UNIVERSITÀ DEGLI STUDI DI SASSARI

SCUOLA DI DOTTORATO DI RICERCA
Scienze e Biotecnologie
dei Sistemi Agrari e Forestali
e delle Produzioni Alimentari

Indirizzo in Scienze e Tecnologie Zootecniche

Ciclo XXV

Nutritional value and utilization of plants with antimicrobial properties as components of the diet of dairy sheep

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A Fabio

Ringraziamenti

Questa tesi segna la conclusione di un percorso al quale hanno partecipato numerose persone che standomi vicine, mi hanno aiutato a dare il meglio durante tutto il dottorato di ricerca.

Per questo motivo vorrei ringraziare:

IlProf. Antonello Cannas, relatore di questa tesi, che mi ha consentito la realizzazione di questo lavoro introducendomi come parte attiva in un importante progetto Europeo ed essere stato una costante guida durante tutto il mio percorso di dottorato;

il Prof. Giuseppe Pulina, direttore della Sezione di Scienze zootecniche e della Scuola di dottorato per avermi messo a disposizione i mezzi necessari per portare a termine questo importante traguardo;

il Prof. Nicolò P.P. Macciotta, in qualità di coordinatore di questo corso di dottorato di ricerca, per la sua assistenza durante tutto il dottorato di ricerca;

il Prof. Pier Giacomo Rassu, il dott. Corrado Dimauro e il dott. Gianni Battacone, la dott.ssa Anna Nudda, il dott. Antonio Pais per la loro disponibilità e cortesia dimostrate durante questi tre anni.

Un affettuoso ringraziamento va a tutti i ricercatori, dottorandi e tesisti che mi hanno aiutato durante le prove sperimentali e hanno reso divertenti e piacevoli le giornate di lavoro:

la dott.ssa Maria Grazia Manca che è stata indispensabile durante i difficili momenti in laboratorio spiegandomi e aiutandomi costantemente;

la dott.ssa Roberta Boe per il suo preziosissimo e costante aiuto in laboratorio;

i dott.ri Gabriella Serra, Ligia Barreto, Oscar Boa ventura Neto anch'essi indispensabili per il raggiungimento di questo importante traguardo, che con la loro simpatia e amicizia, mi sono stati sempre vicini sia durante i momenti bui del lavoro che nei momenti felici di svago.

Ringrazio tutti gli altri colleghi e colleghe del dottorato i dott.ri Matteo Sini, Alberto Stanislao Atzori, Giusitno Gaspa, Claudio Carzedda, Gianfranco Gaias, Elia Pintus, Gabriele Marras, Fabio correddu, Roberto Steri e le dott.sse Maria Annunziata Pintus, Sara Saba, Maura Lovicu, chiara Sulas e Natalia Castañares Castro per l'aiuto, la simpatia, l'amicizia e il supporto forniti. Vorrei inoltre ringraziare Tutto il Personale Tecnico della Sezione di Scienze Zootecniche per l'attiva e indispensabile collaborazione nelle prove sperimentali, in particolare i sig.ri Roberto Rubattu e Antonio Mazza; la dott.ssa Ana Francesconi per i suoi consigli e la sua disponibilità durante lo svolgimento della tesi di dottorato. Ringrazio inoltre i Sig.ri Gavino Biddau e Emilio Usai per l'aiuto nello svolgimento delle pratiche amministrative. Un Enorme Grazie alla mia Famiglia che sempre mi ha incoraggiata e sostenuta.

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ABSTRACT

The aim of these thesis was to evaluate the nutritional characteristics and the productive and health effects of several aromatic plants with known high antimicrobial activity supplied to sheep.

The review of the literature explored the characteristics and nutritional the effects of aromatic plants and of their extracts.

In the first chapter, 18 herbs with known antimicrobial activity -- *Achillea millefolium L.* (aerial parts), *Anethum graveolens L.* (seeds), *Carum carvi L.* (seeds), *Chamaemelum nobile All.* (aerial parts), *Coriandrum sativum L.*(seeds), *Hyssopus officinalis L.* (aerial parts), *Lavandula angustifolia Mill. (*vegetative parts*), Lavandula angustifolia Mill*. (flowers), *Melissa officinalis L*. (vegetative parts), *Ocimum basilicum L*. (vegetative parts), *Origanum vulgare L*. (vegetative parts), *Pimpinella anisum L*. (seeds), *Pinus Silvestris L*. (young shoots), *Rosmarinus officinalis L.* (vegetative parts), *Salvia officinalis L*. (vegetative parts), *Satureja montana L.* (vegetative parts), *Thymus serpyllum L.* (vegetative parts) and *Thymus vulgaris L.* (vegetative parts) – were evaluated to asses their nutritional characterisitics and values utilizing chemical analysis and in vitro fermentation studies. Based on the results of this Chapter, 7 plant species were selected for further studies. In the second and in the third chapters these 7 species were used in two experiments to assess their palatability when used alone or mixed with other concentrates. Based on the results of these studies, one plant was discarded and 5 plants were used in a productive short term experiment. Indeed, the fourth chapter deals with the experiments carried out on lactating ewes in order to evaluate the intake of the mixes including the plants, and the

effects on milk production and composition, health status of the animals, and rumen function. Based on the results of the short term productive experiment, 3 plants were considered superior: *Carum carvi, Coriandrum sativum* and *Satureja Montana*.

These plants need to further studies to asses their productive and health effects when used in long term studies and to assess their ability to transfer active microbial compounds to the milk and the cheese.

INTRODUCTION

Defects of cheese due to bacterial and fungal infections

Cheeses, especially those from raw milk, can have defects due to contamination by fungi and bacteria that degrade the product, with unpleasant economic consequences.

The defects of cheeses can be derived from several factors, such as: i) incorrect animal feeding or use of milk derived from diseased animals (e.g. mastitis); ii) use of milk contaminated before or during the course of processing; iii) incorrect processing techniques, and iv) use of facilities unfit for milk processing, maturation and aging of cheese.

The most common cheese defects are swelling, mold, bitter or acidic tastes, and dough hardness of the dough.

Swelling is the most serious defect that can be found in dairy livestock cheese: swollen cheese has a rounded shape due to the production of gases, such as carbon dioxide and hydrogen, produced by gas generating microbes. Swelling is classified as early or late depending on whether it occurs in the first days after manufacture or during ripening (Vizzardi and Maffeis, 1990). In particular, early swelling occurs in soft semi-hard and hard cheeses, due to milk contamination with microbes of the *Coli-Aerogenes* group (i.e. *Escherichia* and *Aerobacter* genera), which attack lactose producing lactic acid and also considerable quantities of carbon dioxide or hydrogen. The dough becomes spongy, does not allow bleeding and acquires unpleasant tastes and odors. Delayed swelling is a typical defect of cooked and long-aged cheeses. It occurs after the transformation of lactose into lactic acid, even after 15 days or months of cheese

making. The microbial agent of delayed swelling is *Clostridium tyrobutirricum*, which attacks lactate giving origin to butyric acid, carbon dioxide and hydrogen. The cheese presents irregular holes, ball-shaped and cracked dough, which can change the texture and give a rancid taste. It should be noted that the spores of *Clostridium* resist to pasteurization (Vizzardi and Maffeis, 1990).

To prevent late swelling it is necessary to prevent milk contamination with soil or silage, where Clostridia grows, lowering the pH with lactic acid bacteria to prevent the development of this microbe (Vizzardi and Maffeis, 1990).

Another problem of cheese is the presence of fungi which is always facilitated by an excess of humidity in the cheese ripening environment. If they remain outside the cheese rind they are almost never harmful and can be easily removed by washing the cheese periodically. If, instead they are located inside of the cheese, they cause a major depreciation of the product due to the presence of air bubbles in the dough or due to cracking and breaking of the skin. Therefore, the curd must be well compressed during the working phase, to prevent air bubbles from remaining in the dough, and the humidity of the seasoning rooms must be controlled. In longer cheese-maturation periods, it is useful to treat the crust with oil (ash oil) and tomato paste. Fungi can be prevented and eliminated with proper drainage of the curd and salting (the salt has an inhibitory action) and with the use of potassium sorbate as an anti-mold

Another defect during the maturation of sheep cheese, which should not be underestimated, is the attack of parasites such as mites and flies (*Piophila casei*). Mites make powdered cheese, whereas flies lay their eggs in the dough, from which

the so-called worms of cheese originate, being almost impossible to sell Pecorino cheese with these characteristics (Portolano, 1986).

