejorana. Esta disminución puede ser debida al hecho que las bacterias lácticas pueden consumir grasa para la producción de compuestos aromáticos durante la fermentación.

El pH no varió significativamente entre los yogures procedentes de la leche obtenida con distintas raciones (CO, CS y TM); sin embargo, los yogures procedentes de la dieta control tenían una acidez total más elevada que los yogures procedentes de los tratamientos con colza o con mejorana. Esta diferencia puede estar asociada al contenido en sólidos totales que puede influir en la capacidad tampón, y en consecuencia, en la producción de ácido, que es acorde con los resultados observado en los yogures del tratamiento control, dado que tienen el más alto contenido en sólidos totales. Otro factor puede ser la tasa de crecimiento de los micro-organismos y sus capacidades para producir más ácido láctico, como en el caso de los yogures procedentes del tratamiento con mejorana que tenían el más alto recuento de *L. bulgaricus*, al día 1 de fabricación, lo que permitió que tuviera la misma acidez total que los yogures procedente de las ovejas que tenían colza en su ración, a pesar de tener menos contenido en sólidos totales.

6.3.3.1.8. Análisis microbiológicos de los yogures

El recuento de *Streptococcus thermophilus* (ST) y de *Lactobacillus bulgaricus* (LB) en los yogures no estuvo afectado por las dietas recibidas por las ovejas (CO, CS y TM). No obstante, se observaron diferencias significativas en el recuento *L. bulgaricus*

durante el periodo de almacenamiento (28 días). Esta diferencia no fue significativa en el caso de *S. thermophilus*.

El hecho de que no haya cambios en el recuento de bacterias lácticas en los yogures confirmó que la inclusión de colza o de mejorana en la ración de las ovejas no afectó el crecimiento total de las bacterias. Pero un estudio de la evolución del crecimiento de estas bacterias durante 28 días de almacenamiento, mostró que sí hubo modificaciones en el recuento de *L. bulgaricus.*

Independientemente de la dieta, se observó una disminución en el recuento de *L. bulgaricus* durante el periodo de almacenamiento, debido a su alta sensibilidad a las bajas temperaturas. No obstante, esta disminución fue únicamente significativa en el caso de yogures procedentes de la leche obtenida del tratamiento con mejorana, lo que significa que la substitución de 7.5% con mejorana en la dieta de colza, afectó significativamente el crecimiento de los *L. bulgaricus* a los 28 días de fabricación

6.3.3.1.9. Perfil lipídico de los yogures

El perfil de ácidos grasos y el perfil de triglicéridos de los yogures estuvieron influidos por las modificaciones en la dieta de las ovejas (CO, CS and TM). Las diferencias observadas en los yogures fueron asociadas a las diferencias encontradas en la leche, dado que la mayoría de los ácidos grasos y triglicéridos en yogures siguieron el mismo patrón que se observó en la leche. Este último implica que las razones de estas diferencias entre los grupos de yogures fueron las mismas que las descritas para la leche. El contenido en ácidos grasos poli-insaturados disminuyó en los yogures del tratamiento con colza con respecto a los yogures del grupo control. Sin embargo, la reducción en los ácidos grasos saturados no fue significativa, contrariamente a lo que se observó en la leche. No obstante, los yogures con mejorana tuvieron un perfil de ácidos grasos muy similar al perfil descrito en la leche.

La única diferencia entre el perfil de la leche y el perfil de los yogures fue con respecto al contenido en ácido ruménico *cis*-9, *trans*-11 CLA, que aumentó significativamente ente leche y yogurt, independientemente de la dieta con la que se alimentó a las ovejas. Este incremento puede estar asociado a varios factores durante el proceso de fabricación y de fermentación como el tratamiento térmico, los cultivos de bacterias y el almacenamiento.

6.3.3.1.10. Aspectos organolépticos de los yogures

La modificación en la dieta de las ovejas dio lugar a diferentes cambios en los aspectos organolépticos de los yogures. Se observaron diferencias significativas entre yogures con respecto a los parámetros de color (L*, b* y a*). Están diferencias se manifestaron también durante el periodo experimental.

Los yogures elaborados con leche derivada de la dieta con mejorana tenían significativamente ¿ más luminosidad y menos color amarillo, en comparación con los yogures procedentes de los otros dos tratamientos (CO y CS), debido probablemente al menor contenido en grasa. El parámetro a* presentó valores relativamente bajos siendo el valor más alto en los yogures con leches derivadas de la dieta control. En general existen correlaciones ente los parámetros de color y los parámetros fisicoquímicos como en el caso del parámetro b* y el contenido en grasa. Sin embargo, en el caso del parámetro a* no se encontraron correlaciones estadísticamente significativas.

La mayoría de los atributos de textura (firmeza y viscosidad aparente) estuvieron influidos por las dietas que recibieron las ovejas, no obstante, las diferencias no fueron significativas en el caso de la adhesividad. Los yogures procedente de la dieta de las ovejas con colza tenían la textura menos firme y menos viscosa. Los yogures con mejorana tenían una textura similar a la de los yogures control (P>0.05). Estas diferencias en textura se asociaron principalmente a la composición fisicoquímica de la leche de origen. Los yogures con mayor firmeza y viscosidad tuvieron también el más alto contenido en proteína. Esto supone una correlación positiva entre estos dos parámetros, debido a que las interacciones entre las micelas de caseína que forman agregados afectan la firmeza y hacen que la textura del gel sea más rígida. La firmeza y la adhesividad de los yogures disminuyeron durante el periodo experimental. Sin embargo, las modificaciones en la viscosidad aparente no fueron significativas.

En el análisis sensorial no se apreciaron diferencias significativas entre los yogures independientemente de la dieta que recibieron las ovejas para producir los diferentes tipos de leche.

6.3.3.1.11. Caracterización de los compuestos fenólicos de los yogures

La caracterización de los compuestos fenólicos se realizó utilizando la cromatografía líquida-espectrometría de masas (LC-MS), un método igualmente utilizado para

caracterizar los polifenoles en los extractos de mejorana. El objetivo fue tratar de verificar si hubo traspaso de los compuestos fenólicos de la planta a los yogures.

Comparando los resultados cromatografícos de los extractos de yogures con los observados en los extractos de planta, se pudo deducir que los compuestos identificados no fueron compuestos fenólicos. Por otro lado, dado que el análisis se hizo en el sobrenadante de los yogures, utilizando únicamente la fracción hidrofílica, y basándose en el índice masa/carga, se pudo concluir que los compuestos identificados correspondieron a péptidos bioactivos o a complejos formados por péptidos y fenoles, En general, los compuestos fenólicos aparecen unidos a otros compuestos, como los glicosilados, como en el caso de la planta, y pocos estudios investigaron las interacciones que hay entre fenoles y proteínas en productos lácteos.

6.3.3.1.12. Propiedades bioactivas de los yogures

La evaluación de las propiedades bioactivas de los yogures engloba el análisis de la capacidad antioxidante y de la capacidad anti-inflamatoria de los diferentes yogures con respecto a la dieta utilzada en la alimentación de las ovejas.

La capacidad antioxidante se midió en la matriz entera y en el sobrenadante para evaluar las diferentes fracciones (hidrofílica y lipofílica). Además, se evaluó el efecto del solvente (metanol y agua) utilizado para la extracción de los compuestos fenólicos de los extractos de yogures. Estos factores de variación se evaluaron en los diferentes yogures (CO, CS y TM).

La capacidad antioxidante de los yogures medida en el sobrenadante fue significativamente mayor que en la matriz entera. Esta diferencia puede estar asociada a las interacciones que pueden existir entre los compuestos fenólicos, lipídicos y proteicos y que pueden afectar a la capacidad neutralizadora de radicales libres de los poli-fenoles.

Las diferencias entre los yogures fabricados a partir de las leches que provienen de las ovejas alimentadas con diferentes dietas (CO, CS y TM) fueron significativas ($P<0.05$). Se observó que los yogures del tratamiento con mejorana tenían la más elevada capacidad antioxidante en comparación con los yogures del tratamiento con colza. Sin embargo, la capacidad antioxidante de los yogures del tratamiento control fue similar al valor observado en los yogures con mejorana ($P>0.05$). Se observó una correlación positiva entre los resultados de la capacidad antioxidante y los valores observados para

el contenido en ácidos grasos poli-insaturados. No obstante, no se apreció una relación significativa entre el contenido en grasa y los valores de la capacidad antioxidante.

Por otra parte, se observó una correlación positiva entre los resultados de la capacidad antioxidante y los resultados del contenido en proteína de los yogures. Por consiguiente, fue necesario realizar un estudio más extenso de la fracción hidrofílica para detectar los componentes responsables de este incremento en la capacidad antioxidante. Para evaluar esta capacidad antioxidante, se utilizaron diferente métodos (ABTS, DPPH y ORAC).

En la fracción hidrofílica y con respecto a la leche, la capacidad antioxidante de los yogures fue significativamente superior ($P<0.05$). Este incremento fue debido a la actividad proteolítica de las bacterias lácticas (*S. thermophilus* y *L. bulgaricus*) durante la fermentación a través del aumento del nivel de compuestos de nitrógenos solubles, responsables de la liberación de los amino ácidos y péptidos bioactivos de bajo peso molecular.

En los yogures extraídos con metanol y agua durante el periodo experimental (inicio, mitad y final), el efecto de la dieta que recibieron las ovejas fue significativo ($P<0.05$). Se observó un efecto significativo del solvente, con el metanol como mejor solvente, independientemente del método utilizado para medir la capacidad antioxidante (ABTS, DPPH y ORAC). Las diferencias entre los yogures, según la dieta recibida por las ovejas, fueron significativas. Los yogures del tratamiento con mejorana tuvieron la más alta capacidad antioxidante, independientemente del método de extracción utilizado. Sin embrago, las diferencias ente los yogures del tratamiento control y los yogures del tratamiento con colza variaron. Utilizando el ABTS y el ORAC, se observó una alta capacidad antioxidante en los yogures del grupo control con respecto a los yogures del tratamiento con colza pero cuando se utilizó el método DPPH no se encontraron diferencias significativas ($P>0.05$). La diferencia en los resultados puede ser debida a que los método ABTS y ORAC fueron más específicos con las fracciones hidrofílicas que el método DPPH. Las modificaciones en la capacidad antioxidantes de los yogures durante el periodo experimental fueron significativas pero las respuestas variaron de un método a otro sobre todo para los yogures de los tratamientos control y con colza. No obstante, los yogures del tratamiento con mejorana, independientemente del método utilizado tenían siempre el valor más elevado al final del periodo experimental. Las

diferencias observadas en los resultados, según el método utilizado, fueron previstas dado que cada ensayo refleja únicamente la reactividad química bajo las específicas condiciones aplicadas durante el ensayo.

Por otro lado, se observó una correlación significativa entre los valores medidos con el método DPPH y los resultados evaluados con el método ABTS. Sin embargo, no se pudo determinar la correlación entre estos métodos y el método ORAC debido a que utiliza otra unidad de medida.

Debido a estas razones fue imprescindible la utilización de un índice adimensional (RACI) que combina los resultados evaluados con los diferentes métodos.

Se observó que los resultados de los tres métodos estuvieron en concordancia con los valores del RACI. Los yogures obtenidos del tratamiento con mejorana tenían la más alta capacidad antioxidante, seguidos por los yogures del grupo control y finalmente por los yogures del tratamiento con colza. Comparando los yogures de los tratamientos control y con colza, estas diferencias pueden ser debidas al alto contenido en proteínas de los yogures del grupo control y por tanto a la alta concentración en péptidos bioactivos. Sin embargo, comparando los yogures del tratamiento con mejorana y los yogures del grupo control, la diferencia se pudo asociar también a los compuestos fenólicos presentes en los yogures debido a la inclusión de la mejorana en la dieta de las ovejas.

Se observó también que entre yogures del grupo control y yogures con mejorana no se encontraron diferencias significativas en el contenido en proteína. Basándose en estas observaciones, se pudo suponer que estas diferencias en la capacidad antioxidante fueron probablemente debidas a los compuestos fenólicos presentes en los yogures. Sin embargo, visto que no se identificaron compuestos fenólicos en el cromatograma de los extractos de yogures provenientes del tratamiento con mejorana y que los picos correspondieron a péptidos bioactivos o a complejos de péptidos y fenoles, este incremento en la capacidad antioxidante se pudo asociar a la combinación entre todos estos compuestos.

La capacidad anti-inflamatoria se midió con el método SSAO únicamente en la fracción hidrofílica, extraída con metanol en los yogures fabricados al final del periodo experimental debido a que tuvieron la mayor capacidad antioxidante.

Se observó que los yogures elaborados a partir de la leche procedente del tratamiento con mejorana tuvieron la más alta capacidad antioxidante con respecto a los demás yogures (P<0.05). Esta alta capacidad anti-inflamatoria supone que los compuestos antioxidantes actuaron como compuestos anti-inflamatorios e inhibieron la amino oxidasa sensible al semicarbazide (SSAO). Se observó una correlación significativa entre la actividad inhibitoria de la SSAO y los resultados de la capacidad antioxidante medida con los diferentes métodos (DPPH, ABTS y ORAC).

Chapter 7: Conclusiones

7. Conclusiones

- La planta entera de *Thymus mastichina,* de origen silvestre, mostró una alta capacidad antioxidante y anti-inflamatoria, como consecuencia del alto contenido en fenoles totales de la planta, especialmente flavonoides, mayoritariamente luteolina y quercetina.
- La inclusión de semilla de colza (2.85%) en la dieta de las ovejas secas no afectó ni a la ingestión ni a la digestibilidad de los nutrientes ni al balance de nitrógeno. La incorporación de planta entera de mejorana (7.5%) en la dieta con colza de las ovejas dio lugar a un incremento en la digestibilidad de la grasa total de la ración.
- La producción de leche de las ovejas en lactación no se vio afectada de manera significativa por el aporte de colza o mejorana en las raciones. La ingestión de materia seca fue significativamente menor en las raciones que incorporaron colza y menos aún con mejorana.
- La incorporación de colza y mejorana en la ración de las ovejas en lactación dio lugar a una reducción en el contenido en grasa, sólidos totales y ácidos grasos saturados de la leche. Ambas dietas incrementaron el contenido en CLA de la leche en comparación con la dieta control, siendo más marcado el aumento con la mejorana. El contenido en proteína, caseína, ácidos grasos poli-insaturados y fosfatidiletanolamina de la leche fue mayor cuando las ovejas recibieron mejorana en la ración.
- La leche producida a partir de las ovejas que recibieron la dieta con mejorana mostró una capacidad antioxidante más alta que las otras leches.
- Los quesos fabricados a partir de la leche de las ovejas alimentadas con la dieta con colza mostraron un contenido más bajo en proteína en comparación con los otros quesos. La utilización de la dieta con mejorana aumentó el contenido en proteína y disminuyó el contenido en grasa y en sólidos totales. El perfil lipídico de los quesos procedentes de leche de las ovejas que recibieron ambas dietas fue similar al perfil lipídico de la leche.
- La dieta con colza aumentó la adhesividad de los quesos. La dieta con mejorana disminuyó la dureza de los quesos, que mostraron un color amarillo menos intenso. La utilización de ambas dietas no afectó a las características sensoriales de los quesos.