A very serious cheese defect is the presence of antibiotic residues in milk, caused by errors in therapeutic dosage or withdrawal time. These substances negatively select the bacterial flora of milk and can promote the development of *E. coli* and the formation of loosely compacted clots, which take longer to aggregate, bleed with difficulty and are, therefore, more susceptible to fungi and other defects. In extreme cases, if antibiotics residues are at very high levels, the milk does not even coagulate under the action of rennet (Nisha, 2008).

Antibiotics in livestock production

In livestock production systems, antibiotics are commonly used to treat and prevent diseases in order to achieve the highest levels of production from the animals. For the last 35 years, the use of antibiotics as feed additives has been effective in increasing animal weight gain and feed conversion and reducing environmental pollution (Vanbelle, 2002).

Antibiotics are used to improve animal growth performance and feed efficiency, but their indiscriminate use can cause toxicity in consumers. In fact, in recent years, the use of antibiotics is not be permitted in feed intended for human consumption, because of both the observed increase of drug resistance among pathogenic microorganisms and the risk of the presence of residues derived from drugs in human feed (Nisha, 2008).

The World Health Organization (WHO) and the Feed and Agriculture Organization

(FAO) have established tolerance levels for drugs, pesticides or other chemicals in relevant tissues of feed producing animals. Tolerance is the tissue concentration below which a marker residue for the drug or chemical must fall in the target tissue before that edible tissue of the animal is considered safe for human consumption. The maximum values allowed are based on extensive toxicological studies of potential hazards of consumption to human (Nisha, 2008).

To ensure feed safety, the Regulation (EC) n. 470/2009 (European Union, 2009), classifies the pharmacologically active substances used in veterinary medicine, determines the level permitted in feedstuffs of animal origin and establishes maximum residue limits of veterinary medicinal products in these feeds.

Scientists are seeking efficient solutions to reduce the use of drugs in animal husbandry in order to respect the residue limits permitted by law. For example, ruminant microbiologists and nutritionists have been exploring alternative methods of favorably altering rumen metabolism to improve feed efficiency and animal productivity (Benchaar et al., 2008).

From antiquity until the early 20th century, aromatic plants were widely used in medicine as drugs afterwards, their use rapidly decreased due to the introduction of novel and effective synthetic medicines that were cheaper and had benefits to human health (Greathead, 2003).

Recently, the pharmaceutical and scientific communities have increased their interest for medicinal plants, because some plants had antimicrobial activity, without causing resistance in some drug-resistant micro-organisms. This has led researchers to

investigate their mechanisms of action and to isolate their active compounds (Ncube et al, 2008).

Plant extracts are very important in this regard, because they produce saponins and tannins, which have antimicrobial properties (Wallace, 2004). Plant secondary metabolites are named "Essential Oils" (EO), which have an antimicrobial activity that makes them potential alternatives to antibiotics, in order to manipulate microbial activity in the rumen with consequent improvement of feed efficiency (Benchaar et al., 2008).

The antimicrobial properties of EO against a wide range of germs, including bacteria, protozoa and, fungi have been widely demonstrated (Dean and Ritchie, 1987; Sivropoulou et al., 1996; Chao et al., 2000). Essential oils have also been exploited for their activity against a wide variety of feed-borne pathogens. For example, oregano oil and its two main components, carvacrol and thymol, inhibited *Escherichia coli* (O157:H7) (Helander et al., 1998; Elgayyar et al., 2001; Janczyk et al., 2008).

In recent years, the emergence of multi-drug resistant bacteria and the risk that this resistance represents to human health have stimulated the interest of pharmacologists on the use of plant extracts (Benchaar et al., 2008).

Aromatic plants

Aromatic plants are defined as those characterized by one or more active ingredients and essential oils which confer special aromas or flavors. These herbs are used in the production of beverages, perfumes, cosmetics, or as a seasoning for feeds. In nature the active substances produced by the plant itself serve to protect it against phytophagous insects, to stimulate plant metabolism and to attract pollinating insects during anthesis, facilitating the process of pollination of flowers.

The aromatic can be present throughout the plant or in some parts of it such as seeds, roots or leaves. The aromatic plants present structures and tissues which secrete secondary metabolites. These active ingredients are essential compounds for the qualitative characterization of these plants. These compounds are not widespread in the plant kingdom, being usually found in a species or in a group of closely related species (Raven, 2002). The secondary metabolites are produced in the flowers, leaves, fruits, seeds, roots, bulbs, rhizomes, and bark (Figueiredo et al., 2008).

In the 1980 the World Health Organization (WHO) defines "*medicinal plant*" as any plant that contains, in one or more of its organs, substances that can be used for therapeutic purposes or which are precursors of chemo-pharmaceutical synthesis.

The term "medicinal plants" comprises all species of plants with aromatic substances that are used in medicine, cosmetics, liquor, culinary, and also for the production of dyes, tanning agents and insecticides (Scarpa, 2009).

The active substances which are produced by aromatic herbs have a different chemical nature. The principal ones are alkaloids, glycosides, tannins, glucosinolates, vitamins and minerals, resins and balsams, lectins, and essential oils.

Alkaloids

It is an important and large group of nitrogen-containing bases produced by plants. These substances are structurally complex and pharmacologically active at very low doses too, are often toxic and have a low therapeutic index. They can be used in therapy as such.

Alkaloids are not common in the inferior plants but are found frequently in fungi (derived from *Claviceps purpurea*) and Dicotyledons, and rarely in Monocotyledons (Firenzuoli, 2002). They undergo compositional changes into the plant, and can protect the plant against viruses, several microorganisms, insects, animals and also physical agents. With the exception of steroidal alkaloids, alkaloids are synthesized from amino acids. The first alkaloid, isolated in 1803, was the morphine, and the following were caffeine and nicotine (Firenzuoli, 2002)

Heterosides

The *Heterosides* are complex molecules characterized by the combination of a sugar (glucose, fructose, rhamnose, galactose or arabinose) with a non-sugar molecule (genina), being also called *glycosides* (Firenzuoli, 2002).

Often heterosides behave like pro-drugs because, in the organism, the bond is broken down by hydrolysis, thus releasing Genina, which is the pharmacologically active fraction. They may be named according to their sugar molecule, for example: glucosides when the sugar is glucose (e.g. cardioactive glycosides), ramnosidi if the sugar is rhamnose (e.g. galactosides, fruttosides and arabinosides). Flavonoids, saponins and anthocyanins also belong to the family of glycosides (Firenzuoli, 2002).

Tannins

Tannins are structurally complex compounds of the polyphenolic type, initially used for the processing of hides in leather ("tanning action") because it eases the precipitation of proteins.

The tannins are used in medicine for their anti-inflammatory, antiedemigene and vasoconstrictor properties. They also possess other biological properties, in particular disinfectant action against bacteria and viruses, antidiarroica action and preventive action on carcinogenesis. High doses of tannins may be hepatotoxic and irritant for the mucous membranes (Firenzuoli, 2002).

Glucosinolates

The term "glucosinolates" identifies a group of substances containing sulfur, characterized by the bond between the sugar molecule and the aglicone group through a sulfur atom, whose hydrolysis releases isothiocyanates, nitrites or thiocyanates. The prolonged and excessive use of plants containing these substances may be responsible of abortion, formation of goitre and hypothyroidism. On the other hand, they have a protective effect against some forms of cancer, by inhibiting several stages of carcinogenesis (Firenzuoli, 2002).

Vitamins and minerals

The ergosterol exists in some fungi and can be used for the synthesis of vitamin D typical of animal organisms (Firenzuoli, 2002). It belongs to the class of tetra terpenes

(C40) carotenoids, which are yellow-orange, such as β-carotene (provitamin A) and vitamin A (lycopene).

The importance of vitamin preparations and natural mineral extracts compared to those of synthesis must be highlighted. In fact, the composition of extracted minerals often favors the absorption of vitamins and the mineral itself or promotes their synergistic activity. For example, the antioxidant activity of ascorbic acid increases if it is associated with flavonoids and carotenoids, as present in the extract of *Rosa canina*, compared to that of the same dose of pure ascorbic acid (Firenzuoli, 2002).

Resins and balsams

The resins are spontaneously excreted from plants, are amorphous and are chemically complex, whereas the balsams are characterized by mixtures of essential oils and resins, dense and viscous and aromatic, with disinfectant and expectorant actions (Firenzuoli, 2002).

Lectins

The glycoproteins belong to the group of phytochemical lectins, can be found in the seeds of plants, and have various biological activities, such as haemagglutinating, miotic and cytotoxic activities.

Glycoproteins are degraded by the heat of cooking, thus losing their enzymatic activity. They are often toxic both parenterally, and eaten. The cytotoxicity of lectins

inhibits human leukemic cells thanks to their anti-cancer properties. They are also interesting for the immunostimulating properties (Firenzuoli, 2000).

Essential oils

The essential oil (EOs) represent volatile organic oily substances which are extracted from the plant with different techniques such as distillation and extraction. The chemical molecules more common in essential oils are terpene hydrocarbons, alcohols, phenols, aldehydes, ketones, ethers, esters and acids. Aromatic substances are not soluble in water but are important because they are in a well-defined pharmaceutical form. The composition of the EOs and the quantity of their individual components may be modified by many factors that are divided into intrinsic, such as the stage of growth, and extrinsic, such as climatic and edaphic conditions and the extraction method (Marzi, 2003).

In herbs, EOs are produced in the chlorophyll parenchyma of the plant, being formed following the degradation of the primary products; the accumulation of EOs depends on the stage of development of the organ, tissue and cells (Marzi and De Mastro, 2008).

Sometimes the essential oils may be present in different organs of the same plant, but with a different composition (Dorman and Deans, 2000); for example the essential oil obtained from the seeds of *Coriandrum sativum L.* has a different composition from the essential oil obtained from the leaves of the same plant (Delaquis et al., 2002).

It was demonstrated that herbs and spices that contain essential oils in a range between 0.05% and 1% have antimicrobial activity against some pathogens such as *Salmonella typhimurium, Escherichia coli, Listeria monocytogenes* and *Staphylococcus aureus* (Tajkarimi et al., 2010). In addition, several studies have shown the antimicrobial

properties of essential oils against a wide range of microorganisms, including bacteria, protozoa and fungi (Dean and Ritchie, 1987; Sivropoulou et al., 1996 , Chao et al., 2000).

EOs are formed by different volatile organic compounds, have an oily feel, are extracted from aromatic plants by distillation and extraction, have antiseptic, antiparassitic, diuretic, depurative, antiviral, anti-inflammatory actions, and also stimulate the nervous system (Borio, 1981).

The activity of these substances is also very important for feed safety. In fact, Razzaghi-Abyaneh et al. (2009) indicated that the EOs of some medicinal plants may be considered as potential candidates to protect feeds and feeds from toxigenic fungus growth and from subsequent aflatoxin contamination.

In addition to their medical properties, essential oils are also used in feed. In fact, for their content of aromatic components, some plants are used to make feed more palatable, in the preparation of liqueurs, syrups and other beverages, in confectionery, in oral hygiene and in body massage.

Common uses of herbs

The herbs have different medicinal properties but are used mostly in cooking, to flavor feeds or increase their shelf life, in herbal medicine, for the preparation of herbal teas or soft drinks, in the industry, for the preparation of liqueurs and bitters, in perfumery, for the production of ointments, creams and perfumes and also in the chemical industry, for the extraction of substances designed to the feed, cosmetic and pharmaceutical industries (Yusufoğlu et al., 2004).

In recent decades, laboratory studies have demonstrated that some of these plants, or edible parts of them, have significant protective effects on human carcinogenesis (Suhr and Ferguson, 2003).

Their use is changing because they are used increasingly as alternatives to conventional antibiotics, responsible for resistance phenomena in animals subjected to prolonged treatment over time.

For decades, synthetic anthelmintics were used worldwide to minimize losses caused by helminthes infestations. However, in many countries, their resistance to synthetic products has become more serious than expected. The parasite resistance to these products increases business costs, reduces the efficiency of production and increases the risk of contamination. The frequent use and increasing doses of synthetic anthelmintics have caused the decline of their effectiveness (Donald, 1994).

The extracts of herbs are employed in the fight against helminthes, which are parasites that can cause serious gastrointestinal infestations in animals, with consequent economic losses especially in grazing production.

A survey conducted in Ethiopia showed that diseases due to helminthes are responsible

for 28% mortality and 3-8% of weight loss in sheep bred in the plateau of Ethiopia (Bekele et al., 1992). In addition, gastrointestinal worms can compromise animal's health causing lack of appetite, diarrhea and anemia (Athanasiadou and Kyriazakis, 2004).

In fact, the screening and the correct evaluation of these plants can reveal the presence of bioactive compounds with low environmental impact and low bacterial resistance to the active ingredient (Eguale et al., 2007). Thanks to these properties many medicinal plants are used to reduce or eliminate pathogenic bacteria and improve the overall quality of feed production (Tajkarimi et al., 2010). For example, oregano and thyme, which contain terpenes, have antimicrobial and antifungal activity (Bendahou et al., 2008; Naidu, 2000), whereas cinnamon, cloves and caraway seeds have strong antimicrobial effects against *Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, Escherichia coli* and *Candida albicans* (Agaoglu et al., 2007).

Several herbs, such as oregano, rosemary, thyme, sage, basil and garlic, have been successful as antimicrobials alone or in combination with other methods of conservation. However, the efficiency of many antimicrobial agents of medicinal plants depends on pH, storage temperature, amount of oxygen present, and concentration of essential oils and active substances of the plant (Burt et al., 2007; Du and Li, 2008; Koutsoumanis et al., 1999; Svoboda et al., 2006).

In animal feeding, the use of herbs and the substances extracted from them is valid primarily to regulate rumen fermentation, influencing decisively the development of microbial populations by reducing or eliminating pathogenic microorganisms. In this

context, some herbs or their extracts have assumed the role of growth promoters (Brenes and Roura, 2010) or stimulators of lipid metabolism (Acamovic and Brooker, 2005).

In recent years, an increasing number of studies on the potential use of medicinal plants or their extracts as an alternative to antibiotics in feed for ruminants have been conducted (Benchaar et al., 2007b). Nutritional strategies seek to maximize livestock production by reducing the production costs; for example, by using additives, such as probiotics, ionophores and, especially, antibiotics, in ruminant feeding, (Nagaraja, 1995). The habitual use of additives in animal feed in farms has been criticized by consumer organizations, and therefore their use inside the European Union has been limited (European Union, 1998).

As a result, medicinal plants or their extracts have been marketed as plant origin additives, which can be accepted by the consumer and proposed to farmers, even if the potential efficacy of these additives in the regulation of the digestive physiology and functions of the rumen has yet to be deeply evaluated (Broudiscou et al., 2002).

In some scientific studies, plant extracts, such as saponins, were evaluated for their antimicrobial effects and for their ability to improve both rumen fermentation and nutrient use by ruminant animals (Hristov et al., 1999; Wang et al., 2000). In addition, other studies have shown that the essential oils modify the growth and metabolism of different species of bacteria, including the rumen bacteria (Wallace, 2004). Many of these studies have been conducted in the laboratory (in vitro) (McIntosh et al., 2003; Newbold et al., 2004), whereas few of them evaluated in vivo the actual changes in

rumen fermentation and the improvement of nutrient use and performance of dairy cows caused by essential oils (Benchaar et al., 2006).

In Italy, the consumption of herbs is increasing, but the cultivation and harvest of these plants have not increased equally.

The amount of collected wild herbs has declined in recent years primarily for reasons connected with indiscriminate collection and environmental pollution.

Moreover, heavy pollution can harm the health and purity of the plants collected near factories, busy high streets or along the ditches to drain.

In Italy, the progressive abandonment of herb collection has not been replaced by an increase of cultivations, probably because of a lack of politically supported prices, a lack of corporate facilities for initial processing and processing facilities able to give the product a market added value. Italy depends 80-90% on foreign in the supply of herbs.