- La capacidad antioxidante de los quesos fue más alta que la de la leche. Los quesos fabricados a partir de la leche de las ovejas que recibieron la dieta con mejorana mostraron una capacidad antioxidante más alta que los demás quesos.
- Los yogures fabricados a partir de la leche de las ovejas que recibieron colza y mejorana en su dieta mostraron un contenido menor en grasa y sólidos totales. La leche procedente de las ovejas que consumieron mejorana incrementó el contenido en proteína de los yogures. El perfil lipídico de los yogures fue similar al perfil lipídico de la leche y los quesos. Además, el contenido en CLA aumentó durante la fermentación.
- La firmeza y la viscosidad de los yogures fue menor cuando se utilizó colza en la dieta de las ovejas y aumentaron con la dieta con mejorana, sin que hubiera cambios en las propiedades sensoriales. Los yogures procedentes de leche de ovejas alimentadas con mejorana mostraron más luminosidad.
- La capacidad antioxidante de los yogures fue dos veces más ata que la capacidad antioxidante de la leche. La utilización de la dieta con mejorana incrementó la capacidad antioxidante y anti-inflamatoria de los yogures en comparación con la dieta con colza y la dieta control.

Como conclusión final, la presente tesis demuestra que la combinación entre la semilla de colza y la planta de mejorana en la dieta de las ovejas no afecta la producción de leche e induce la producción de leche y productos derivados con una perfil lipídico más saludable y una mayor capacidad antioxidante y anti-inflamatoria.

Chapter 8: References

8. References

Abad-García, B., Berrueta, L.A., Garmón-Lobato, S., Gallo, B and Vicente, F. (2009). A general analytical strategy for the characterization of phenolic compounds in fruit juices by high-performance liquid chromatography with diode array detection coupled to electrospray ionization and triple quadrupole mass spectrometry. *Journal of Chromatography A.* 1216: 5398-5415.

Abbeddou, S., Rischkowsky, B., Richter, E.K., Hess, H.D and Kreuzer, M. 2011. Modification of milk fatty acid composition by feeding forages and agro-industrial byproducts from dry areas to Awassi sheep. *J. Dairy Sci.* 94: 4657-4668.

Abdullah, D., Musa Türker, M and Izabela, K. 2012. Antioxidant capacity and phenolic constituents of *Malva neglecta* Wallr. and *Plantago lanceolata* L. from Eastern Anatolia Region of Turkey. *Journal of Herbal Medicine.* 2: 42-51.

Abu-Reidah, I.M., Contreras, M.M., Arráez-Román, D., Fernández-Gutiérrez, A., Segura-Carretero, A. 2013. UHPLC-ESI-QTOF-MS-based metabolic profiling of Vicia faba L. (Fabaceae) seeds as a key strategy for characterization in foodomics. *Electrophoresis*. 35(11): 1571-1581.

Abu-Jdayil, B. 2003. Modelling the time-dependent rheological behavior of semisolid foodstuffs. *Journal of Food Engineering*. 57(1): 97-102.

Additives for Polymers. 2008. Antioxidant market forecast to grow at 5.2%/year to US$5.8 billion in 2016. 9: 9-10.

Adolfsson, O., Meydani, S.N and Russell, R.M. Yogurt and gut function. 2004. *Am J Clin Nutr.* 80: 245-256.

Aganga, A.A., Amarteifio, J.O and Nkile, N. 2002. Effect of stage of lactation on nutrient composition of Tswana sheep and goat's milk. *Journal of food composition and analysis.* 15: 533-543.

Ahmad, S., Gaucher, I., Rouseau, F., Beaucher, E., Piot, M., Grongnet, J.F and Gaucheron, F. 2008. Effects of acidification on physicochemical characteristics of bufalo milk: A comparison with cow's milk. *Food Chem*. 106: 1-17.

Albano, S.M and Miguel, M.G. 2011. Biological activities of extracts of plants grown in Portugal. *Industrial Crops and Products.* 33: 338-343.

Albenzio, M and Santillo, A. 2011. Biochemical characteristics of ewe and goat milk: Effect on the quality of dairy products. *Small Ruminant Research.* 101:133-140.

Albenzioa, M., Taibib, L., Caropresea, M., De Rosac, G., Muscioa, A and Sevia, A. 2003. Immune response, udder health and productive traits of machine milked and suckling ewes. *Small Ruminant Research.* 48:189-200.

Alexander, G., Singh, B., Sahoo, A and Bhat, T.K. 2008. In vitro screening of plant extracts to enhance the efficiency of utilization of energy and nitrogen in ruminant diets. *Animal Feed Science and Technology.* 145: 229-244.

Aloglu, H.S and Oner, Z. 2011. Determination of antioxidant activity of bioactive peptide fractions obtained from yogurt. *J. Dairy Sci.* 94: 5305-5314.

Alonso, L., Fontecha, J., Lozada, L., Fraga, M.J., Juárez, M. 1999. Fatty acid composition of caprine milk: major, branched-chain, and trans fatty acids. *J Dairy Sci*. 82(5): 878-884.

Amiri, H. 2012. Essential oils composition and antioxidant properties of three thymus species. *Evidence-Based Complementary and Alternative Medecine*.

Aneja, R. P and Murthy, T. N. 1990. Conjugated linoleic acid contents of Indian curds and ghee. *Indian Journal of Dairy Science*. 43: 231–238.

Andreotti, G., Trivellone, E and Motta, A. 2006. Characterization of buffalo milk by 31P-nuclear magnetic resonance spectroscopy. *Journal of Food Composition and Analysis*. 19(8): 843-849.

Andrews, J., Vazquez-Anon, M and Bowman, G. 2006. Fat stability and preservation of fatty acids with AGRADO® antioxidant in feed ingredients used in ruminant rations. *J. Dairy Sci*. 89(1): 60. (Abstr.)

Ankeman Granata, L and Morr, C.V. 1996. Improved Acid, Flavor and Volatile Compound Production in a High Protein and Fiber Soymilk Yogurt-like Product. *Journal of Food Science*. 61(2): 331-336.

Annunzioata, A and Vecchio, R. 2011. Functional foods development in the European market: A consumer perspective. *Journal of Functional Foods*. 3: 223-228.

Antunac, M., Havranek, J and Samarzua, D., 2001. Freezing point of goat 571 milk. *Milchwissensch*. 56: 14-16.

AOAC. 2006. Official Methods of Analysis. 18th ed. Assoc. Off. Anal. Chem., Gaithersburg, MD.

Apak, R., Guclu, K., Demirata, B., Ozyurek, M., Celik, S.E., Bektasoglu, B., Berker, K.I and Ozyurt, D. 2007. Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC assay. *Molecules*. 12: 1496-1547.

Appeddu, L.A., Ely, D.G., Aaron, D.K., Deweese, W.P and Fink, E. 2004. Effects of supplementing with calcium salts of palm oil fatty acids or hydrogenated tallow on ewe milk production and twin lamb growth. *J Anim Sci*. 82: 2780-2789.

Arranz, J.J., Bayón, Y and SanPrimitivo, F. 2001. Differentiation among Spanish sheep breeds using microsatellites. *Genet. Sel. Evol*. 33: 529-542.

Ashraf, R., Shah, N.P. 2011. Selective and differential enumerations of *Lactobacillus delbrueckii* subsp. bulgaricus, *Streptococcus thermophilus*, *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacterium* spp. in yoghurt – a review. *Int. J. Food Microbiol.* 149: 194-208.

Bakker, G.C.M., Van Erk, M.J., Pellis, L., Wopereis, S., Rubingh, C.M., Cnubben, N.H.P., Kooistra, T., Ommen, B.V., and Hendriks, H.F.J. 2010. An antiinflammatory dietary mix modulates inflammation and oxidative and metabolic stress in overweight men: a nutrigenomics approach. *Am J Clin Nutr*. 91: 1044-1059.

Barillet, F. 1997. Genetics of milk production. In: The genetics of sheep, (eds, Piper, L and Ruvinsky, A), pp 539-564. Oxford, UK: CAB International.

Barłowska, J., Szwajkowska, M., Litwińczuk, Z and Król, J. 2011. Nutritional value and technological suitability of milk from various animal species used for dairy

production. *Comprehensive Reviews in Food Science and Food Safety*. 10(6): 291-302.

Barros, L., Heleno, S.A., Carvalho, A.M and Ferreira, I.C.F.R. 2010. *Lamiaceae* often used in Portuguese folk medicine as a source of powerful antioxidants: Vitamins and phenolics. *Food Science and Technology.* 43: 544-550.

Bauer, K.D., Garbe, H and Surburg, H. 1997. Common fragrance and flavor materials. Weinheim, Germany: Wiley-VCH Verlag.

Bauman, D.E and Griinari, J.M. 2001. Regulation and nutritional manipulation of milk fat: low-fat milk syndrome. *Livestock Production Science.* 70: 15–29.

Bauman, D.E., Baumgard, L.H., Corl, B.A., Griinari, J.M. 1999. Biosynthesis of conjugated linoleic acid in ruminants. *Proceedings of the American Society of Animal Science*. 1-15.

Bauman, D. E. and Lock, A. L.. 2006. Conjugated linoleic acid: biosynthesis and nutritional significance. In: Advanced Dairy Chemistry, Volume 2: Lipids, 3rd Edition, pp, 93-136. (Eds, Fox, P.F. and McSweeney, P.L.H). New York, USA: Kluwer Academic/Plenum Publishers.

Bauman, D. E., Perfield, J.W., de Veth, M.J. and Lock A.L. 2003. New perspectives on lipid digestion and metabolism in ruminants. *Proc. Cornell Nutr. Conf*. 175-189.

Belbury, M.A. 1995. Conjugated dienoic linoleate: A polyunsaturated fatty acid with unique chemoprotective properties. *Nutrition reviews.* 53(4): 83-89.

Bell, J.A., Griinari, J.M and Kennelly, J.J. 2006. Effect of safflower oil, flaxseed oil, monensin, and vitamin E on concentration of conjugated linoleic acid in bovine milk fat. *J. Dairy Sci.* 89: 733-748.

Bell, J.M. 1983. Nutrients and toxicants in rapseed meal: A review. *Journal of Animal Science*. 58(4): 996-1010.

Bellenand, J.F., Baloutch, G., Ong, N and Lecerf, J. 1980. Effects of coconut oil on heart lipids and on fatty Acid utilization in rapeseed oil. *Lipids.* 15(11): 938–943.

Ben Farhata, M., Landoulsia, A., Chaouch-Hamadaa, R., Sotomayorc, J.A and Jordánca, M.J. 2013. Characterization and quantification of phenolic compounds and andantioxidant properties of *Salvia* species growing in different habitats. *Industrial Crops and Products.* 49: 904-914.

Bennett, R.N. and Wallsgrove, R.M. 1994. Secondary metabolites in plant defense mechanisms. *New Phytol*. 127: 617-633.

Bentes J., Miguel, M.G., Monteiro, I., Costa, M., Figueired, A.C., Barroso, J.G and Pedro, L.G. 2009. Antioxidant activities of the essential oils and extracts of Portuguese *Thymbra capitata* and *Thymus mastichina*. *Italian Journal of Food Science*. 21(2):183.

Bento, F., Galego, L., Gonçalves, V., Cavac, T., Almeida, V., Margarida, C. 2006. Headspace solid-phase microextraction gas chromatography and gas chromatography-mass spectrometry analysis of the volatile compounds during packing of: *Calamintha baetica* , *Thymus mastichina* and *Origanum vulgare*. *Traditional Food Processing and Techological Innovation in the Peripheral Regions*.

Berdahl, D.R., Nahas, R.I and Barren, J.P. 2010. Synthetic and natural antioxidant additives in food stabilization: current applications and future research. In Oxidation

in foods and beverages and antioxidant applications, (ed, Smit, G), pp 273-320. Cambridge: Woodhead Publishing.

Bhat, Z.F and Bhat, H. 2011. Milk and Dairy Products as Functional Foods: A Review. *International Journal of Dairy Science*. 6(1): 1-12.

Bhattacharyaa, A., Banua, J., Rahman, M., Causey, J and Fernandes, G. 2006. Biological effects of conjugated linoleic acids in health and disease. *Journal of Nutritional Biochemistry*. 17: 789-810.

Bholah, K.D and Neergheen-Bhujun, V.S. 2013. An insight of the Mauritian consumers' awareness, perceptions and expectations of functional foods. *International journal of nutrition and food sciences.* 2(2): 52-59.

Bianchi, L., Bolla, A., Budelli, E., Caroli, A., Casoli, C., Pauselli, M and Duranti, E. 2004. Effect of udder health status and lactation phase on the characteristics of Sardinian ewe milk. *J. Dairy Sci.* 87(8): 2401-2408.

Bigliardi, B and Galati, F. 2013. Innovation trends in the food industry: The case of functional foods. Trends in Food Science and Technology. 31:118-129.

Birollo,G.A., Reinheimer, J.A., Vinderola, C.G. 2000. Viability of lactic acid microflora in different types of yoghurt. *Food Res Int.* 33: 799-805.

Biström, M and Nordström, K. 2002. Identification of key success factors of functional dairy foods product development. *Trends in Food Science and Technology*. 13(11): 372–379.

Bitman, J and Wood, D.L. 1991. Changes in milk phospholipids during lactation. Journal of Dairy Science. *Journal of Dairy Science*. 73: 1208-1216.

Blanco, S.J. 2007. *Folia Botanica Extremadurensis.* 1.

Blanco, S.J. 1999. The autonomous behaviour of normal thymus grafts. *Aust. J. Exp*. 99.

Blank-Porat, D. 2007. The anticancer prodrugs of butyri acid AN-7 and AN-9, possess antigiogenic properties. Cancer lett. 259(1): 39-41.

Blaschko, H., Friedman, P.J., Hawes, R and Nilsson, K. 1958. The amine oxidases of mammalian plasma. *J. Physiol.* 145: 384-404.

Blauwiekel, R., Xu, S., Harrison, J.H., Loney, K.A., Riley, R.E. and Calhoun, M.C. 1997. Effect of whole cottonseed, gossypol and ruminally protected lysine supplementation on milk yield and composition. Journal of Dairy Science. 80: 1358-1365.

Bodas, R., Manso, T., Mantecón, A.R., Juárez, M., de la fuente, M.A and Gómez-Cortéz, P. 2010. Comparison of the fatty acid profiles in cheeses from ewes fed diets supplemented with different plant oils. *J. Agric. Food Chem*. 58(19): 10493-10502.

Bogue, J., Delahunty, C and Kelleher, C. 1999. Market-Oriented New Product Development: Consumers' Perceptions of Diet and Health and their Consumption of Reduced-fat and Reduced-calorie Foods. Agribusiness Discussion Paper No. 24.