The intensive cultivation of aromatic plants in Italy concerns mainly the species most widely used. All other aromatic plants, with a lower market and not widely used, are produced in garden cultivations, experimental fields, and ornamental collections. The area planted with herbs could increase if the conditions, especially in the EU, would be less favorable to imports and if strict quality standards would be adopted.

In Sardinia, the heterogeneity of the environment and the richness in medicinal components of native flora is particularly suitable for the enhancement and development of the cultivation of herbs. In fact, herbs are cultivated with appropriate farming techniques, with the aim of getting the drugs in an environment similar to that where they grow naturally (Cicalò, 2011). The yield of cultivation of herbs and their

products depends on environmental parameters, such as soil and climatic conditions, geographic location and pollution; thus the alteration of one of these parameters can cause problems during the cultivation. Only with an increase of rational production of herbs, we can guarantee a steady supply of herbs, in terms of both quantity and quality, to the industries (Cicalò, 2011). The cultivation of herbs is often performed with agronomic techniques which exclude fertilization and protection from pests with the aid of chemical. In fact, biological cultivations of aromatic plants are normally adopted, they are not widespread throughout the territory, being classified as niche crops (Cicalò, 2011).

Effects of aromatic plants on the health status of animals

Toxic and therapeutic effects of plants

Many studies have highlighted the positive and negative effects of herbs supplied as feed to animals.

Among the positive effects of herbs, the literature has demonstrated their antimicrobial power against certain species of microorganisms.

Since the time of the rural indigenous communities, people have used herbs to cure certain diseases. For example, the *Heteromorpha trifoliate* plant was traditionally used to cure stomach diseases. This plant have showed an important antimicrobial activity against *Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella gallinarum* and *Staphilococcus albus* (Desta, 1993, Baker et al. 1995 cited in Korir et al., 2012).

Soković et al. (2007) studied the antibacterial activity, against an epidemiologically relevant group of bacterial feed-borne pathogens, of essential oils from 10 aromatic plants, namely *Matricaria chamommilla, Mentha piperita, Menta spicata, Lavandula angustifolia, Ocimum basilicum, Thymus vulgaris, Origanum vulgare, Salvia officinalis, Citrus limon* and *C.aurantium.* A large variety of naturally occurring and potentially feed-compatible plant-derived oils and oil compounds were tested. The authors concluded that the most active antimicrobial compounds against major feed-borne pathogens identified in that study Soković et al. (2007) , i) were candidates for successive studies of synergism, compatibility, and activity in feed or feed-processing

systems; ii) could replace conventional chemical antimicrobials, and iii) could also be used, at low and non-toxic concentrations, for the prevention and treatment of intestinal diseases in animals and humans caused by Salmonella, Listeria, and other bacterial species (Soković et al., 2007).

Iacobellis et al. (2005) found that the antimicrobial activity of essential oils extracted from certain aromatic plants, i.e. *Cuminum cyminum L.* and *Carum carvi,* was high particularly against *Clavibacter, Curtobacterium, Rhodococcus, Erwinia, Xanthomonas, Ralstonia,* and *Agrobacterium*, which are responsible for cultivated mushroom or plant diseases worldwide. In general, antimicrobial activity was lower against bacteria belonging to the genus *Pseudomonas*. The authors suggested the use of essential oils for the control of bacterial diseases.

In the literature, the possible toxic effects of essential oils supplied to various animal species have also been highlighted.

Janczyk et al. (2008 studied the possible toxic effects on intestinal microflora of a substance contained in the *Thymus* and *Origanum* plants, the Thymol (5-methyl-2-isopropylphenol), which is a phenolic compound used to inhibit oral bacteria and is the main component in thyme oil. Thymol was supplied to a group of 32 piglets: two groups of piglets were fed a standard diet and two groups received the same diet supplemented with 1% free natural identical thymol. The piglets were sacrificed at 49 d of life with the euthanasia protocol which provides for the use of intravenous thiopental and intracardiac Tanax® in order to evaluate microbiological intestinal differences in the various treatment groups. In the groups treated with thymol, there were no reductions in growth but the composition of the microbial population

changed. According to the authors, this could indicate that, in addition its antibacterial effect against pathogens, thymol might also influence bacteria critical to intestinal fermentation.

Evans and Martin (2000) studied the effects of thymol on ruminal bacteria *in vitro*, to determine the effects of thymol on growth and lactate production by the *Streptococcus bovis JB1* and *Selenomonas ruminantium HD4*. *Streptococcus bovis JB1* and *Selenomonas ruminantium HD4* cultures were obtained to determine the effects of different concentrations of thymol on growth and lactate production by *S. bovis JB1* (Gram positive) and *S. ruminantium HD4* (Gram negative), and to examine the effects of thymol on glucose fermentation by mixed ruminal microorganisms in vitro. Thymol was a potent inhibitor of L-lactate production by *S. bovis JB1* and *S. ruminantium HD4* and inhibited glucose uptake by whole cells of both bacteria. Thymol (400 mg/ml) was also a strong inhibitor of CH4 and lactate production when the glucose fermentation by mixed ruminal microorganisms was examined. However, thymol treatment also inhibited acetate and propionate, and these changes in fermentation and products would not be nutritionally beneficial to the host animal.

Palatability

The supply of herbs to animals has many difficulties because of particular tastes and odors emanated by the active substances contained in the plants, which inhibit intake by the animal. In other words, some herbs can be scarcely appetizing.

In fact, before approaching the feed, the animals are strongly influenced by the aroma that it emanates.

To understand the physiological behavior of animals towards the feed, the concept of palatability must be considered.

The concept of attractiveness is not easy to describe, because it contains in a single word a complex set of characteristics related to feed, to senses which stimulates and to response of animals during supply of a certain feed. According to Greenhalgh and Reid (1971), the term attractiveness includes the features of palatability and dietary conditions that stimulate a selective response by animals. Forbes (1986, 1995), instead, argued that the desirability cannot regard only the quality of the feed, because it depends on the experience and the metabolic state of the animal.

Mertens (1996), instead, describes the palatability as a characteristic of the feed which, together with the gustatory, olfactory and visual perception, determines the choice by the animal. So even if palatability is a characteristic of the feed, it is partially the result of a learned behavior of the animal.

Other authors, such as Rolls (1986), Provenza (1995, 1996) and Provenza et al. (1995), seem to have a more comprehensive point of view, because they describe the palatability as the interrelationship between taste and the effects that result from feed

intake, as influenced by the chemical characteristics of the feed, by the nutritional status of the animal and by the past experiences at the time of supply of the feed.

The selection of the feed by the animal is a complex physiological phenomenon, where factors related to the feed, animal and external environment contribute decisively to influence animal behavior towards the feed. In fact, what is defined as "taste" of a feed is actually a complex sensory system that includes the combination of taste, odor, appearance, texture, temperature, sensitivity, and subjective experience, which can be direct or inherited by the individual animal (Dal Maso, 2004). The nervous system and the mechanisms that control sensory perceptions are the tools that allow the animal to choose the best essences. The peripheral nervous system allows the organism to recognize the many external stimuli that are encompassed by sight, hearing, touch, taste and smell. (Bortolami and Callegari, 1999)

In general, we can say that the smell and the taste of feed are the main factors that influence the feed choice because they can determine acceptance or refusal of a meal.

The body's response to external stimuli determined by the supply of a meal is divided into two phases: the cephalic and gastric phase. In the cephalic phase, the volatile components of feeds cause the excitation of the olfactory mucosa, resulting in an increase of production of saliva and gastric juices. In the following gastric phase, the soluble components of saliva stimulate taste buds, causing excitation of the nerve centers of taste, where the stimulus of each specific essence of the feed is recognized. This triggers a series of cascade processes (positive feedback) such as the increase of the secretion of gastric and pancreatic enzymes. In general, the combination of these

actions regulates the digestive process and consequently the intake of feed (Dal Maso, 2004).