Bonanno, A., Tornambè, G., Bellina, V., De Pasquale, C., Mazza, F., Maniaci, G and Di Grigoli, A. 2013. Effect of farming system and cheesemaking technology on the physicochemical characteristics, fatty acid profile, and sensory properties of Caciocavallo Palermitano cheese. *J. Dairy Sci.* 96(1): 710-772.

Boomsma, F., Dijk, J.V., Bhaggoe, U.M., Bouhuizen, A.M.B and Van den Meiracker, A.H. 2000. Variation in semicarbazide-sensitive amine oxidase activity in plasma and tissues of mammals. *Comparative Biochemistry and Physiology.* 126(1): 69-78.

Boroski, M., Giroux, H.J., Sabik, H., Petit, H.V., Visentainer, J.V., Matumoto-Pintro, P.T and Britten, M. 2012. Use of oregano extract and oregano essential oil as antioxidants in functional. *Food Science and Technology.* 47(1): 167-174.

Bounatirou, S., Smiti, S., Miguel, M.G., Faleiro, L., Rejeb, M.N., Neffati, M., Costa, M.M., Figueiredo, A.C., Barroso, J.G and Pedro, L.G. 2007. Chemical composition, antioxidant and antibacterial activities of the essencial oils isolated from Tunisian *Thymus capitatus* Hoff. et Link. *Food Chemistry*. 105(1): 146-155.

Bourne, M.C. 1978. Texture profile analysis. *Food Technology*. 32: 62- 72.

Boutoial, K., Ferrandini, E., Rovira, S., Garcia, V and Bélen López, Ma. 2013. Effect of feeding goats with rosemary (*Rosemarinus officinalis* spp.) by-producton milk and cheese properties. *Small Ruminant Research*. 112(1-3): 147-153.

Boylston, T.D and Beitz, D.C. 2002. Conjugated linoleic acid and fatty acid composition of yogurt produced from milk of cows fed soy oil and conjugated linoleic acid. *Journal of food science*. 67(5): 1973-1978.

Bravo, L. 1998. Polyphenols: Chemistry, dietary osurces, metabolism, and nutritional significance. *Nutrition Reviews.* 56(11).

Brewer, M.S. 2011. Natural Antioxidants: Sources, Compounds, Mechanisms of Action, and Potential Applications. *Comprehensive Reviews in Food Science and Food Safety*. 10(4): 221-247.

Bristrom, M and Nordstrom, K. 2002. Identification of key success factors of functional dairy foods product development. *Trends in Food Science and Technology*. 13(11): 372-379.

Buccioni, A., Rapaccini, S., Antongiovanni, M., Minieri, S., Conte, G and Mele, M. 2010. Conjugated linoleic acid and C18:1 isomers content in milk fat of sheep and their transfer to Pecorino Toscano cheese. *International Dairy Journal.* 20(3): 190-194.

Cai, Y., Luo, Q. Sun M. and Corke, H. 2004. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sci*. 74: 2157-2184.

Caja, G and Rancourt, M. 2002. Situation actuelle et perspectives de la production des ovins laitiers en Espagne. *Options Méditerraéennes*. 39 (A): 57-66.

Castro, T., Manso, T., Jimeno, V., Del Alamo, M and Mantecón, A.R. 2009. Effects of dietary sources of vegetable fats on performance of dairy ewes and conjugated linoleic acid (CLA) in milk. *Small Ruminant Research.* 84(1-3): 47-53.

Casoli, C., Duranti, E., Morbidini, L., Panella, F and Vizioli, V. 1989. Quantitative and compositional variations of Massese sheep milk by parity and stage of lactation. *Small Rumin. Res*. 2: 47-62.

CFR, 2013. Code of Federal Regulations. Title 21, food and drugs. Chapter 1, food and drug administration. Part 131, milk and cream.

Chahar, M.K., Sharma, N., Dobhal, M.P and Joshi, Y.C. 2011. Flavonoids: A versatile source of anticancer drugs. *Pharmacogn Rev*. 5(9): 1-12.

Chandan, R.C and O'Rell, K.R. 2006. Ingredients for Yogurt manufacture. In: Manufacturing of yogurt and fermented milk (ed, Chandan, R.C), chap 11, pp 247-264. Ames, IA: Blackwell Publishing.

Chichlowski, M.W., Schroeder, J.W., Park, C.S., Keller, W.L and Schimek, D.E. 2005. Altering the fatty acids in milk fat by including canola seed in dairy cattle diets. *J Dairy Sci*. 88(9): 3084-94.

Chillard, Y., Ferlay, A., Mansbridge, M and Doreau, M. 2000. Ruminant milk fat plasticity: Nutritional control of saturated, polyunsaturated, trans and conjugated fatty acids. *Ann. Zootech.* 49: 181–205.

Chilliard, Y., Ferlay, A., Rouel, J., and Lamberet, G. 2003. A review of nutritional and physiological factors affecting goat milk lipid synthesis and lipolysis. *J. Dairy Sci*. 86: 1751-1770.

Chilliard Y., Glasser, F., Ferlay, A., Bernard, L., Rouel, J. and Doreau, M. 2007. Diet, rumen biohydrogenation and nutritional quality of cow and goat milk fat. *European Journal of Lipid Science and Technology*. 109: 828-855.

Chilliard, Y and Ferlay, A. 2004. Dietary lipids and forages interactions on cow and goat milk fatty acid composition and sensory properties. *Reprod. Nutr. Dev.* 44: 467-492.

Chilliard, Y., Martin, C., Rouel, J and Doreau, M. 2009. Milk fatty acids in dairy cows fed whole crude linseed, extruded linseed, or linseed oil, and their relationship with methane output. *J. Dairy Sci*. 92(10): 5199-5211.

Chiofalo, V., Liotta, L., Fiumanò, R., Benedetta, E and Chiofalo, B. 2012. Influence of dietary supplementation of *Rosmarinus officinalis* L. on performances of dairy ewes organically managed. *Small Ruminant Research.* 104(1-3): 122-128.

Coakley, M., Barrett, Murphy, J.J., Ross, R.P., Devery, R and Stanton, C. 2007. Cheese manufacture with milk with elevated conjugated linoleic acid levels caused by dietary manipulation. *J. Dairy Sci.* 90(6): 2919-2927.

Collomb, M., Schmid,, A., Sieber, R., Wechsler, D and Ryhänen, E-L. 2006. Conjugated linoleic acids in milk fat: Variation and physiological effects. *International Dairy Journal*. 16(11): 1347-1361.

Contarini, G and Povolo, M. 2013. Phospholipids in milk fat: composition, Biological and technological significance, and analytical strategies. *Int.* J. Mol. Sci. 14(2): 2808-2831.

Coppa, M., Ferlay, A., Monsallier, F., Verdier-Metz, I., Pradel, P., Didienne, R., Farruggia, A., Montel, M.C and Martin, B. 2011. Milk fatty acid composition and cheese texture and appearance from cows fed hay or different grazing systems on upland pastures. *J. Dairy Sci*. 94: 1132-1145.

Coppens, P., Fernandes da Silva, M and Pettman, S. 2006. European regulations on nutraceuticals, dietary supplements and functional foods: A framework based on safety. *Science Direct*. 3: 59-74.

Cowan, C.M. and Larson, L.L. 1979. Relationship of the estrous cylce to milk composition. *Journal of Dairy Science*. 62: 546-550.

Coward, J., Kulbe, H., Chakravarty, P., Leader, D., Vassileva, V., ALeinster, D., Thompson, R., Schioppa, T., Nemeth, J., Vermeule, N., Cummings, J., Exhepaj, E.,

Jirström, K., Gallagher, W.M., Brennan, D.J., McNeish, I.A and Balkwill, F. 2011. Interleukin-6 as a therapeutic target in human ovarian cancer. *Clin Cancer Res.* 17(18): 6083-6090.

Cruz-Hernandez, C., Kramer, J.K.G., Kennelly, J.J., Glimm, D.R., Sorensen, B.M., Okine, E.K., Goonewardene, L.A. and Weselake, R.J. 2007. Evaluating the conjugated linoleic acid and trans 18:1 Isomers in milk fat of dairy cows fed increasing amounts of sunflower oil and a constant level of fish oil. *J. Dairy Sci.* 90: 3786-3801.

Cueva, O and Aryana, K.J. Quality attributes of a heart healthy yogurt. *Science Direct*. 41: 537-544.

Dalar, A., Turker, M and Konczak. 2012. Antioxidant capacity and phenolic constituents of *Malva neglecta* Wallr. and *Plantago lanceolata* L. from Eastern Anatolia Region of Turkey. *Journal of Herbal Medicine*. 2(2): 42-51.

Dapkevicius, A. 2002. Isolation and structure elucidation of radical scavengers from *Thymus* vulgaris leaves . *Journal of Natural Products*. 65(6): 892-896.

Dario, C, Carnicella, D., Dario, M and Bufano, G. 2008. Genetic polymorphism of (β-lactoglobulin gene and effect on milk composition in Leccese sheep. *Small Ruminant Research*. 74: 270-273.

Dashwood, R.H. 2007. Frontiers in polyphenols and cancer prevention. *J. Nutr.* 137: 267S-269S.

Decker, E. A., Elias, R. J and McClements, D. J. 2010. Oxidation in foods and beverages and antioxidant applications. Cambridge, UK: Woodhead Publishing.

De Gordoa, R., Bustamante, J.C.R ., de Renobales, M., Virto, M., Barron, L.J.R., Nájera, A.I., Albisu, M., Pérez Elortondo, F.J., Oregi, L., Ruiz, R and Mandaluniz, N. 2008. Total antioxidant capacity of sheep's milk. 5th IDF symposium on cheese ripening.

Delahunty, C.M. and Drake, M.A. Sensory character of cheese and its evaluation. in: Cheese: Chemistry, Physics and Microbiology General Aspects. 1. 3rd ed. Elsevier, London, UK; 2004: 455–487

Dell'Aquila, S., Pilla, A.M., Catillo, G., Scardella, G and Taibi, L. 1993. Milk yield of Comisana, Delle Langhe Massese, Sarda and crossbred ewes. *Zootecnica Nutrizione Animale*. 14: 95-102.

De la Fuente, L.F., San Primitivo, F., Fuertes, J.A and Gonzalo, C. 1997. Daily and between-milking variations and repeatabilities in milk yield, somatic cell count, fat and protein of dairy ewes. *Small Ruminant Research*. 24: 133-139.

De La Torre, A., Debiton, E., Durand, D., Chardigny, J-M., Berdeaux, O., Loreau, O, Barthomeuf, C., Bauchart, D and Gruffat, D. 2005. Conjugated linoleic acid isomers and their conjugated derivatives inhibit growth of human cancer cell lines. *Anticancer Research*. 25: 3943-3950.

Delbecchi, L., Ahnadi, C. E., Kennelly, J. J and Lacasse, P. 2001. Milk Fatty Acid Composition and Mammary Lipid Metabolism in Holstein Cows Fed Protected or Unprotected Canola Seeds. *J. Dairy Sci*. 84(6):1375-1381.

Delgado, T., Marinero, P., Asensio-S-Manzanera, M. C., Asensio, C., Herrero, B., Pereira, J.A and Ramalhosa, E. 2014. Antioxidant activity of twenty wild Spanish

Thymus mastichina L. populations and its relation with their chemical composition. *Food Science and Technology*. 57(1): 412-418.

De Mejia, E.G., Ramirez-Mares, M.V and Puangraphant, S. 2006. Bioactive components of tea: Cancer, inflamation and behavior. *Brain, Behavior, and Immunity*. 23(6): 721-731.

De Ondarza, M.B., Wilson, J.W and Engstrom, M. 2009. Case study: Effect of supplemental β-carotene on yield of milk and milk components and on reproduction of dairy cows. *The Professional Animal Scientist.* 25: 510-516.

DePeters, E.J., German, J.B., Taylor, S.J., Essex, S.T and Perez-Monti, H. 2001. Fatty Acid and triglyceride composition of milk fat from lactating holstein cows in response to supplemental canola oil. *J. Dairy Sci.* 84(4): 929-936.

Dhiman, T.R., Helmink, E.D., Mcmahon, D.J., Fife, R.L and Pariza, M.W. 1995. Conjugated linoleic acid content of milk and cheese from cows fed extruded oilseeds. *J Dairy Sci.* 82(2): 412-419.

Dibner, J. J., Kitchell, M. L., Atwell, C. A and Ivey, F. J. 1996. The effect of dietary ingredients and age on the microscopic structure of the gastrointestinal tract in poultry. *J. Appl. Poult. Res.* 5: 70-77.

Directiva 89/104/CEE del Consejo de 21 de diciembre de 1988 relativa a la Aproximación de las Legislaciones de los Estados Miembros en materia de Marcas.

Disenhaus, C., Jammes, H., Hervieu, J., Ternois, F and Sauvant, D. 1995. Effects of recombinant bovine somatotropin on goat milk yield, composition and plasma metabolites. *Small Rum. Res*. 15: 139-148.

Doreau, M., Aurousseau, E and Martin, C. 2009. Effects of linseed lipids fed as rolled seeds, extruded seeds or oil on organic matter and crude protein digestion in cows. *Anim. Feed Sci. Technol*. 150: 187-196.

Doreau, M and Chilliard, Y. 1997. Digestion and metabolism of dietary fat in farm animals. *British Journal of Nutrition.* 78(1): S15-S35.

Doreau, M and Ferry, A. 1995. Digestion and utilisation of fatty acids by ruminants. Animal Feed Science Technology. 45: 379-396.

Dorman, D.H.J., Bachmayer, O., Kosar, M and Hiltunen, R. 2004. Antioxidant Properties of Aqueous Extracts from Selected *Lamiaceae* Species Grown in Turkey. *J. Agric. Food Chem.* 52(4): 762-770.

Eastridge, M.L and Firkins, J.L. 2000. Feeding tallow triglycerides of different saturation and particle size to lactating dairy cows. *Anim. Feed Sci. Tech*. 83: 249-259.

Enjalbert, F., Moncoulon, R., Vemay, M and Griess, D. 1994. Effects of different forms of polyunsaturated fatty acids on rumen fermentation and total nutrient digestibility of sheep fed prairie hay based diets. *Small Ruminant Research.* 14(2): 127-135.

Erkkilä, A., De Mello, V.D.F., Riserus, U and Laaksonen, D.E. 2008. Dietary fatty acids and cardiovascular disease: An epidemiological approach. *Progress in Lipid Research.* 47(3): 172-187.

Escarpa, A and Gonzalez, M. C. 2001. Approach to the content of total extractable phenolic compounds from different food samples by comparison of chromatographic and spectrophotometric methods. *Analytica Chimica Acta.* 427(1): 119-127.

Estevez, A.M., Mejia, J., Figuerola, F and Escoba, B. 2010. Effect of solid content and sugar combinations on the quality of soymilk based yogurt. *Journal of Food Processing and Preservation.* 34(1): 87-97.

Falguera, V., Aliguer, N and Falguera, M. 2012. An integrated approach to current trends in food consumption: Moving toward functional and organic products. *Food Control.* 26(2): 274-281.

Fadela, C., Abderrahim, C and Ahmed, B. 2009. Sensorial and Physico-Chemical Characteristics of Yoghurt Manufactured with Ewe's and Skim Milk. *World Journal of Dairy and Food Sciences*. 4 (2): 136-140.