The palatability of a feed may also be influenced by factors linked to the feed itself. In particular, the morphology of plants is very important for herbivores, because characteristics such as the presence or absence of pins or the capacity of the plant to produce unpleasant odors depresses their palatability. These particular morphological structures have well defined functions that allow plant species, both herbaceous and woody, to protect themselves from the action of herbivores .

Anatomically, herbivores are adapted to ingest plants that contain large spines, like Cardus, belonging to the family of Asteraceae, because their oral cavity is covered with a mucous membrane (epithelium and lamina propria) characterized by a protective squamous epithelium (Barone, 1994).

The volatile component of the feed (odor emanated) is also very important because it is immediately perceived by the animal; in fact, compounds containing sulfur and terpenes are responsible for unpleasant odors that can induce the rejection of the meal, whereas the aldehydes increase palatability (Mereu, 2009). After the olfactory perception, other factors which can cause feed acceptance or rejection come into play, such as the taste, the size of the fiber and the nutritional characteristics of feed. In fact, the pleasing flavors accentuate the palatability of a meal evoking positive post-ingestive feelings that lead increased intake by the animal (Avondo and Bordonaro, 2001).

If you consider the effects of fiber characteristics on the palatability of the ration, animals prefer fibers slightly lignified, less bulky and more rich in nutrients feed

(Avondo and Bordonaro, 2001). This behavior regards physiological mechanisms associated to the overall volume of the feed, because when a very bulky feed arrives in the rumen, it causes the expansion of rumen walls, thus activating mechanisms of satiety which cause disruption of the meal (negative feedback) (Aguggini et al., 1998).

Animal behavior towards feeds

Many animal factors influence the selection and palatability of feed.

The age is one of the main factors, with adult animals being more selective towards feeds than young animals. Adult animals, which have more experience, recognize more palatable feeds, because they have memorized odors, flavors, fragrances and flavors over the years; young animals, instead, have a limited digestive capacity which affects feed selection. In fact, full maturity in terms of requirements is reached after several months, with differences between species (Bittante et al., 2006).

Another important factor is the physiological state of an animal, because when the requirements for lactation, gestation and movement are elevated, the animal ingests and select more attractive and more nutritive feeds, to allow it to accumulate more energy and protein reserves.

Animals with high milk production and multiple pregnancies have increased requirements but ingest a smaller amount of feed because the capacity of the rumen is reduced by the presence of the fetus which compresses it. For this reason, the animal must ingest feeds of better quality, so that the rumen is not filled abruptly with coarse feed (Cannas, 2009).

The environmental factors are also very important and can affect the selection and palatability of feeds.

The environment in which an animal lives can change its lifestyle routine and its intrinsic characteristics. Because some environmental conditions of the farm can cause stress in the animal, which can change its eating habits, in recent years rearing techniques which improve livestock welfare have been adopted.

Environmental factors such as cold and hot make the animal suffer from low or high temperatures, thus generating physical stress.

The cold stress and heat stress affect feed differently. The cold stress increases feed intake and reduces digestibility, whereas heat stress reduces intake and increases digestibility (Cannas, 2009).

Effects of aromatic plants on monogastric production

Osman et al. (2006) conducted a study to investigate the effects of *Eucalyptus*, pomegranate, lime and thyme, supplied at two different ration levels, on growth performance, carcass traits, blood constituents and economic efficiency of broiler chicks. In general, the addition of the aromatic plants in the ration caused higher live weight gain of chicks, especially those fed with *Eucalyptus*, which had the highest body weight increments at 28 and at 42 days of age. Chicks fed rations with the addition of thyme had an increase of dietary intake. These plants did not have significant effects on feed conversion, carcass characteristics and blood constituents.

Effects of essential oils on ruminant production

The effects of extracts of aromatic plants on performance of ruminants are extensively treated in the literature.

Effects of essential oils on production and rumen of beef livestock

Benchaar et al. (2006) conducted two experiments on the effects of monensin and different doses of a mixture of essential oils in the diet on feed intake, growth performance and feed efficiency (Experiment 1), as well as on nutrient digestibility and N retention (Experiment 2) in beef cattle. The mix of essential oils consisted primarily in natural natural-like compounds, which included *thymol, vanillin, eugenol and limonene*. The first experiment evaluated the growth performance of 20 heifers and 20 steers (Angus × Hereford, initial body weight = 369 ± 10 kg) which were fed a ration supplemented with monensin (33 mg / kg DM), or with a mixture of essential oils (2, 3, 4 g/d), or a ration without supplementation (control). The second experiment evaluated digestibility and nitrogen balance of five steers (Angus × Hereford, initial body weight = 244 ± 4 kg) fed diets supplemented with monensin (33 mg/kg DM), with a mixture of essential oils (2 and 4 g/d) or without integration (control), in a Latin Square experimental design 5x5. In the Experiment 1, DM intake was not influenced by the addition of essential oils, whereas it was 10% lower in cattle fed monensin than in control. Average daily gain was similar in all three groups. Feed efficiency was not affected by treatments, but diets with essential oils had a quadratic effect, which was maximum at the dose of 2 g of essential oil per day. In the Experiment 2, the DM intake did not differ between cattle fed monensin and those of control, but was higher in steers fed essential oils than in those of the control group (6.30 vs. 5.85 kg/d and

increased linearly with increasing amounts of essential oils in the diet. Apparent digestibility and nitrogen retention did not change with the addition of monensin or essential oils in the diet.

Effects of aromatic plants on the milk production and composition in lactating ruminants

With regard to milk production and composition in ruminants fed essential oils, Benchaar et al. (2007b) performed a study in Holstein cows fed a basal ration (alfalfa or corn silage) supplemented or not with 750 mg/d of a mixture of essential oils containing *thymol, eugenol, vanillin*, *guaiacol* and *limonene.* Milk production (normalized to 4% of fat) was not influenced by the addition of essential oils, whereas the concentration of lactose was higher in cows fed the diet supplemented with essential oils than in the control group (4.78% vs. 4.58% of lactose in the milk). Milk fat, protein and urea were no significantly differences between the two groups. Milk fatty acid profile was not significantly influenced, suggesting that the essential oils added to the ration did not influence lipid metabolism in the rumen.

Malecky et al. (2009), instead, tested the effects of the addition, to the basal ration (control), of a mixture of monoterpenes at two different doses (0.043 and 0.43 g/kg of dry matter eaten) on the milk production and composition in Alpine and Saanen goats. The intake of dry matter and the production of milk were not affected by the addition of the mixture of monoterpenes. Milk protein was lower in the group fed the highest

dose of monoterpenes, but this effect was attributed to a greater milk production. The somatic cell count was slightly higher in the group fed the highest dose, whereas the milk fat content did not differ between groups (Malecky et al., 2009).

Effects of essential oils on the rumen

The literature reports several studies showing the effects of essential oils in the feed on the rumen. For example, Nagy and Tengerdy (1968) observed that essential oils extracted from *Artemisia Tridentata* caused an inhibition of activities of rumen bacteria *in vitro*. Most of the evaluated compounds including oxygenated Monoterpenes, especially aldehydes and alcohols of Monoterpenes, strongly inhibited the growth and metabolism of rumen microbes, whereas the monoterpenes hydrocarbons slightly inhibited and, sometimes, stimulated the activity of rumen germs. These results represented the first demonstration of how the chemical composition of essential oils greatly influences the activities of rumen microorganisms (Nagy and Tengerdy, 1968). Any changes in the rumen bacterial population can modify the normal process of biohydrogenation and consequently fatty acid composition of milk fat (Nagy and Tengerdy, 1968).

In addition, Van Soest (1994) noted that a reduction in rumen pH induces variations in the bacterial population, and change the end products of fermentation.

Hristov et al. (2008) evaluated the effectiveness of 40 individual EO in inhibiting ammonia release from native and hydrolyzed casein in the rumen, and their overall effects on ruminal fermentation *in vitro*. In that study, 2 Holstein cows in early lactation were used as donors of rumen inoculum, by withdrawing the liquid from 4

different locations using a rumen cannula. The liquid was taken to assess the effects of the following essential oils *in vitro*: *Basil, Bergamot, Camphor, Caraway, Cedarwood, Cinnamon, Citronella, Clove, Eucalyptus, FrankMyrrh, Gardenia, Hibiscus, Honeysuckle, Jasmine, Juniper, Lavender, Lemongrass, Lilac, Lily, Magnolia, Musk, Neroli, Nutmeg, Oregano, Patchouli, Peppermint, SweetOrange, Petitgrain, Rosemary, RosedeProv, RoseGeran, Sage, Sandalwood, SiberianPine, Spearmint, Tangerine, TeaTree, Thyme, Wintergreen, YlangYlang*. The effects of EO on fermentation observed in the study were subtle. FrankMyrrh, Gardenia, Hibiscus, Eucalyptus and Peppermint oils slightly decreased deamination (i.e. ammonia release from native and hydrolyzed casein), whereas Rose, Geran, Sandalwood, and TeaTree, slightly increased acetate and total VFA concentrations compared to the control. So it was shown that it is unlikely that these moderate *in vitro* effects would correspond to any substantial impact on ruminal fermentation *in vivo* (Hristov et al., 2008).

The concentration of ammonia contained in the ruminal fluid, according to Patra (2011), sometimes decreases because the essential oils inhibit the growth of Hyper-Ammonia Producing (HAP) bacteria. The HAP bacteria comprise only around 1% of the rumen bacterial populations, but they have a high deamination activity (Russell et al., 1988; Wallace, 2004). This high deamination activity could decrease the rate of ammonia production in the rumen, which may be nutritionally beneficial increasing the efficiency of protein utilization in the rumen (Wallace et al., 2002). Newbold et al. (2004) reported a 25% reduction in bacterial deaminative activities *in vitro*. In this regard, however, the type and amount of essential oil used is very important. In fact, ammonia concentrations decreased *in vitro* with oregano oil at 30 and 300 mg/l -1,

with cinnamon oil at 0.3-300 mg/L (pH 7.0; Cardozo et al., 2006) and with cinnamaldehyde at 3000 mg/L (Busquet et al., 2006). However, these effects were not observed in other *in vitro* studies using anethol up to 3000 mg/L, and carvacrol and carvone up to 300 mg/L (Busquet et al., 2006) and in *in vivo* studies (Castillejos et al., 2006; Benchaar et al., 2007a).

Processes of biohydrogenation and lipolysis

In ruminants, the dietary fat in the diet is low and fatty acids in the rumen can change because they undergo the processes of ruminal biohydrogenation. The lipids eaten with the diet undergo lipolysis and biohydrogenation by the rumen microflora in the rumen (Harfoot, 1978; Palmquist and Jenkins, 1980; Jenkins, 1993).

In lipolysis, dietary fat, mainly triglycerides, are hydrolyzed in free fatty acids and glycerol, which origins propionic acid, after fermentation. Among the 74 bacterial strains capable of hydrolyzing the ester linkages (Fay et al., 1990), there is the *Anaerovibrio lipolytica.*

In biohydrogenation, unsaturated fatty acids, liberated by lipolysis, are transformed into saturated forms by rumen microorganisms through hydrogenation. This process is useful for the microorganisms to protect themselves from the toxic effects of unsaturated fatty acids (Antongiovanni et al., 2003).

In the unsaturated fatty acids with one of the double bonds in position cis-12, there is before the enzymatic isomerization with the transformation of the *cis* bond in trans-12-11 by either bacterial isomerase; is needed to free carboxyl group and this is made possible by lipolysis, for the isomerization of polyunsaturated fatty acids is necessary

the presence of isolated diene cis-9, cis-12. At the isomerization process follows the hydrogenation of the bond cis-9, by a microbial reductase and the acid is obtained trans-11 octadecanoic (or vaccenic acid) which can be further hydrogenated to stearic acid (Antongiovanni et al., 2003).

Effects of essential oils on the production of milk fatty acids

The effect of essential oils on the production of milk can change depending on the nutrition. In fact, Santos et al. (2010) observed that a ration supplied with a mixture of EO containing eugenol, geranyl acetate and coriander oil as main components increased the percentage of fat on milk fat, but had no effect on the production of milk and other milk components.

According to Benchaar et al. (2007b) and Agarwal et al. (2009), an increase in fat synthesis could be due to a higher production of acetate in relation to production of propionate in the rumen, as a result of supplementation with essential oils or because of a energy shift of the ration (Santos et al., 2010). In addition, Santos et al. (2010) argued that a diet with essential oils reduced dry matter intake, without any effects on milk production, suggesting a better nutrient efficiency. Some studies on essential oils showed changes in the process of bio-hydrogenation of fats in the rumen. For example, Lourenço et al. (2008) reported that cinnamaldehyde, a component of essential oils, added at a concentration of 500 mg/l in a dual-flow continuous bacterial culture fermenter influenced the process of bio-hydrogenation of polyunsaturated fatty acids. In a similar study, Benchaar et al. (2007a) found that the profile of acidic milk of cows was not influenced by supplementation with 750 mg per day with a mixture of

compounds of essential oils. However, when the same mixture was given at a higher concentration (2 g/day), the concentrations of conjugated linoleic acid (CLA cis-9, trans 11 18:02) increased.

Essential oils or their metabolic products may be present in milk and meat products, depending on the ration supplied to the animals. In fact, according to Molnar et al. (1997), the supply of cumin seeds and chamomile to goats caused the presence of limonene and carvone in milk, whereas the meatabolites of essential oils, present in chamomile, were not detected in the milk.

Several monoterpenes, such as α-pinene, β-pinene, β-myrcene, sabinene, camphene, limonene and δ3-hulls, were found in the milk of cows grazing on pasture (Noni and Battelli, 2008; Chion et al, 2010). So, the presence of essential oils or their derivatives may enhance the nutritional and organoleptic properties of dairy products, thus providing an added value to the product (Chion et al., 2010).

With regard to *Volatile Fatty Acids* (VFA), it was showed that a dietary supplementation with essential oils or their metabolites has increased the total concentration of volatile fatty acids, although this has been demonstrated in a limited number of studies (Benchaar et al., 2008). In two studies *in vivo*, a mixture of essential oils (MEO) commercially available had no effects on the total concentration of the VFA, when sheep were fed 100 mg/d and the cows with 1 g/d of MEO (Newbold et al., 2004; Beauchemin and McGinn, 2006). It is possible that the effects of MEO on the concentration of the AGV may depend on the composition of the diet; in fact, Benchaar et al. (2008) reported that concentrations of 750 mg/d of MEO tended to increase the total concentration of the AGV in the rumen of lactating cows when their

diet contained alfalfa silage, and tended to decrease it when their diet contained corn silage.

Busquet et al. (2006) and Castillejos et al. (2006), instead, found that none of the tested essential oils or their compounds increased the total concentration of the VFA, except for the highest concentrations of most treatments caused a decrease in the total concentration of the VFA as a possible result of a lack of feed digestion.

The lack of change in the total concentration of AGV could be considered beneficial, if accompanied by changes such as a decrease in the concentration of ammonia and methane. By contrast, a reduction in total production of the AGV as a result of the addition with essential oils could be seen as an unfavorable nutritional factor (Benchaar et al., 2008).

TECheese Project

The Techeese project is supported by the European Union and has the main objective of increasing the competitiveness of European livestock and dairy products, by reducing cheese losses due to the presence of undesirable microorganisms, following two basic guidelines: prevention and control.

The preventive action is focused on the formulation of feed containing specific herbs with antiseptic properties, which would transfer their properties to milk and cheese. In this way it should be possible to produce sheep cheese made from raw milk free from undesirable microorganisms that can adversely affect the quality of cheese. One cheese that has been targeted in this project is the Fiore Sardo, which is produced with raw

milk. Another objective is to asses possible effects of the utilization of aromatic plants on the health status of sheep.

The control action is responsible for developing inspection systems based on X-ray equipment at low energy, in order to detect internal defects of the product before it leaves the factory to be placed on the market.

The research has a duration of three years and involves three research centers (University of Sassari, Italy; University of Castilla La Mancha, Spain; University of Brasov, Romania), four associations of small businesses (including the Consortium of Fiore Sardo of Sardinia) and four private companies (including one from Sardinia, Italy).