FAO. 2013. FAOSTAT producción agrícola. Available in internet: http://www. faostat.fao.org/ [agosto de 2012].

Favaro, C.S., Terzi, S.C., Trugo, L.C., Della Modesta, R.C., Couri, S. 2001). Development and sensory evaluation of soymilk based yoghurt. Archivos Latino Americanos De Nutricion. 51: 100-104.

FENIL. 2012. Federación Nacional de Industrias Lácteas. Available in internet: http://www.fenil.org/.

Fernandez, S.V., Robertson, F.M., Pei, J., Aburto-Chumpitaz, L., Mu, Z., Chu, K., Alpaugh, R.K., Huang, Y., Cao, Y., Ye, Z., Cai, K.Q., Boley, K.M., Klein-Szanto, A.J., Devarajan, K., Addya, S and Cristofanilli, M. 2013. Inflammatory breast cancer: clues for targeted therapies. *Breast Cancer Res Treat.* 140(1): 23-33.

Fisher, Pand Windhad, E.J. 2011. Rheology of food materials. Curr. Opin. Colloid Interface Sci. 16: 36-40.

Frankel, E.N and Meyer, A.S. 2000. The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. *J Sci Food Agric*. 80(13):1925-1941.

Franz, C., Baserb, K.H.C and Windischc, W. 2010. Essential oils and aromatic plants in animal feeding: European perspective. *Flavour Fragr.* 25(5): 327–340.

Fresno, A.M and Sergio, A. 2012. Chemical, textural and sensorial changes during the ripening of Majorero goat cheese. *International Journal of Dairy Technology*. 65(3): 393-400.

Foegeding, E.A., Daubert, C.R., Drake, M.A., Essick, G., Trulsson, M., Vinyard, C.J and Van De Velde, F. 2011. A comprehensive approach to understanding textrual properties of semi and solid foods. *Journal of Textural Studies*. 42(2): 103-129.

Fontecha, J., Goudjil, H., Ríos, J.J., Fraga, M.J and Juárez, M. 2005. Identity of the major triacylglycerols in ovine milk fat. *International Dairy Journal*. 15(12): 1217–1224.

Fontecha, J., Rodriguez-Alcala, L.M., Calvo, M.V and Juárez, M. 2011. Bioactive Milk Lipids. *Currentl Nutrition and Food Science.* 7(3):155-159(5).

Fox, P.F and McSweeney, P.L.H. 2004. Cheese: an overview. In Cheese: Chemistry, Physics and Microbiology, (eds Fox, P.F., McSweeney, P.L.H., Cogan T.M and Guinee, T.P), Vol 1. London,UK: Elsevier, General Aspects.

Garcia, C and Innis, S. 2013. Structure of the human milk fat globule. *Lipid Technology*. 25(10): 223-226.

García-Rodríguez, M.C., Carvente-Juárez, M.M and Altamirano-Lozano, M.A. 2013. Antigenotoxic and apoptotic activity of green tea polyphenol extracts on hexavalent chromium-induced DNA damage in peripheral blood of CD-1 mice: Analysis with differential acridine orange/ethidium bromide staining. *Oxidative Medicine and Cellular Longevity*.

Garnsworthy, P.C., Masson, L.L., Lock, A.L and Mottram, T.T. 2006. Variation of milk citrate with stage of lactation and de novo fatty acid synthesis in dairy cows. *Journal of Dairy Science.* 89: 1604-1612.

Gaynor, P.J., Erdman, R.A., Teter, B.B., Sampugna, J., Capuco, A.V., Waldo, D.R and Hamosh. M. 1994. Milk fat yield and composition during abomasal infusion of cis or trans octadecenoates in Holstein cows. *J Dairy Sci*. 77(1): 157-165.

Glasser, F., Ferlay, A and Chilliard, Y. 2008. Oilseed lipid supplements and fatty acid composition of cow Milk: A meta-Analysis. *J. Dairy Sci.* 91: 4687-4703.

Gómez-Cortés, P., Bach, A., Luna, P., Juárez, M and de la Fuente, M.A. 2009. Effects of extruded linseed supplementation on n-3 fatty acids and conjugated linoleic acid in milk and cheese from ewes. *J. Dairy Sci*. 92(9): 4122-4134.

Gómez-Cortés, P., Bodas, R., Mantecón, A.R and de la Fuente, M.A. 2011a. Milk composition and fatty acid profile of residual and available milk from ewes fed with diets supplemented with different vegetable oils. *Small Ruminant Research*. 97(1-3): 72-75.

Gómez-Cortés, P., Frutos, P., Mantecon, A. R., Juárez, M., de la Fuente, M. A and Hervas, G. 2008a. Milk production, conjugated linoleic acid content, and in vitro ruminal. *J. Dairy Sci*. 91: 1560-1569.

Gómez-Cortés, P., Frutos, P., Mantecon, A.R., Juárez, M., Hervas, G and de la Fuente. 2008b. Addition of olive oil to dairy ewe diets: effect on Milk. *J. Dairy Sci*. 91(4): 3119–3127.

Gómez-Cortés, P., Toral, T G., Frutos, P., Juárez, M., de la Fuente, M. A and Hervás, G. 2011b. Effect of the supplementation of dairy sheep diet with incremental amounts of sunflower oil on animal performance and milk fatty acid profile. *Food Chemistry*. 125(2): 644-651.

Gonthier, C., Mustafa, A.F., Ouellet, D.R., Chouinard, P.Y., Berthiaume, R and Petit, H.V. 2005. Feeding micronized and extruded flaxseed to dairy cows: Effects on blood parameters and milk fatty acid composition. *J Dairy Sci*. 88: 748-756.

Gordon, M.H. 2012. Significance of Dietary Antioxidants for Health. *Int. J. Mol. Sci.* 13(1):173-179.

Goudjil, H., Fontecha, J., Luna, P., de la Fuente., M.A., Alonso, L and Juárez, M. 2004. Quantitative characterization of unsaturated and trans fatty acids in ewe's milk fat. *Lait.* 84: 473-482.

Greathead, H. 2003. Plants and plant extracts for improving animal productivity. *Proceeding of Nutritional Society*. 62: 279-290.

Grieve, M. 1931. Thyme, Garden. *Botanical. com*.

Griinari, J.M., Bauman, D.E and Mikko, J. 2003. Nutritional regulation of milk fat synthesis. *Annu. Rev. Nutr*. 23: 203-227.

Griinari, J.M., Corl, A., Lacy, S.H., Chouinard, P.Y., Nurmela, K.V.V and Bauman, D.E. 2000. Conjugated Linoleic Acid Is Synthesized Endogenously in Lactating Dairy Cows by Δ9-Desaturase[1,2]. *J. Nutr.* 130(9): 2285-2291.

Grinari, J.M., D. A. Dwyer, D.A., McGuire, M.A., Bauman, D.E., Palmquist, D.L and Nurmel, K.V.V. 1998. trans-octadecenoic acids and milk fat depression in lactating dairy cows. *J Dairy Sci.* 81: 1251-1261.

Grummer, R.R. 1991. Effect of feed on the composition of milk fat. *J. Dairy Sci*. 74: 3244-3257.

Grygorczyk, A., Lesschaeve, I., Corredig, M., Duizer, L. 2013. Extraction of consumer texture preferences for yogurt: Comparison of the preferred attribute elicitation method to conventional profiling. *Food Quality and Preference.* 27(2): 215–222.

Guesnet, P., Alessandri, J.M., Vancassel, S., Denis, I and Lavia.lle, M. 2005. Acides gras oméga 3 et fonctions cérébrales. *Nutrition clinique et métabolisme.* 19(3): 131-134.

Guo, M., Park, Y.W., Dixon, P.H., Gilmore, J.A and Kindstedt, P.S. 2003. Relationship between the yield of cheese (Chevre) and chemical composition of goat milk. Small Ruminant Research. 52(1-2): 103-107.

Hala, M.F., El-Din, E.I., Sanaa, G., Badran, M.A., Gad, A.S and El-Said, M. 2010. Manufacture of low fat UF-soft cheese supplemented with rosemary extract (as natural antioxidant). *Journal of American Science.* 6(10): 570-579.

Haenlein, G.F.W. 1995. Nutritional value of dairy products of ewe and goat milk. Production and utilization of ewe and goat milk. Proceedings of the IDF/Greek National Committee of IDF/ CIRVAL Seminar held in Crete (Greece), 19-21 October. 159-178.

Haenlein, G.F.W. 1998.The value of goats and sheep to sustain mountain farmers. *International Journal of Animal Sciences.* 13: 187-194.

Haenlein, G.F.W. 2002. Relationship of somatic cell counts in goat milk to mastitis and productivity. *Small Rumin. Res*. 45: 163-178.

Haenlein, G.F.W and Wendorff, W.L. 2006. Sheep milk: production and utilization. In: Handbook of Milks of Non-bovine Mammals, Chapter 3 (eds, Park,Y. and Haenlein, G.F.W), pp. 137-194. Oxford Clarendon: Blackwell Publ.

Halliwell, B and Gutteridge, J.M.C. 2007. Free radicals. In: Biology and medicine. Oxford Clarendon: Blackwell Publ.

Han, J., Wang, Y., Yu, C.L., Yan Y.S and Xie, X.Q. 2011. Extraction and determination of chloramphenicol in feed water, milk, and honey samples using an ionic liquid/sodium citrate aqueous two-phase system coupled with high-performance liquid chromatography. *Analytical and Bioanalytical Chemistry*. 399(3): 1295-1304.

Harborne, J. B. 1999. The comparative biochemistry of phytoalexin induction in plants. *Biochemical Systematics and Ecology*. 27(4): 335-367.

Harfoot, C. G., and Hazlewood, G. P. 1997. Lipid metabolism in the rumen. In: The Rumen Microbial Ecosystem, (eds, Hobson P.N. and Stewart C.S), pp 382-426. London, UK: Chapman and Hall.

Hart, K.J., Yánéz-Ruiz, D.R and Duval, S.M. 2008. Plant extracts to manipulate rumen fermentation. *Animal Feed Science and Technology*. 147(1-3): 8-35.

Harvatine, K.J., Boisclair, Y.R. and Bauman, D.E. 2009. Recent advances in the regulation of milk fat synthesis. *Animal*. 3(1): 40-54.

Hatziminaoglou, I., Georgoedidus, A and Karalasos, A. 1990. Factors affecting milk yield and prolificacity of Karagouniko sheep in west thelassy (Greece). *Livestock Production Science*. 24: 181-186.

Hazzit, M., Baaliouamer, A., Veríssimo, A.R., Faleiro, M.L and Miguel, M.G. 2009. Chemical composition and biological activities of Algerian *Thymus* oils. *Food Chemistry*. 116(3): 714-721.

He, M and Armentano, L.E. 2011. Effect of fatty acid profile in vegetable oils and antioxidant supplementation on dairy cattle performance and milk fat depression. *J. Dairy Sci*. 94(5): 2481-2491.

Helmut, S. 1997. Oxidative stress: Oxidants and antioxidants. *Experimental Physiology*. 82(2): 291-295.

Hernandez, M.M., Esteban, M., Szabo, P., Boada, M and Unzeta, M. 2005. Human plasma semicarbazide sensitive amine oxidase (SSAO), β-amyloid protein and aging. *Neuroscience Letters*. 384(1–2): 183–187.

Hernández-Ledesma, B., Mercedes, R., Gómez-Ruiz, J.A. 2011.Bioactive components of ovine and caprine cheese whey. *Small Ruminant Research*. 101: 196-204.

Herrero, M., Plaz, M., Cifuentes, A and Ibáñez, E. 2010. Green processes for the extraction of bioactives from Rosemary: Chemical and functional characterization via ultra-performance liquid chromatography-tandem mass spectrometry and in-vitro assays. *Journal of Chromatography A.* 1217(16): 2512-2520.

Herrero, A.M and Requena, T. The effect of supplementing goats milk with whey protein concentrate on textural properties of set-type yoghurt. *International Journal of Food Science and Technology*. 41(1): 87-92.

Hervás, G., Luna, P., Mantecón, A.M., Castañares, N., de la Fuente, M.A., Juárez, M and Frutos, P. 2008. Effect of diet supplementation with sunflower oil on milk production, fatty acid profile and ruminal fermentation in lactating dairy ewes. Journal of dairy research. 74(4): 399-405.

Hilali, M., Iniguez, L., Knaus, W., Schreiner, M., Wurzinger, M and Mayer, H.K. 2011. Dietary supplementation with nonconventional feeds from the Middle East: Assessing the effects on physicochemical and organoleptic propeties of Awassi sheep milk yogurt. *J. Dairy Sci*. 94(12): 5737-5749.

Hilali, M., 2001. Study of the physico-chemical properties and coagulation parameters of Awassi sheep milk collected in Maragha region, Syria, According to Pasture. Thesis. University of Aleppo, Aleppo, Syria.

Hinneburg, I., Dorman, D and Hiltunen, R. 2006. Antioxidant activities of extracts from selected culinary herbs and spices. *Food Chemistry.* 97(1): 122-129.

Hino, T., Andoh, N and Ohgi, H. 1993. Effects of β-Carotene and α-tocopherol on rumen bacteria in the utilization of long-chain fatty acids and cellulose. *J Dairy Sci.* 76(2): 600-605.

Hossain, M. B., Brunton, N.P., Barry-Ryan, C., Martin-Diana, A.B and Wilkinson, M. 2010. Characterization of phenolics composition in Lamiaceae spices by LC-ESI-MS/MS. *J. Agric. Food Chem*. 58 (19): 10576-10581.

Hossain, M.B., Barry-Ryan, C., Martin-Diana, A.B and Brunton, N.P. 2010. Effect of drying method on the antioxidant capacity of six *Lamiaceae* herbs. *Food Chemistry*. 123(1): 85-91.

Hossain, M.B., Brunton, N.P., Barry-Ryan, C., Martin-Diana, A.B and Wilkinson, M. 2008. Antioxidant activity of spice extracts and phenolics in comparison to synthetic antioxidants. *Rasayan J. Chem.* 1(4): 751-756.

Houseknecht, K., Vanden I., Heuvel, J.P., Moya-Camarena, S.Y., Portocarrerro, C.P., Peck, L.W., Nickel, K.P and Belury, M.A. 1998. Conjugated linoleic acid normalizes impaired glucose tolerance in the Zucker diabetic fatty fa/fa rat. *Biochem. Biophys. Res. Commun*. 244: 678-682

Hristov, A.N., Lee, C., Cassidy, T., Heyler, K., Tekkipe, J.A., Varga, G.A., Corl, B and Brandt, R.C. 2013. Effect of *Origanum Vulgare* L. leaves on rumen fermentation, production, and milk fatty acid composition in lactatin dairy cows. *Journal of Dairy Science*. 96(2): 1189-1202.

Huang, D., Ou, B and Prior, R.L. 2005. The chemistry behind antioxidant capacity assays. *Journal of Agriculture and Food Chemistry*. 53(6): 1841-1856.

Huang, Y., Schoonmaker, J.P., Oren, S.L., Trenkle, A and Beitz, D.C. 2009. Calcium salts of CLA improve availability of dietary CLA. *Livestock Science.* 122(1): 1-7.

Hurtaud, C., Peyraud, J.L., Michel, G., Berthelot, D and Delaby, L. 2009. Winter feeding systems and dairy cow breed have an impact on milk composition and flavour of two Protected Designation of Origin French cheeses. *Animal.* 3(9): 1327-1338.

Hyder, P.W., Fredrickson, E.L., Estell, R.E and Lucero, M.E. 2002. Transport of phenolic compounds from leaf surface of creosotebush and tarbush to soil surface by precipitation. *J Chem Ecol*. 28(12): 2475-82.

Imaizumi, K., Sekihara, K., Sugano, M. 1991. Hypocholesterolemic action of dietary phosphatidylethanolamine in rats sensitive to exogenous cholesterol. *The Journal of Nutritional Biochemistry*. 2(5): 251-254.

Ip, C., Banni, S., Angioni, E., Carta, G., McGinley, Thompson, H., Barbano, D and Bauman, D. 1999. Conjugated linoleic acid-enriched butter fat alters mammary gland morphogenesis and reduces cancer risk in rats. *Journal of Nutrition*. 129: 2135-2142.

Ip, C., Singh, M., Thompson, H.J and Scimeca, J.M. 1994. Conjugated linoleioc acid suppresses carcinogenesis and proliferative activity of the mammary gland in the rat. *Cancer research*. 54: 1212.

Ismaili, H., Milella, L., Fkih-Tetouani, S., Ilidrissi, A., Camporese, A., Sosa, S., Altinier, G., Della Loggia, R and Aquino, R. 2004. In vivo topical anti-inflammatory and in vitro antioxidant activitiesof two extracts of Thymus stureioides leaves. Journal of Ethnopharmacology. 91(1): 31-36.

ISO 8586-1:1993. Sensory analysis. General guidance for the selection, training and monitoring of assessors. Part 1: Selected assessors.

ISO 11869: 1997. Yogurt. Determination of titratable acidity. Potentiometric method.

ISO 14156: 2001. Milk and milk products. Extraction methods for lipids and liposoluble compounds.

ISO 8968-1: 2001. Milk and milk products. Determination of nitrogen content. Part 1: Kjeldahl principle and crude protein calculation.

ISO 7889: 2003. Yogurt. Enumeration of characteristic microorganisms. Colony-count technique at 37 degrees C.

ISO 5534: 2004. Cheese and processed cheese. Determination of the total solids content (Reference method).

ISO 1735: 2004. Cheese and processed cheese products. Determination of fat content -- Gravimetric method (Reference method).

ISO 13580: 2005. Yogurt. Determination of total solids content (Reference method).

ISO 1211: 2010. Milk. Determination of fat content. Gravimetric method (Reference method).

Issaa, A.Y., Volatea, S.R and Wargovich, M.J. 2006. The role of phytochemicals in inhibition of cancer and inflammation: New directions and perspectives. *Journal of Food Composition and Analysis.* 19(5): 405-419.

Jahreis, G., Fritsche, J., Mockel, P., Schbne, F., Moller, U and Steinhart, H. 1999. The potential anticarcinogenic conjugated linoleic acid, cis-9,trans-11 C 18:2 in milk of different species: cow, goat, ewe, sow, mare, women. *Nutrition Research.* 19(10): 1541-1549.

Jain, P., Pareek, A., Ratan, Y., Sharma, S and Paliwal, S. 2013. Free radicals and dietary antioxidants: A potential review. *Int. J. Pharm. Sci. Rev. Res.* 18(7): 34-48.

Jan, A.T., Kamli, M.r., Murtaza, I., Singh, J.B., Ali, A and Haq, Q.M.R. 2010. Dietary flavonoid quercetin and associated health benefits: An overview. *Food Review International*. 26(3): 302-317.

Jayawardena, V.P. 2000. Observations on the ruminal protein degradation products and the absorption of ruminally derived free and peptide-bound amino acids via ovine for estomach epithelia in vitro. A dissertation submitted to Virginia Polytechnic Institute and State University for P.h.D.

Jaworska, D., Waszkiewicz-Robak, W., Kolanowski, W and Swiderski, F. 2005. Relative importance of texture properties in the sensory quality and acceptance of natural yoghurts. *International Journal of Dairy Technology*. 58(1): 39-46.

Jaziri, I., Ben Slama, M., Mhadhbi, H., Urdaci, M.C and Hamdi, M. 2009. Effect of green and black teas (*Camellia sinensis* L.) on the characteristic microflora of yogurt during fermentation and refrigerated storage. *Food Chemistry.* 112(3): 614-620.

Jenkins, T.C., Klein, C.M and Lee, Y.J. 2002. New insights on the pathways of lipid biohydrogenation in the rumen with possible implications on animal performance. 90-105.

Jenkins, T. C and McGuire, M. A. 2006. Major advances in nutrition: Impact on milk composition. *J. Dairy Sci*. 89(4): 1302-1310.

Jenkins, T.C., Wallace, R.J., Moate, P.J and Mosley, E.E. 2008. Board-invited review: Recent advances in biohydrogenation of unsaturated fatty acids within the rumen microbial ecosystem. *J Anim Sci.* 86: 397-412.

Jensen, Robert G.2000. The Composition of Bovine Milk Lipids: January 1995 to December 2000. *J. Dairy Sci.* 85(2):295-350 .

Jia, H.L., Ji, Q.L., Xing, S.L., Zhang, P.H., Zhu, G.L and Wang, X.H. 2010. Chemical composition and antioxidant, antimicrobial activities of the essential oils of Thymus marschallianus Will. and Thymus proximus Serg. Journal of food science. 75(1): 59-65.

Jiang, J., Björck, L and Fondéna, R. 1997. Conjugated linoleic acid in swedish dairy products with special reference to the manufacture of hard cheeses. Int. Dairy Journal. 7(12): 863-867.

Jiménez, A.M., Murcia, M.A., Parras, P and Martínez-Tomè, M. 2008. On the importance of adequately choosing the ingredients of yoghurt and enriched milk for their antioxidant activity. *International Dairy Journal of Food Science and Technology*. 43(8): 1464-1473.

Johnson, M and Law, B.A. 2010. Technology of cheesemaking. Washington, D.C: Blackwell Published.

Jones, E.L., Shingfield, K.J., Kohen, C., Jones, A.K., Lupoli, B., Grandison, A.S., Beever, D.E., Williams, P.C., Calder, P.C and Yacoob, P. 2005. Chemical, physical, and sensory properties of dairy products enriched with conjugated linoleic acid. *J. Dairy Sci*. 88(8): 2923-2937.

Jouany, J.P and Morgavi, D.P. 2007. Use of 'natural' products as alternatives to antibiotic feed additives in ruminant production. *Animal*. 1(10): 1443-1466.

Justesen, U., Knuthsen, P., Andersen, N.L., Leth, T. 2000. Estimation of daily intake distribution of flavonols and flavanones in Denmark. *Food and Nutrition*. 40: 1654-661.

Kahn, A., Szczesniak, S and Earl, L. 1971. Consumer awarness of and attitudes to food texture. *Journal of Texture studies.* 2(3): 280-295.

Kaiser, A., Carle, R and Kammerer, D.R. 2013. Effects of blanching on polyphenol stability of innovative paste-like parsley (*Petroselinum crispum* Mill., *Nym ex A.* W Hill) and marjoram (*Origanum majorana* L.) products. *Food Chemistry.* 138(2-3): 1648-1656.

Karaaslan, M., Ozden, M.,Vardin, H and Turkoglu, H. 2011. Phenolic fortification of yogurt using grape and callus extracts. *Food Science and Technology.* 44(4): 1065-1072.

Karagül-Yüceer, Y., Drake, M.A and Cadwallader, K.R. 2013. Aroma-active Components of Liquid Cheddar Whey. *Journal of Food Science.* 68(4): 1215–1219.

Kemp, M.Q., Jeffy, B.D., and Romagnolo, D.F. 2003. Conjugated linoleic acid inhibits cell proliferation through a p53-dependent mechanism: effects on the expression of G1-restriction points in breast and colon cancer cells. *J Nutr.* 133(11): 3670-3677.

Kerio, L.C., Wachira, F.N., Wanyoko, J.K and Rotich, M.K. 2013. Total polyphenols, catechin profiles and antioxidant activity of tea products from purple leaf coloured tea cultivars. *Food Chemistry.* 136(3-4): 1405–1413.

Kim, E.J., Huws, S.A., Lee, M.R.F and Scollan, N.D. 2009. Dietary transformation of lipid in the rumen microbial ecosystem. *Asian-Aust. J. Anim. Sci*. 22(9): 1341-1350.

Kim, J.H., Kim, C.H and Ko, Y.D. 2002. Influence of dietary addition of dried wormwood (Artemisia sp.) on the performance and carcass characteristics of hanwoo steers and the nutrient digestibility of sheep. *Asian-Aust. J. Anim. Sci*. 15(3): 390-399.

Kim, S.C., Adesogan, A.T., Kim, J.H and Ko, Y.D. 2006. Influence of replacing rice straw with wormwood (*Artemisia montana*) silage on feed intake, digestibility and ruminal fermentation characteristics of sheep. *Animal Feed Science and Technology*. 128(1-2): 1-13.

Kim, S.E and Wijesekara, I. 2010. Development and biological activities of marine-derived bioactive peptides: A review. *Journal of Functional Foods.* 2(1): 1-9.

Kim K.J. Lee H.L. 2004. Complete chloroplast genome sequences from Korean ginseng (*Panax schinseng* Nees) and comparative analysis of sequence evolution among 17 vascular plants. *DNA Research.* 11: 247-261.

Kim, Y.J and Liu, R.H. 2002. Increase of conjugated linoleic acid content in milk by fermentation with lactic acid bacteria. *Journal of Food Science.* 67(5): 1731-1737.

Kindleysides, S., Quek, S-Y and Miller, M.R. 2012. Inhibition of fish oil oxidation and the radical scavenging activity of New Zealand seaweed extracts. *Food Chemistry*. 133(4): 1624-1631.

Kırca, A and Arslan, E. 2008. Antioxidant capacity and total phenolic content of selected plants from Turkey. *International Journal of Food Science and Technology*. 43(11): 2038–2046.

Ko, Y.D., Kim, J.H., Adesogan, A.T., Ha, H.M and Kim, S.C. 2006. The effect of replacing rice straw with dry wormwood (*Artemisia* sp.) on intake, digestibility, nitrogen balance and ruminal fermentation characteristics in sheep. *Animal Feed Science and Technology.* 125(1-2): 99-110.

Korhonen, H and Pihlanto-Leppälä, A. 2004. Bioactive peptides: Production and functionality. *International Dairy Journal*. 16(9): 945-960.

Komprda, T., Dvorak, R., Fialova, M., Šustova, K and Pechova, A. 2005. Fatty acid content in milk of dairy cows on a diet with high fat content derived from rapeseed. *Czech J. Anim. Sci.* 50(7): 311-331.

Konwar, B.K., Ahmed, H.F., Phukan, B., Das, A and Medhi, A.K. 1990. Effect of feeding decaffeinated tea waste (*camellia assamica*) in cross-bred growing calves. *International J. Anim. Sci*. 5: 53

Kotilainen, L., Rajalahti, R., Ragasa, C and Pehu, E. Health enhancing foods: Opportunities for strengthening the sector in developing countries. Discussion Paper 30. Washington, DC: World Bank.

Kumar, V.V., Umar, V., Sharma, V and Bector, B.S. 2006. Effect of ripening on total conjugated linoleic acid and its isomers in buffalo Cheddar cheese. *International Journal of Dairy Technology*. 59(4): 257-260.

Lagouri, V., Bantouna, A and Stathopoulos, P. 2010. A comparison of the antioxidant activity and phenolic content ofnonpolar and polar extracts obtained from four endemic *Lamiaceae* species grown in Greece. *Journal of Food Processing and Preservation.* 34(5): 872-886.

Laskaridis, K., Serafeimidou, A., Zlatanos, S., Gylou, E., Kontorepanidou, E and Sagredos, A. 2013. Changes in fatty acid profile of feta cheese including conjugated linoleic acid. *J Sci Food Agric*. 93(9): 2130-2136.

Leatherhead Food International. 2012.Antioxidant market report. Available in internet: http://www.leatherheadfood.com/antioxidants-market-report.

Lee, K.N., Kritchevsky, D and Pariza, M.W. 1994. Conjugated linoleic acid and atherosclerosis in rabbits. *Atherosclerosis*. 108: 19-25.

Lee,W.J and Lucey, J.A. 2010. Formation and physical properties of yogurt. Asian-Aust. J. Anim. Sci. 23(9): 1127-1136.

Levander, O.A., Fontela, R., Morris, V.C and Ager, A.L. 1995. Protection against murine cerebral malaria by dietary induced oxidative stress. *stress. J Parasitol.* 81(1): 99-103.

Lewinsohn, R. 1984. Mammalian monoamine-oxidizing enzymes with special reference to benzylamine oxidase in human tissues. *Braz J Med Biol Res.* 17(3-4): 223-256.

Lin, L.Z, Harnly, J.J and Wang, C.C. 2007. Screening Method for the Identification of Glycosylated Flavonoids and Other Phenolic Compounds Using a Standard Analytical Approach for All Plant Materials. *Agric Food Chem*. 55: 1084-1096.

Lin, S.Y., Wang, C.C., Lu, Y.L., Wud, W.C and Hou, W.C. 2008. Antioxidant, anti-semicarbazide-sensitive amine oxidase, and anti-hypertensive activities of geraniin isolated from phyllanthus urinaria. *Food and Chemical Toxicology.* 46(7): 2485-2492.

Lin, T.Y., Lin, C.W and Lee, C.H. 1999. Conjugated linoleic acid concentration as afected by lactic cultures and added linoleic acid. *Food Chemistry* 67(1): 1-5.

Liu, H., Xu, X.M and Guo, S.D. 2008. Comparison of full-fat and low-fat cheese analogues with or without pectin gel through microstructure, texture, rheology, thermal and sensory analysis. *International Journal of Food Science and Technology*. 43(9): 1581-1592.

Liu, R., Suna, J., Bi, K and Guoa, D-A. 2005. Identification and determination of major flavonoids in rat serum by HPLC–UV and HPLC–MS methods following oral administration of *Dalbergia odorifera* extract. *Journal of Chromatography B.* 829(1-2): 35-44.

Lobo, V., Patil, A., Phatak, A and Chandra, N. 2010. Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacognosy Reviews*. 4(8): 118-126.

Lovely, M.C and Meullenet, J.F. 2009. Comparison of reference mapping techniques for the optimization of strawberry yogurt. *Journal of Sensory Studies*. 24(4): 457-478.

Lu, W and Foo, Y. 2001. Antioxidant capacity of phenolic phytochemicals from various cultivars of plums. Food Chemistry. 81: 321-326.