Aromatic plants utilized in the research project

The project involves the use of eighteen species of herbs below described (http://www.agraria.org/):

- *Achillea millefolium*. Its common name is Achillea Mille-Feuille. It belongs to the Compositae family. It is a perennial herbaceous plant present in all European continent, and also found in the Himalayas and Siberia. It has an erect stem, striated, pubescent. The leaves are alternate and tripennate, the flowers are in corymbs and have yellow-green egg-shaped enclosures. The fruits are achenes compressed. Achillea contains an essential oil consisting of terpene compounds and azulene. It has also a bitter substance, the achilleina. This plant was formerly used as a tonic and antispasmodic, and is currently

used mainly in the formulation of bitter liqueurs and vermouth, but also in cosmetics. Its flowering tops or the whole plant, dried in the shade, are used.

– *Anethum graveolens*. Its common name is Aneto. It belongs to the family of Ombrelliferae. It is an annual herbaceous plant native to West Africa. In Italy it is cultivated and often naturalized. It has erect stems that can be up to one meter in height. The leaves are alternating and green-blue, the little yellow flowers are grouped in inflorescences of umbels with 15-30 rays. The seeds are oblong oval. It has an aroma reminiscent of fennel. Anetum contains essential oil, nitrogenous substances, tannins and resins. It contains anethole, an oil much used in the pharmaceutical field. Its properties are antispasmodic, stomachic, diuretic and vermifuge. From the plant, roots and fruits (improperly called seeds) are used, with the umbels being picked in late summer, dried in ventilated dark places and then beaten to extract the seeds.

– *Carum carvi*. Its common name is Caraway. It belongs to the family of Ombrelliferae. It is a biennial or perennial herbaceous plant widespread in Central Europe. In Italy, it is present in fields and pastures of the Alps (from 800 to 2400 meters), it is more rare in the Northern Apennines, and it is absent in remaining areas. It has an erect stem, up to 60-cm high. The inflorescences are umbels with 7-12 rays. The seeds are oblong-ovoid and strongly aromatic. Caraway contains essential oils consisting in carvone and limonene, fatty acids, proteins, carbohydrates, tannins and cellulose. It is a plant that has carminative, digestive and galactogogues properties. Caraway

fruits (called seeds) are used, they are collected when ripe and let dry in a ventilated dark place.

- *Chamaemelum nobile*. Its common name is Chamomile. It belongs to the family of Asteraceae (or Compositae). It is an herbaceous perennial evergreen. It is native to Western Europe, but in Italy it is not found in the wild. The stems can grow up to 30 centimeters tall. The essential oil of this plant contains flavonoids, coumarin, alcohol, fatty acids, glycosides, vitamin C. The flowers are collected at the beginning of flowering. It is preferable to collect them in dry days in order to facilitate the drying process. If the plant is destined to distillation to obtain essential oils, it is better to use the fresh product.
- *Coriandrum sativum*. Its common name is Coriander. It belongs to the Ombrelliferae family. It is an annual herbaceous plant originated in the south-western Mediterranean regions. It is cultivated in Italy. Its shaft is cylindrical, branched and 50 centimeter-tall. The flowers, white-pink, are very small and are grouped into umbels of 4-6 rays. The main component of coriander is coriandrol, but it also has monoterpenic alcohols, coumarins and alkaloids. It has stimulant, neurotonic and euphoric properties. If supplied at high doses, it can cause symptoms of agitation and intoxication, followed by depression. The coriander seeds have a higher content of volatile oils than the leaves.
- *Hissopus officinalis*. Its common name is Hyssop. It belongs to the family of Labiatae. Hyssop is an herbaceous perennial plant native of the southern and western Asia. It is spontaneous in many mountain areas of northern Italy, and

occurs sometimes in the plains in the rest of the peninsula. It is a plant that has been cultivated since ancient times. It is bushy with stems erect, slender, and woody at the base, up to 60 cm tall. The aromatic leaves, from which oil is extracted, are small, lanceolate, slightly hairy and deep green. The flowers are small, intense blue or, rarely, white or pink. The fruit is a tetrachenium containing a single seed black and wrinkled. It contains essential oil, tannins, choline, glycosides and flavonoids. It has carminative, antispasmodic, depurative and healing properties. The leaves and flower tops are collected at the beginning of flowering, freshly picked and dried immediately.

– *Lavandula angustifolia*. Its common name is Lavender. It belongs to the family of Labiatae, it is a very hardy plant native of ùMediterranean countries. It is widespread in Italy where you can find it spontaneous or cultivated from the plains up to 1800 meters, especially in arid areas. It is a perennial plant that can reach one meter in height. Its stems are erect, branched, woody at the base. The leaves are linear, lanceolate, with narrow gray-green color. The inflorescences are ears and each contains a variable number of flowers, which are very aromatic and with a strong aroma characteristic of the plant. The lavender essential oil is very active, giving to it antiseptic, disinfectant, healing, diuretic, properties. It is also considered a mild sedative. The typical aroma of lavender is given by the essential oils that are produced by glands located in all green parts of the plant but more concentrated in the flowers. Lavender flowers are

used after they are dried, preferably in a dark and ventilated place. The fresh flowers are used to extract the essential oils.

- *Melissa officinalis*. Its common name is Melissa. It belongs to the family of Labiatae. It is a spontaneous herbaceous plant, perennial, and hardy. It is native of southern Europe and western Asia. In Italy it is present in all regions, from the lowlands to 1000 meters. It is bushy and its stems that can reach 80 centimeters tall. The leaves are large. Melissa contains essential oils based on citronella, central, linalool and geraniol. It also contains tannins and resins and succinic acid. Its has antispasmodic, tonic and carminative properties.
- *Ocimum basilicum*. Its common name is Basil. It belongs to the Labiatae family. It is an annual herbaceous plant, native of tropical Asia and across the Middle East. It has become widespread in Europe, particularly Italy and southern France, from which it spread out throughout Europe. It has erect stems that can reach 60 centimeters in height. The leaves are rich in essential oils that give it its characteristic flavor. The flowers are white or pink, together in spikes. Basil is used in cooking for its pleasant aroma. It therapeutic properties are stimulating, antispasmodic, digestive and disinfectant. The leaves collected from spring to summer and the flowers collected in summer are used.
- *Origanum vulgare*. Its common name is Oregano. It belongs to the Labiatae family. It is an herbaceous perennial, native to Europe and western Asia. In Italy, it is present in almost all regions, from the lowlands to 1300 meters. It

can reach a height of 80 cm. Oregano has well-known medicinal properties. The combination of active constituents is variable, depending on the time of picking, the growing conditions and storage conditions. The main constituent are phenols, particularly the thymol and the carvacrol of oregano, are responsible for its properties; other constituents, in addition to the essential oils, are fats, proteins, minerals (calcium, iron, magnesium, zinc, sodium, and potassium), carbohydrates and vitamins such as thiamine. Oregano is antiseptic, expectorant and attenuates intestinal pain.

– *Pimpinella anisum*. Its common name is Anise. It belongs to the family of Ombrelliferae. It is an annual herbaceous plant. It is native to the Middle East. and it is the most known species of anise in the West. It is so widespread in Italy that now it can be considered a spontaneous species. Its stem is round, hollow inside and can reach one meter in height. The flowers are small, whitish and arranged in umbrella-shaped inflorescences. The fruits are achenes. The anise is rich in essential oils and proteins. It has antispasmodic, expectorant and digestive properties.

– *Pinus silvestris*. Its common name is Scotch Pine. It belongs to the family Pinaceae. It is a widespread species in the Eurasian continent. In Italy, it is widespread in all mainland valleys. It is a tree-like plant that rarely reaches 30 feet high. Its foliage is light, with leaves (needles) in pairs, up to 5 cm long, rigid and pungent, green glaucescent. The pine has expectorant and diuretic activities. The parts of the pine most often used are the buds, which are

employed after maceration in alcohol and with the addition of heated water, as syrup for the respiratory tract. Its main product is the resin, whose production can be greatly increased through wounds on the trunk. The resin is harvested mainly in summer and autumn, because in early winter its flow stops.