Lucey, J.A., Tamehana, M., Singh, H and Munro, P.A. 1998. A comparison of the formation, rheological properties and microstructure of skim milk gels made with a bacterial culture glucono-δ-lacton. *Food Research International*. 31: 147-155.

Luna, P., Bach, A., Juárez, M and de la Fuente, M.A. 2008. Influence of diets rich in flax seed and sunflower oil on the fatty acid composition of ewes' milk fat especially on the level of conjugated linoleic acid, n-3 and n-6 fatty acids. *International Dairy Journal.* 18(1): 99-107.

Luna, P., Juárez, M and de la Fuente, M.A. 2005. Validation of a rapid milk fat separation method to determine the fatty acid profile by gas chromatography. *J. Dairy Sci* 88(10): 3377-3381.

Lykkesfeldt, J, and Svendsen, O. 2007. Oxidants and antioxidants in disease: oxidative stress in farm animals. *Vet J*. 173(3): 502-511.

Lyles, G. A. 1996. Mammalian plasma and tissue-bound semi sensitive Amine Oxidases: Pharmacological and toxicological aspects. *J. Biochem. Cell Bid.* 28(3): 259-214.

Magenis, R.B., Prudencio, E.S., Amboni, R.D.M.C., Júnior, N.G.C., Oliveira, R.V.B., Soldi, V and Benedet, H.D. 2006. Compositional and physical properties of yogurts manufactured from milk and whey cheese concentrated by ultrafiltration. *International Journal of Food Science and Technology.* 41(5): 560-566.

MAGRAMA (2011). Avance Anuario de Estadística 2011. Disponible desde internet:<http://www. magrama.gob.es/es/estadistica/temas/estadpublicaciones/ anuario-de-estadistica/2011/ default.aspx> [consulta: septiembre de 2012].

MAGRAMA (2012). Avance Anuario de Estadística 2011. Disponible desde internet:<http://www. magrama.gob.es/es/estadistica/temas/estadpublicaciones/ anuario-de-estadistica/2012/ default.aspx[consulta: septiembre de 2013].

Majewska, M., Skrzycki, M., Podsiad, M and Czeczot, H. 2011. Evaluation *Pharmaceutica Drug Research.* 68(4): 611-615.

Makkar, H.P.S., Francis, G and Becker, K. 2007. Bioactivity of phytochemicals in some lesser-known effects and potential applications in livestock and aquaculture production systems. *Animal.* 1(9): 1371-1391.

Martín-Diana, A.B., Janer, C., Peláez, C and Requena, T. 2004. Effect of milk fat replacement by polyunsaturated fatty acids on the microbiological, rheological and sensorial properties of fermented milks. *Journal of the Science of Food and Agriculture*. 84(12): 1599-1605.

Martínez-Marín, A.L., Pérez Hernández, M., Pérez Alba, L., Gómez Castro, G and Garzón Sígler, A.I. 2010. Efecto de la grasa de la dieta sobre la grasa láctea de los rumiantes: Una revisión. *Interciencia*. 35(10): 723-729.

Martini, M., Altomonte, I and Salari, F. 2013. Evaluation of the fatty acid profile from the core and membrane of fat globules in ewe's milk during lactation. *Food Science and Technology.* 50(1): 253-258.

Materska, Małgorzata. 2008. Quercetin and its derivates: Chemical structure and bioactivity. *Pol. J. Food Nutr. Sci.* 58(4): 407-413.

Mathew, B.B., Archana, T and Jataw, S.K. 2010. Free Radicals and Antioxidants : A Review. *Journal of Pharmacy Research.* 4(12): 4340-4434.

Mazhri, M., Mesgaran, M.D and Moussavi, A.H 2009. Effect of diet caontaining a variety of Iranian Rapeseed meal (SLM sp.) on high producing lactating Holstein cow responses. *Journal of Animl and Veterinary Advances*. 8(2): 265-269.

McKusick, B.C., Thomas, D.L., Berger, Y.M., and Marnet, P.G. 2002. Effect of milking interval on alveolar versus cisternal milk accumulation and milk production and composition in dairy ewes. *J. Dairy Sci*. 85(9): 2197-2206.

McLeod, R.S., Le Blanc, A.M., Langille, M.A., Mitchell, P.L and Currie, P.L. 2004. Conjugated linoleic acids, atherosclerosis, and hepatic very-low-density lipoprotein metabolism. *Am J Clin Nutr.* 79(6): 1169S-1174S.

Meira, S.M.M., Daroit, D.J., Helfer, V.E., Correa, A.P.F., Segalin, J., Carro, S and Brandelli, L. 2012. Bioactive peptides in water-soluble extracts of ovine cheeses from Southern Brazil and Uruguay. *Food Research International*. 48(1): 322-329.

Mele, M., Contarini, G., Cercaci, Serra, A., Buccioni, A., Conte, M.P., Funaro, A., Banni, S., Lercker, G and Secchiari, P. 2011. Enrichment of Pecorino cheese with conjugated linoleic acid by feeding dairy ewes with extruded linseed: Effect on fatty acid and triglycerides composition and on oxidative stability. *International Dairy Journal.* 21(5): 365-372.

Meydani, M and Ha, S. 2006. Dietary Polyphenols and Obesity. *Nutrients*. 2: 737-751.

Miguel, G., Simões, M., Figueiredo, A.C., Barroso, J.G., Pedro, L.G and Carvalho, L. 2004. Composition and antioxidant activities of the essential oils of *Thymus caespititius, Thymus camphoratus* and *Thymus mastichina*. *Food Chemistry*. 86(2): 183-188.

Mikolayunas, C.M., Thomas, D.L., Dahl, G.E, Gressley, T.F and Berger, Y.M. 2008. Effect of prepartum photoperiod on milk production and prolactin concentration of dairy ewes. *J. Dairy Sci*. 91(1): 85-90.

Miller, J.K., Brzezinska-Slebodzinska, E and Madsen, F.C. 1993. Oxidative stress, antioxidants, and animal function. *J Dairy Sci.* 76(9): 2812-2823.

Mink, P.J., Scrafford, C.G., Barraj, L.M., Harnack, L., Hong, C.P., Nettleton, J.A and Jacobs, D.R. 2007. Flavonoid intake and cardiovascular disease mortality: A prospective study in postmenopausal women. *Am J Clin Nutr.* 85(3): 895-909.

Mishina, V., Gray, J.P., Heckc, D.E., Debra, L., Laskin, D.L and Laskind, J.D. 2010. Application of the Amplex red/horseradish peroxidase assay to measure hydrogen peroxide generation by recombinant microsomal enzymes. *Free Radical Biology & Medicine.* 48(11):1485-1491.

Mistry, V.V and Kasperson, K.M. 1998. Influence of salt on the quality of reduced fat Cheddar cheese. *J. Dairy Sci*. 81: 1214-1221.

Mitjavila, M.T and Moreno, J.J. 2012. The effects of polyphenols on oxidative stress and the arachidonic acid cascade. Implications for the prevention/treatment of high prevalence diseases. *Biochemical Pharmacology.* 84(9): 1113-1122.

Morales, V.M. 2005. Floraliteraria del Quijote alusiones almundo vegetalen las obras completas de cervantes. *Instituto de Estudios Albacetenses "Don Juan Manuel"*. 200.

Morand-Fehr, P. 1981. Nutrition and feeding of goats: Application to the temperate climatic conditions. In: Goat production (ed, Gall, C), London, UK: Academic Press.

Morand-Fehr, P., Fedele, V., Decandia, M and Le Frileux, Y. 2007. Influence of farming and feeding systems on composition and quality of goat and sheep milk. *Small Ruminant Research.* 68(1-2): 20-34.

Morgavi, J.P and Jouany, D.P. 2007. Use of 'natural' products as alternatives to antibiotic feed additives in ruminant production. *Animal* 1(10): 1443-1466.

Morton, L.W., Abu-Amsha Caccetta, R., Puddey, I.B and Croft, K.D. 2000. Chemistry and biological effects of dietary phenolic compounds: Relevance to cardiovascular disease. *Clin Exp Pharmacol Physiol.* 27(3): 152-159.

Mughetti, L., F. Sinesio, F., Acuti, G., Antonini, C., Moneta, E., Peparaio, M., Trabalza-Marinucci, M. 2012. Integration of extruded linseed into dairy sheep diets: Effects on milk composition and quality and sensorial properties of Pecorino cheese. *Animal Feed Science and Technology.* 178(1-2): 27-39.

Nagashima, A.I., Pansiera, P.E., Baracat, M.M and Castro-Gómez, R.J.H. 2013. Development of effervescent products, in powder and tablet form, supplemented with probiotics *Lactobacillus acidophilus* and *Saccharomyces boulardii*. *Food Sci. Technol, Campinas.* 33(4): 605-611.

Nagy, T.O., Solar, S., Sontag, G and Koenig, J. 2011. Identification of phenolic components in dried spices and influence of irradiation. *Food Chemistry.* 128(2): 530-534.

Nalin Siriwardhana, N., Nishan S. Kalupahana, N.S., Cekanova, M., Greer, M.L.B and Moussa, N.M. 2013. Modulation of adipose tissue inflammation by bioactive food compounds. *Journal of Nutritional Biochemistry.* 24(4): 613-623.

Nair, H.B., Sung, B., Yadav, V.R., Kannappan, R., Chaturvedi, M.M and Aggarwal, B.B. 2010. Delivery of antiinflammatory nutraceuticals by nanoparticules for the prevention and treatment of cancer. *Biochemical Pharmacology*. 80(12): 1833-1843.

Nafikov, R.A and Beitz, D.C. 2007. Carbohydrate and Lipid Metabolism in Farm Animals. *J. Nutr*. 137(3): 702-705.

Nedyalka V. Yanishlieva, Emma Marinova, Jan Pokorny. 2006. Natural antioxidants from herbs and spices. *Eur. J. Lipid Sci. Technol.* 108(9): 776-793.

Neves, C. A., Dos Santos, W. B. R., Santos, G. T. D., Da Silva, D. C., Jobim, C. C., Santos, F. S., Visentainer, J. V and Petit, H. V. 2009. Production performance and milk composition of dairy cows fed extruded canola seeds treated with or without lignosulfonate. *Animal Feed Science and Technology*. 154(1-2): 83-92.

Neves, L., Pereira, M.A., Mota, M and Alves, M.M. 2009. Detection and quantification of long chain fatty acids in liquid and solid samples and its relevance to understand anaerobic digestion of lipids. *Bioresource Technology.* 100(1): 91-96.

Nickavar, B and Esbati, N. 2012. Evaluation of the antioxidant capacity and phenolic content of three *Thymus* species. *J Acupunct Meridian Stud.* 5(3): 119-125.

Nicoletta, P., Serafini, M., Colombi, B., Del Rio, D., Salvatore, S., Bianchi, M and Brighenti, F. 2003. Total antioxidant capacity of plant foods, beverages and oils consumed in Italy assessed by three different in vitro assays. *J. Nutr.* 133(9): 2812-2819.

Nicolosi, R.J., Rogers, E.J., Kritchevsky, D., Scimeca, J.A and Huth, P.J. 1997. Dietary conjugated linoleic acid reduces plasma lipoproteins and early aortic atherosclerosis in hypercholesterolemic hamsters. *Artery*. 22: 266-277

Nieto, G., Bñón, S and Garrido, M.D. 2011. Effect of supplementing ewes' diet with thyme (*Thymus zygis* ssp. *gracilis*) leaves on the lipid oxidation of cooked lam meat. *Food Chemistry*. 125(4): 1147-1152.

Nieto, G., Díaz, P., Bañón, S and Garrido, M.D. 2010. Dietary administration of ewe diets with a distillate from rosemary leaves (*Rosmarinus officinalis* L.): Influence on lamb meat quality. *Meat Science*. 84(1): 23-29.

Nijveldt, R., Van Nood, E., Van Hoor, E.C.P., Boelens, G., Van Norren, K., Van Leeuwen, P.A.M. 2001. Flavonoids: a review of probable mechanisms of action and potential applications. *Am Soc Clin Nutr*. 74(4): 48–25.

Niki, E. 2011. Antioxidant capacity: which capacity and how to assess it. *Journal of Berry Research*. 1(4): 169-176.

Norat, E and Riboli, T. 2003. Epidemiologic evidence of the protective effect of fruit and vegetables on cancer risk1,2,3,4. *The American Journal of Clinical Nutrition*. 78(3): 559S-569S.

O'Brien, N.M and O'Connor, T.P. 2004. Nutritional aspects of cheese. In: Cheese: Chemistry, Physics and Microbiology, Volume 2 (eds, Fox, F., McSweeney, P.L.H., Cogan, T.M and Guinee, T.P), pp 420-434. London, UK :Hardcover.

O´Callaghan, T.P and Guinee, D.G. 2004. Rheology and Texture of Cheese. In: Cheese: Chemistry, Physics and Microbiology (ed, Guinee, D.G), pp 395-421. Amesterdam: Elseiver Academic Press.

O'Connell, J.E. and Fox, P.F. 2001. Significance and applications of phenolic compounds in the production and quality of milk and dairy products: A review. *International Dairy Journal*. 11(3): 103-120.

Ong, L., Henriksson, A and Shah, N.P. 2007. Angiotensin converting enzyme-inhibitory activity in Cheddar cheeses made with the addition of probiotic *Lactobacillus casei* sp. *Lait.* 87: 149–165.

Osuna, D.R., Casals, R., Caja, G and Peris, S. 1998. Effects of feeding whole oilseeds to partially replace calcium soaps of fatty acids on dairy ewes intake and milk production and composition. *J. Dairy Sci*. 81(1): 302. (Abstr. 1179).

Ould Eleya, M.M., Desobry Banon, S.D and Hardy, J. 1995. A Comparative study of pH and temperature effects on the acidic coagulation of milks from cows, goats, and sheep. *J Dairy Sci*. 78(12): 2591-2988.

Othmane, M.H., Fuertes, J.A and San Primitivo, F. 1995. Estimacion indirecta del rendimiento quesero individual en ganado ovino. ITEA. 16: 741-743.

Othmane, M.H. 2000. Parametres genetiques de la composition du lait de brebis et du rendement fourrager en laboratoire. PhD Thesis, University of Leon.

Ozcan, T. 2013. Determination of yogurt quality by using rheological and textural parameters. *2nd International Conference on Nutrition and Food Sciences.* 53.

Özer, B.H and Kirmaci, H.V. 2010. Functional milks and dairy beverages. *International Journal of Dairy Technology.* 63(1): 1-15.

Özgen, U., Mavi, A., Terzi, Z., Kazaz, C., Asçı, A., Kaya, Y and Seçen, H. 2011. Relationship between chemical structure and antioxidant activity of luteolin and its glycosides isolated from *Thymus sipyleus* subsp. *sipyleus* var. *sipyleus*. *Rec. Nat. Prod.* 5(1): 12-21.

Palmquist, D.L. 1991. Influence of source and amount of dietary fat on digestibility in lactating cows. *J Dairy Sci*. 74(4): 1354-1360.

Palmquist, D.L. 2006. Milk Fat: Origin of fatty acids and influence of nutritional factors thereon. In: Advanced Dairy Chemistry, Lipids, 3rd edition, (eds, Fox P.F. and McSween P.L.H). New York: Springer.

Palmquist, D. L., Lock, A. L., Shingfield, K. J. and Bauman, D. E. 2005. Biosynthesis of conjugated linoleic acid in ruminants and humans. In: Advances in Food and Nutrition Research, Vol. 50, (ed, Taylor, S.L), pp 179-217. San Diego, CA: Elsevier Inc.

Pandya, A.J and Ghodke, K.M. 2007. Goat and sheep milk products other than cheeses and yoghurt. *Small Ruminant Research.* 68(1-2): 193-206.

Pandey, K.B and rizvi, K.K. 2009. Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative Medicine and Cellular Longevity*. 2(5): 270-278.

Park, Y.W., Júarez, M., Ramos, M and Haenlein, G.F.W. 2007. Physico-chemical characteristics of goat and sheep milk. *Small Ruminant Research.* 68(1-2): 88-113.

Parodi, P. 2005. Dairy product consumption and the risk of breast cancer. *J. Am. Coll. Nutr*. 24:556S-568S.

Pastorino, A.J., Hansen, C.L. and McMahon, D.J. 2003. Effect of pH on the Chemical Composition and Structure-Function Relationships of Cheddar Cheese. *J. Dairy Sci.* 86(9): 2751-2760.

Pellegrini, O., Remeuf, F and Rivemale, M. 1994. Évolution des caractéristiques physico-chimiques et des paramètres de coagulation du lait de brebis collecté dans la région de Roquefort. *Lait*. 74: 425-442.

Pérez-Jiménez, J., Arranz, S., Tabernero, M., Díaz-Rubio, M.E., Serrano, J., Goñi, I and Sauri-Calixto, F. 2008. Updated methodology to determine antioxidant capacity in plantfoods, oils and beverages: Extraction, measurement and expression of results. *Food Research International*. 41(3): 274-285.

Petit, H. 2009. Antioxidants and dairy production: The example of flax. *R. Bras. Zootec*. 38: 352-361.

Petit, H.V., Germiquest, C and Lebel D. 2004. Effect of feeding whole, unprocessed sunflower seeds and flaxseed on milk production, milk composition, and prostaglandin secretion in dairy cows. *J. Dairy Sci.* 87: 3889-3898.

Petrovic, D.M., Skalicki, Z., Bogdanovic, V., Petrovic, M.M., Kurcubic, V. 2005. The Effect of Paragenetic Factors on Performance Traits in Complete Lactations in Simmenual Cows. *Biotechnology in Animal Husbrandy*. 21(5-6): 7-12.

Pihlanto, A. 2006. Lactic fermentation and bioactive peptides. In: Biochemistry, genetics and molecular biology , chapter 13 (ed, Kongo, M). INTECH. http://www.intechopen.com/books/lactic-acid-bacteria-r-d-for-food-health-and livestock-purposes/lactic-fermentation-and-bioactive-peptides

Pinho, O., Mendes, E., Alves, M.M and Ferreira, I. M. P. L. V.O. 2004. Chemical, physical and sensorial characteristics of Terrincho ewe cheese: Changes during ripenning and intravarietal comparation. *J. Dairy Sci*. 87(2): 249-257.

Pinto, G., Caira, S., Cuollo, M., Lilla, S., Chianese, L and Addeo, F. 2013. Bioactive Casein Phosphopeptides in Dairy Products as Nutraceuticals for Functional Foods. In: Milk protein, chapter 1. INTECH. http://cdn.intechopen.com/pdfs-wm/38840.pdf.

Pintus, S., Murru, E., Carta, G., Cordeddu, L., Batetta, B., Accossu, S., Pistis, D., Uda,S., Ghiani, M.E., Mele, M., Secchiari, P., Almerighi, G., Pintus, P and Banni, S.2013. Sheep cheese naturally enriched in α-linolenic, conjugated linoleic and vaccenic acids improves the lipid profile and reduces anandamide in the plasma of hypercholesterolaemic subjects. *British Journal of Nutrition*. 109(8): 1453-1462.

Pottier, J., Focant, M., Debier, C., De Buysser, G., Goffe, C., Mignolet, E., Froidmont, E and Larondelle, Y. 2006. Effect of dietary vitamin E on rumen biohydrogenation pathways and milk fat depression in dairy cows fed high-fat diets. *J. Dairy Sci.* 89(2): 685-692.

Pratt, D.E. 1990. Natural antioxidants not exploited commercially. In: food antioxidant, (ed, Hudson, B.J.F), pp 171-192. Elsevier Science Publishers.

Precht, D.1992. Detection of foreign fat in milk fat I. Qualitative detection by triacylglycerol formulae. European Food Research and Technology. 194(1): 1-8.

Precious, E and Lyles, G.A. 1988. Properties of a semicarbazide-sensitive amine oxidase in human umbilical artery. Journal of Pharmacy and Pharmacology. 40(9): 627-633.

Pereira, O. R., & Cardoso, S. M. (2013). Overview on Mentha and Thymus polyphenols.*Current Analytical Chemistry*. 9(3): 382-396.

Prior, R and Wu, X. 2013. Diet antioxidant capacity: relationships to oxidative stress and Health. *American Journal of Biomedical Sciences*. 5(2): 126-139.

Prior, R.L., Hoang, H., Gu, L., Wu, X., Bacchiocca, M., Howard, L., Hampsch-Woodill, M., Huang, D., Ou, B and Jacob, R. 2003. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity (ORAC)) of plasma and other biological and food samples. *Journal of Agriculture and Food Chemistry*. 51(11): 3273-3279.

Pulido, F., Giráldez, J., Bodas, R., Andrés, S and Prieto, N. 2012. Effect of reduction of milking frequency and supplementation of vitamin E and selenium above requirements on milk yield and composition in Assaf ewes. *J. Dairy Sci*. 95(7): 3527-3535.

Pulido, R., Hernández-García, M and Saura-Calixto, F. 2003. Contribution of beverages to the intake of lipophilic and hydrophilic antioxidants in the Spanish diet. *Eur J Clin Nutr*. 57(10): 1275-82.

Pulina, G., Nudda, A., Battacone, G., Cannas, A. 2006. Effects of nutrition on the contents of fat, protein, somatic cells, aromatic compounds, and undesirable substances in sheep milk. *Animal Feed Science and Technology.* 131(3-4): 255-291.

Pulina, G., Nudda, A. 2004. Milk production. In: Dairy sheep nutrition, (ed Pulina, G) , pp 1-13. Oxfordshire: CABI Publishing.

Rababah, T.M., Banat, F., Rababah, A., Ereifej, K and Yang, W. 2010. Optimization of Extraction Conditions of Total Phenolics, Antioxidant Activities and Anthocyanin of Oregano, Thyme Terebinth, and Pomegranate. *Journal of Food Science*. 75(7): C626-C632.

Rahman, K. 2007. Studies on free radicals, antioxidants, and co-factors. *Clinical Interventions in Aging.* 2(2): 219-236.

Ramchoun, M., Harnafi, H., Alem, C., Büchele, B., Simmet, T., Rouis, M., Atmani, F and Amrani, S. 2012. Hypolipidemic and antioxidant effect of polyphenol-rich extracts from Moroccan thyme varieties. e-SPEN Journal. 7(3): 119-124.

Rauter, A.P., Martins, A., Borges, C., Ferreira, J., Maria-Ros´ario Bronze, J.J., Coelho, A.V., Choi, Y.H and Verpoorte, R. 2005. Liquid chromatography–diode array detection–electrospray ionisation mass spectrometry/nuclear magnetic resonance analyses of the anti-hyperglycemic flavonoid extract of Genista tenera Structure elucidation of a flavonoid-C-glycoside. *Journal of Chromatography A.* 1089(1-2): 59-64.

Raymer, P.L. 2002. Canola: An emerging oilseed crop. In: trends in new crops and new uses, (eds, Janick, J and Whipkey, A), pp 122-126. Alexandria, VA: ASHS Press.

Re, R., Pellegrin, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic Biol Med.* 26(9-10): 1231-1237.

Real decreto 176/2013, de 8 de marzo, por el que se derogan total o parcialmente determinadas reglamentaciones técnico-sanitarias y normas de calidad referidas a productos alimenticios (boe de 29 de marzo de 2013).

Real Decreto 271/2014, de 11 de abril, por el que se aprueba la Norma de Calidad para el yogur o yoghourt.

Reboul, E., Thap, S., Perrot, E., Amiot, M.J., Lairon, D and Borel, P. 2007. Effect of the main dietary antioxidants (carotenoids, c-tocopherol, polyphenols, and vitamin C) on a-tocopherol absorption. *European Journal of Clinical Nutrition.* 61(10): 1167-1173.

Redeuil, K., Bertholet, R., Kussmann, M., Steiling, H., Rezzi, S and Nagy, K. 2009. Quantification of flavan-3-ols and phenolic acids in milk-based fodd products by reversed-phase liquid chromatography-tandm mass spectrometry. *Journal of Chromatography A.* 1216(47): 8362-8370.

Reddy, G.B., Khandare, A.L., Reddy, P.Y., Rao, G.S., Balakrishna, N and Srivalli, I. 2003. Antioxidant defense system and lipid peroxidation in patients with skeletal fluorosis and in fluoride-intoxicated rabbits.*Toxicol Sci*. 72(2): 363-368.

Regulation on the implementation of Law No. 17/2001 of December 7, 2001 on Trademarks (approved by Royal Decree No. 687/2002 of July 12, 2002, and last amended by Royal Decree No. 1431/2008 of August 29, 2008).

Regulation (EC) No. 853/2004 of the European Parliament and of the Council laying down specific hygiene rules for the hygiene of foodstuffs.

Regulation (EC) No 1924/2006 of the European Parliament and of the council of 20 December 2006 on nutrition and health claims made on foods.

Regulation (EC) No 1662/2006 of 6 November 2006 amending Regulation (EC) No 853/2004 of the European Parliament and of the Council laying down specific hygiene rules for food of animal origin.

Regulation (EC) No 92/46/EC laying down the health rules for the production and placing on the market of raw milk, heat-treated milk and milk-based products.

Reis Giada, M.D. 2013. Food Phenolic Compounds: Main Classes, Sources and Their Antioxidant Power. In: Oxidative Stress and Chronic Degenerative Diseases - A Role for Antioxidants, Chapter 4 (ed, Morales-González, J.A). InTech Publisher.

Reynolds, C.K., Cannon, V.L and Loerch, S.C. 2006. Effects of forage source and supplementation with soybean and marine algal oil on milk fatty acid composition of ewes. *Animal Feed Science and Technology.* 131(3-4): 333-357.

Rice-Evans, CA., Miller, NJ., Paganga, G. 1997. Structure–antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic Biol Med.* 20(7): 933-956.

Rincóna, F., Morenoa, R., Zureraa,G and Amaro, M. 1994. Mineral composition as a characteristic for the identification of animal origin of raw milk. *Journal of Dairy Research.* 61(1): 151-154.

Roby, M.H.H, Sarhana, M.A., Selima, K.A-H and Khalela, K.I. 2013. Evaluation of antioxidant activity, total phenols and phenolic compounds in thyme (*Thymus vulgaris* L.), sage (*Salvia officinalis* L.), and marjoram (*Origanum majorana* L.) extracts. *Industrial Crops and Products.* 43: 827-831.

Rodríguez-Alcalá, L.M and Fontecha, J. 2010. Major lipid classes separation of buttermilk, and cows, goats and ewes milk by high performance liquid chromatography with an evaporative light scattering detector focused on the phospholipid fraction. *Journal of Chromatography A.* 1217: 3063-3066.

Rodríguez-Alcalá, L.M., Hart, F and Fontecha, J. 2009. Fatty acid profile and CLA isomers content of cow, ewe and goat milks processed by high pressure homogenization. *Innovative Food Science and Emerging Technologies.* 10 (10): 32-36.

Rodríguez, D., Rocha-Santos, T.a.p., Freitas, A.C., Gomes, A.M.P and Duarte, A.C. 2012. Analytical strategies for characterization and validation of functional dairy foods. *Trends in Analytical Chemistry*. 41:27-45.

Rodríguez Ruiz, L.A. 2013. Analisis de la rentabilidad en las explotaciones de ovino de leche de Castilla y Leon. PhD Thesis.

Rombaut, R and Dewettinck, K. 2006. Properties, analysis and purification of milk polar lipids. International Dairy Journal. 16(11): 1362-1373.

Royal Decree No. 687/2002 of 12 July adopting the Regulations implementing Trademark Law No. 17/2001 of December 7, on Trade Marks.

Royal Decree No. 1113/2006 cheeses and melted cheeses quality regulation

Russo, M., Spagnuolo, C., Tedesco, I., Bilotto, S and Russo, G.L. 2012. The flavonoid quercetin in disease prevention and therapy: Facts and fancies. *Biochemical Pharmacology.* 83(1): 6-15.

Sánchez-Gómez, P, López Espinosa, J.A., Sánchez Saorín, F.J and Valverde, R.M. 2004. *Thymus* x *faustinoi*, híbrido nuevo del sureste de la Península Ibérica. *Anales de Biología* 26: 175-178.

Sánchez-Moreno, C. 2002. Review: Methods used to evaluate the free radical scavenging activity in foods and biological systems. *Food Sci Tech Int.* 8(3): 121-137.

Sanz Sampelayo, M.R., Chilliard, Y., Schmidely, Ph and Bozaa, J. 2007. Influence of type of diet on the fat constituents of goat and sheep milk. *Small Ruminant Research.* 68(1-2): 42-63.

Sanz Sampelayo, M.R., Martín Alonso, J.J., Pérez, L., Extremera, F.G and Boza, J. Dietary supplements for lactating goats by polyunsaturated fatty acid-rich protected fat: Effects after supplement withdrawal. *J. Dairy Sci* 87(6): 1796-1800.

Sarrazin, P., Mustafa, A.F., Chouinard, P.Y., Raghavan, G.S.V and Sotocinal, S.A. 2004. Performance of dairy cows fed roasted sunflower seed. J. *Sci. Food Agric*. 84 (10): 1179-1185.

Saxelin, M., Corpela, R and Marya-Makinen, A. 2003. Functional dairy product. In Dairy Processing (ed, Smit, G), pp 229-260. Cambridge: Woodhead Publishing.

Scalbert, A and Williamson, G. 2000. Dietary intake and bioavaiability of polyphenols. *Journal of Nutrition.* 130(8): 2073S-2085S.

Scandalios, J.G. 2005. Oxidative stress: molecular perception and transduction of signals triggering antioxidant gene defenses. *Brazilian Journal of Medical and Biological Research.* 38(7): 995-1014.

Scintu, M.F and Piredda, G. 2007. Typicity and biodiversity of goat and sheep milk products. *Small Ruminant Research.* 68(1-2): 221-231.

Scolozzi, C., Martini, M. and Abramo, F. 2003. A method for identification and characterization of ewe's milk fat globules. Milchwissenschaft . 58(9): 490-493.

Seaman, D.R. 2002. The diet-induced proinflammatory state: A cause of chronic pain and other degenerative diseases. *J Manipulative Physiol.* 25(3): 168-179.

Sengupta, A and Bhattacharjee, S. 2009. Cardamom (*Elettaria cardamomum)* and its active constituent, 1,8-cineole. In: Molecular targets and therapeutic uses of spices (Modern Uses for Ancient Medecines), (eds, Aggarwal, B.B and Kunnumakkara, A.B). Singapore, London: WorldScientific Publishing.

Serafeimidou, A., Zlatanos, S., Kritikos, G and Tourianis, A. 2013. Change of fatty acid profile, including conjugated linoleic acid (CLA) content during refrigerated storage of yogurt made of cow and sheep milk. *Journal of Food Composition and Analysis.* 31(1): 24-30.

Serafini, M., Testa, M.F., Villaño, D., Pecorari, M., van Wieren, K., Azzini, E., Brambilla, A and Maiani, G. 2009. Antioxidant activity of blueberry fruit is impaired by association with milk. *Free Radic Biol Med*. 46(6): 769-774.

Sevi, A., Taibi, L., Albenzio, M., Muscio, A., Annicchiarico, G. 2000. Effect of parity on milk yield, composition, somatic cell count, renneting parameters and bacteria counts of Comisana ewes. *Small Ruminant Research.* 37(1-2): 99-100.

Sgorlon, S., Stradaioli, G., Zanin, D and Stefanon, B. 2006. Biochemical and molecular responses to antioxidant supplementation in sheep. *Small Ruminant Research.* 64(1-2): 143-151.

Shah, N. P. 2003. Probiotics and prebiotics. *Agro. Food Ind. Hi. Tech*. 15: 13-16.

Shan, B., Cai, Y.Z., Sun, M and Corke, H. 2005. Antioxidant capacity of 26 spice extracts and characterization of their phenolic constituents. *J. Agric. Food Chem.* 53(20): 7749-7759.

Sharma, S.C. 1996. Applied multivariate techniques. New York: JohnWiley and Sons Inc.

Shen, Q., Zhang, B., Xu, R., Wang, Y., Ding, X. and Li, P. 2010. Antioxidant activity in vitro of selenium contained protein from the se-enriched. Bifodobacterium animalis 01. *Anaerobe*. 16: 380- 386.

Shingfield, K.J., Sæbø, A., Sæbø, P.C., Toivonen, V and Griinari, J.M. 2009. Effect of abomasal infusions of a mixture of octadecenoic acids on milk fat synthesis in lactating cows. *J. Dairy Sci*. 92(9): 4317-4329.

Shingfield, K.J., Chilliard, Y., Toivonen, V., Kairenius, P and Givens, D. I. 2008. Trans fatty acids and bioactive lipids in ruminant milk. In: Bioactive components of milk. (ed, Bösze, Z), pp. 3-65. New York, NY: Springer.

Shultz, T.D., Chew, B.P and Seaman, W.R. 1992. Differential stimulatory and inhibitory responses of human MCF-7 breast cancer cells to linoleic acid and conjugated linoleic acid in culture. *Anticancer research*. 12:2143-2146.

Sieber, R., Collomb, M., Aeschlimann, A., Jelen, P and Eyer, H. 2004. Impact of microbial cultures on conjugated linoleic acid in dairy products. *International Dairy Journal.* 14(1): 1-15.

Sies, H. 1997. Oxidative stress: Oxidants and antioxidants. *Exp Physiol*. 82: 291-295.

Sies, H. 1997. Physiological society symosium: Impaired endothelial and smooth muscle cell function in oxidative stress. *Experitnental Physiology*. 82: 291-295.

Singleton, V.L,, Orthofer, R and Lamuela-Raventos, R.M. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Meth Enzymol*. 299: 152-178.

Siriwardhana, N., Kalupahana, N.S., Cekanova, M., LeMieux, M., Greer, B and Moustaid-Moussa, N. 2013. Modulation of adipose tissue inflammation by bioactive food compounds. *Journal of Nutritional Biochemistry*. 24: 613-623.

Siró, I., Kápolna, E., Kàpolna, B and Lugasi, A. 2008. Functional food. Product development, marketing and consumer acceptance: A review. *Appetite*. 51(3): 456-467.

Sivakumaran, M. 2004. Dynamics of white blood cell retention by white blood cell removal filters. *Transfusion*. 44(1): 1271-1272.

Sloan, A.E. 2000. The top ten functional food trends. Food Technology. 54: 33-62.

Sodini, I., Montella, J and Tong, P.S. 2004. Physical properties of yogurt fortified with various commercial whey protein concentrates. *Science of Food and Agriculture*. 85(5): 853-859.

Sørensen, A-D.M., Petersen, L.K., de Diego, S., Nielsen, N.S., Lue, B-M., Yang, Z , Xu, X and Jacobsen, C. 2012. The antioxidative effect of lipophilized rutin and dihydrocaffeic acid in fish oil enriched milk. *Eur. J. Lipid Sci. Technol.* 114(4): 434-445.

Souza Cruz, R., Camilloto, G.P and Dos Santos Pires, A.C. 2012. Oxygen Scavengers: An Approach on Food Preservation. *Structure and Function of Food Engineering.* Chapter 2.

Spence, J. T. 2006. Challenges related to the composition of functional foods. *Journal of Food Composition and Analysis.* 19: S4-S6.

Skrede, G., Bryhn, V., Aaby, K., Skivik Jorgensen, A and Birkeland, S.E. 2004. Antioxidative properties of commercial fruit preparations and stability of bilberry and black currant extracts in milk products. *Sensory and Nutritive Qualities of Food* 69(9): S351-S356.

Sun, T and Tanumihardjo, S. A. 2007. An integrated approach to evaluate antioxidant capacity. *Journal of Food Science.* 72(9): 159-165.

Szakály, Z., Szente, V., Kövér, G., Polereczki, Z and Szigeti, O. 2012. The influence of lifestyle on health behavior and preference for functional foods. *Appetite.* 58(1): 406-413.

Szczesniak, A.S and Kahn, E.L. 1971. Consumer awareness of and attitudes to food texture. *Journal of Texture Studies*. 2(3): 280-295.

Tamime, A.Y., Wszolek, M., Božanić, R and Özer, B. 2011. Popular ovine and caprine fermented milks. Small Ruminant Research. 101(1-3): 2-16.

Tamime, A.Y and Robinson, R.K. 1999. Background to Manufacturing Practice. In: Yogurt: Science and Technology, pp. 6-47. Cambridge, UK: Wodhead Publishing.

Tejada, L., Gómez, R and Fernádez-Salguero, J. 2007. Sensory characteristics of ewe milk cheese made with three types of coagulant: Calf rennet, powdered vegetable coagulant and crude aqueous exctract from Cynara Cardunculus. *Journal of Food Quality*. 30(1): 91-103.

Tijerina-Sáenz, A., Innis, S.M and Kitts, D.D. 2009. Antioxidant capacity of human milk and its association with vitamins A and E and fatty acid composition. *Acta Pediatrica*. 98(11): 1793-1798.

Trouillas, P., Claude-Alain, C., Daovy-Paulette, A., Simon, A., Marfak, A and Delgate, C. 2003. Antioxidant, anti-anti-inflammatory and antiproliferative properties of sixteen water plant extracts used in the limousin country side as herbal teas. *Food chemistry*. 80(3): 399-407.

Tsiplakou, E and Zervas, G. 2008. Comparative study between sheep and goats on rumenic acid and vaccenic acid in milk fat under the same dietary treatments. *Livestock Science.* 119: 87-94.

Tsuda, T., Watanabe, M., Katsumi Ohshima, K., Yamamoto, A., Kawakishi, S and Osawa, T. 1994. Antioxidative Components Isolated from the Seed of Tamarind (*Tamarindus indica* L.). *J. Agric. Food Chem.* 42(12): 2671-2674.

Turumtaya, E.A., İslamoğlu, F., Derya, C., Avus¸ A., Huseyin, S., Turumtayc, A.H and Vanholmeda, B. 2014. Correlation between phenolic compounds and antioxidant activity of Anzer tea (*Thymus praecox* Opiz subsp. *caucasicus* var. *caucasicus*). *Industrial Crops and Products*. 52: 687-694.

Ubertalle, A. 1989. Sheep milk: quality and factors of variation. Il Mondo Del Latte. 7: 278-288.

Ugarte, E., Serrano, M., De la Fuente, L.F., Pérez Guzmán, M.D., Alfonso, L., Gutiérrez, J.P. (2002). Situación actual de los programas de Mejora Genética en Ovino de Leche. *ITEA.* 98: 102-117.

Ulla, J. 2000. Negative atmospheric pressure chemical ionisation low-energy collision activation mass spectrometry for the characterisation of flavonoids in extracts of fresh herbs. *Journal of Chromatography A.* 902(2): 369-379.

Vandeweghe, P., Norquist, P and Flyn, E. 2001. U.S. Patent No. 6,39,12 B2. Washington, DC: U.S. Patent and Trademark Ofice.

Vauzour, D., Rodriguez-Mateos, A., Corona, G., Oruna-Concha, M.J and Spencer, J.P.E. 2010. Polyphenols and human health: Prevention of disease and mechanisms of action. *Nutrients.* 2(11): 1106-1131.

Vázquez-Anón, M and Jenkins, T. 2007. Effects of feeding oxidized fat with or without dietary antioxidants on nutrient digestibility, microbial Nitrogen, and fatty acid metabolism. *J. Dairy Sci*. 90(9): 4361-4867.

Vázquez-Anón, M., Nocek, J., Bowman, G., Hampton, T., Atwell, C., Vázquez, P and Jenkins, T. 2008. Effects of feeding a dietary antioxidant in diets with oxidized fat on lactation performance and antioxidant status of the cow. *J. Dairy Sci*. 91(8): 3165-3172.

Vertuani, S., Angusti, A and Manfredini, S. 2004. The antioxidants and pro-antioxidants network: An overview. *Current Pharmaceutical Design.* 10(14): 1677-1694.

Veskoukis, A.S., Tsatsakis, A.M and Kouretas, D. 2012. Dietary oxidative stress and antioxidant defense with an emphasis on plant extract administration. *Cell Stress and Chaperones*. 17: 11-21.

Vitas, S.J., Malbasa, R.V., Grahovac, J.A and Loncar, E.S. 2012. The antioxidantactivity of kombucha fermented milk productswith stinging nettle and winter savory. *Chemical Industry and Chemical engineering Quarterly*. 19(1): 129-139.

Vivar-Quintana, A.M., De La Mano, E.B and Revilla, I. 2006. Relationship between somatic cell counts and the properties of yoghurt made from ewes' milk. *International Dairy Journal*. 16 (3): 262-267.

Wang, W., Wu, N., Zu, Y.G and Fu, Y.J. 2008. Antioxidative activity of Rosmarinus officinalis L. essential oil compared to its main components. *Food Chemistry*. 108(3): 1019-1022.

Wang, Y., Douglas, G. B., Waghorn, G.C., Barry, T.N. and Foote, A.G. 1996. Effect of condensed tannins in Lotus corniculatus upon lactation performance in ewes. *J. Agric. Sci*. 126: 353-362.

Wang, Y.M., Wang, J.H., Wang, C., Chen, B., Liu, J.X., Cao, H., Guo, F.C and Vázquez-Añón, M. 2012. Effect of different rumen-inert fatty acids supplemented with a dietary antioxidant on performance and antioxidative status of early-lactation cows. *J. Dairy Sci.* 93(8): 3738-3745.

Wang, Y.M., Wang, J.H., Wang, C., Wang, J.K., Chen, B., Liu, J.X., Cao, H and Guo, F.C. 2010. Effect of Dietary Antioxidant and Energy Density on Performance and anti-oxidative Status of Transition Cows. *Asian-Aust. J. Anim. Sci*. 23(10): 1299-1307.

Wilkins, R.J and Jones, R. 2000. Alternative home-grown protein sources for ruminants in the United Kingdom. *Anim. Feed Sci. Tech*. 85: 23-32.

William, G., Christen, S., Debra, A., Schaumberg, S., Glynn, R.J and Buring, J.E. 2011. Dietary-3 fatty acid and fish intake and incident age-related macular degeneration in women. *Arch Ophthalmol . 129(7).*

Wojdyło, A., Oszmianski, J and Czemerys, R. 2007. Antioxidant activity and phenolic compounds in 32 selected herbs. *Food Chemistry.* 105: 940-949.

Wong, S.P., Leong, L.P and Koh, J.H.W. 2005. Antioxidant activities of aqueous extracts of selected plants. *Food Chemistry.* 99(4): 775-783.

Wood, J and Bitman, D.L. 1990. Changes in milk fat phospholipids during lactation. *J Dairy Sci.* 73(5): 208-216.

Yanishielva. N., Pokorny, J and M. Gordon. 2006. Antioxidant in foods. *Food Chem*. 77: 503-520.

Ye, M., Ren, L., Wu, Y., Wang, Y and Liu, Y. 2013. Quality characteristics andantioxidant activity of hickory-black soybean yogurt. *Food Science and Technology*. 51(1): 314-318.

Yin, H and porter, N.A. 2005. New insights regarding the autoxidation of polyunsaturated fatty acids. *Antioxid Redox Signal*. 7(1-2): 170-84.

Yoon, J.H and Baek, S.J. 2005. Molecular targets of dietary polyohenols with anti-inflammatory properties. *Yonsei Medical Journal*. 46(5): 585-596.

Yu, P.H and Deng, Y.L. 2000. Potential cytotoxic effect of chronic administration of creatine, a nutrition supplement to augment athletic performance. *Med Hypothesis* 54(5): 726-728.

Zampatti, S., Ricci, F., Cusumano, A., Tonino, M., Novelli, G and Giardina, E. 2013. Review of nutrient actions on age-related macular degeneration. *Nutrition Research*. 4(2): 95-105.

Zhang, R.H., Mustafa, A.F and Zhao, X. 2006a. Effects of flaxseed supplementation to lactating ewes on milk composition, cheese yield, and fatty acid composition of milk and cheese. *Small Ruminant Research*. 63: 233-241.

Zhang, R.H., Mustafa, A.F and Zhao, X. 2006b. Effects of feeding oilseeds rich in linoleic and linolenic fatty acids to lactating ewes on cheese yield and on fatty acid composition of milk and cheese. *Animal Feed Science and Technology*. 127: 220-233.

Zhang, R.H., Mustafa, A.F., Zhao, X. 2007. Effects of feeding oilseeds on nutrient utilization by lactating ewes. *Small Rumin. Res*. 67(2-3): 307-311.

Zulueta, A., Maurizi, A., Frígola, A., Esteve, M.J., Coli, R and Burini, G. 2009. Antioxidant capacity of cow mil, whey and deproteinized milk. *International Dairy Journal*. 19(6-7): 380-385.

INIA
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria

INSTITUTO TECNOLÓGICO AGRARIO
Junta de Castilla y León
Consejería de Agricultura y Ganadería

CSIC
Consejo Superior de Investigaciones Científicas