– *Rosmarinus officinalis*. Its common name is Rosemary. Belonging to the Labiatae family, it is an evergreen perennial shrub native of the Mediterranean, where you can find it growing wild along the coast and up to 1500 meters in height. Its root system is very deep and helps to contain the soil. It is bushy and can reach 3 meters in height. The leaves are needle-like, pale green, very thick and rich in oil glands. It is considered an excellent balsamic plant. Rosemary is rich in essential oils such as pinene, limonene, conforene, flavonoids, tannins. It also contains rosmarinic acid, which has antioxidant properties. Rosemary has also antiseptic, antispasmodic, antiseptic and anti-inflammatory properties. Both leaves and flowers are gathered at full bloom during the summer. For the production of essential oils, fresh flowering tops are used. It is better to use it fresh, because with the drying process it loses much of its active ingredients.

– *Salvia officinalis*. Its common name is Sage. It belongs to the Labiatae family. It is an herbaceous perennial native of southern Europe. It is present in all regions of Italy and is sometimes cultivated in the wild. It can reach a meter in height. Sage has antiseptic, antibacterial, expectorant, and wound healing properties. It also has tonic activity in the nervous system. Its essential oil has a very complex composition and its main constituents are alpha-and beta-thujone and

camphor. Sage also contains organic acids, flavonoids and saponins. The leaves, fresh or dried, are used in medicine.The dried tops dried are subjected to an extraction process in current steam to obtain oil.

- *Satureja montana*. Its common name is Winter Savory. It belongs to the Labiatae family. It originated in the mountainous regions of Central and South America. It is a perennial plant with a strong aromatic odor. It can reach 40 inches tall. The stem is woody at the base, erect, widely branched, often forming a small bush. Santoreggia contains essential oil, whose main constituents are carvacrol and cimolo, enzymes and hydrocarbons. It has antiseptic, antispasmodic and expectorant activities. The leaves and flowers are dried before use.
- *Thymus serpillum*. Its common name is Wild Thyme. It belongs to the family Labiatae. It is a herbaceous perennial plant, common in all Europe and North Africa. The composition of active ingredients of the thymus is variable depending on the time of picking, collection and storage conditions. The main constituents are the phenols and, in particular thymol (which has antiseptic properties) and the carvacloro (also it has antiseptic properties). Other constituents of the essential oil of thyme are linalool, cimolo, cymene, timene and apinene. If the plant is harvested during the winter, it has a low content of phenols, with a predominance of thymol. If collected in the summer, there is a high concentration of phenols, especially carvacrol. The wild thyme flowering

tops are harvested in April , and the leaves are dried in a shaded and well ventilated place.

– *Thymus vulgaris*. Its common name is Common Thyme. It belongs to the Labiatae family. It is originated in the western areas of the Mediterranean. In Italy, it is present in almost all the territory, in the wild or cultivated, in dry areas, from lowlands to 900 meters in height. It is a perennial shrub, with woody and much branched at the bottom so as to form a very compact bush. Its characteristics, properties and the plant partes used are similar to those of wild thyme.

Objectives of the thesis

This thesis was carried out within the objectives and the research planned for the Techeese Project. The goals of the thesis were to assess the nutritional characteristics of several aromatic plants, to test their palatability and their effects when fed to lactating ewes.

The specific objectives were to:

- Evaluate the nutritional characteristics of eighteen aromatic plants with high antimicrobial activity (*Achillea millefolium, Anethum graveolens, Carum carvi, Chamaemelum nobile, Coriandrum sativum, Hyssopus officinalis, Lavandula angustifolia (herb), Lavandula angustifolia (flowers),Melissa officinalis, Ocimum basilicum, Origanum vulgare, Pimpinella anisum, Pinus silvestris, Rosmarinus officinalis, Salvia officinalis, Satureja montana, Thymus serpyllum and Thymus vulgaris*), by measuring their chemical composition and *in vitro* digestibility, which, together in vitro antimicrobial studies carried out by a partner of the Techeese project, could allow the identification of the best 6-7 plants to be used in subsequent studies on dairy lambs and ewes;
- Conduct palatability tests with Sarda sheep of seven aromatic plants supplied alone or mixed with other ingredients of the ration, to determine the most preferred herbs, based on intake and animal behavior during the meals. Five to six plants would be selected based on the results of this section;

– Evaluate the effects of the aromatic plants selected on the basis of their palatability on the production and characteristics of milk, on rumen metabolism and on the health status of lactating Sarda dairy ewes.

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CHAPTER 1

Assessment of the nutritional characteristics of 18 aromatic plants

INTRODUCTION

Aromatic plants can contain secondary compounds with antimicrobial activity. For example, Matasyoh et al. (2009) found in *Coriandrum sativum* 24 different antimicrobial essential oil components.

Usually these compounds are supplied to productive animals as extracts (Benchaar et al., 2008). When utilized in this form, they are concentrated and can be very effective but are subjected to all the restrictions described in the UE legislation on the utilization of feed additives (Regulation EC n. 1831/2003). For this reason, it might technically and economically viable the supply of active essential oils by feeding the aromatic plants as they are. In this way they would be considered normal animal feedstuffs and their utilization would not require a lengthy and costly authorization process. This approach might require that the animals eat significative amounts of these plants to guarantee adequate supply of essential oils. For this reason, the objective of this Chapter was to assess their nutritional characteristics as dietary ingredients.

MATERIALS AND METHODS

Plants

This study utilized 18 species of aromatic herbs and shrubs previously selected by the research group of the University of Castilla-La Mancha of the TECHeese project, based on information on their anti microbial activity reported in the literature. The plants chosen belonged to the following species: *Achillea millefolium L.* (aerial parts), *Anethum graveolens L.* (seeds), *Carum carvi L.* (seeds), *Chamaemelum nobile All.* (aerial parts), *Coriandrum sativum L.*(seeds), *Hyssopus officinalis L.* (aerial parts), *Lavandula angustifolia Mill. (*vegetative parts*), Lavandula angustifolia Mill.* (flowers), *Melissa officinalis L.* (vegetative parts), *Ocimum basilicum L.* (vegetative parts), *Origanum vulgare L.* (vegetative parts), *Pimpinella anisum L.* (seeds), *Pinus Silvestris L.* (young shoots), *Rosmarinus officinalis L.* (vegetative parts), *Salvia officinalis L.* (vegetative parts), *Satureja montana L.* (vegetative parts), *Thymus serpyllum L.* (vegetative parts) and *Thymus vulgaris L.* (vegetative parts).

Sampling and chemical analyses of aromatic plants

Air-dried samples of the eighteen aromatic plants were analyzed in the laboratory of chemical analysis of the Department of Agricultural Sciences, Section of Animal Science, of the University of Sassari. The dried plants were coarsely ground with a mill having a grid of 1 mm in diameter for chemical analysis of DM, crude protein (CP), ether extract (EE), ash, neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL). Non-fibrous carbohydrates (NFC) and ADL/NDF were then calculated.

The percentage of DM was determined by oven drying at 100°C for 24 h. The concentration of NDF and ADL were determined with the method of Van Soest et al. (1991). Ether extract was determined according to AOAC (1990a). In accordance with the method of Van Soest et al. (1991), the samples of plants with an EE content higher than 4% were submitted to a pre-treatment with ethanol before NDF analysis, in order to solubilize the lipids of the same plant and to prevent interference with the NDF analysis. Ash and CP were determined following the AOAC (1990b). The various protein fractions were calculated according to the Cornell Net Carbohydrate and Protein System (CNCPS) model, as described by Sniffen et al. (1992), and analyzed with the methods described by Licitra et al. (1996). The following fractions were determined: A = non-protein nitrogen, B1 = true soluble protein, B2 = insoluble protein - insoluble protein in neutral detergent, B3 = neutral detergent insoluble protein (NDIP) - acid detergent insoluble protein (ADIP), and C = insoluble protein in acid detergent.

The concentration of non fibrous carbohydrates (NFC) of the ration was calculated using the formula: NFC = 100 - (NDF-NDIP) - CP - EE - Ash.

The energy value was calculated using the summative equations of method of Van Soest and Fox, (1992). They were expressed in terms of net energy of lactation, (NEL) assuming a nutritional level of energy intake 3 times higher than maintenance, and then converted in milk forage units (UFL) assuming that 1 UFL = 1700 kcal of NEL.

In vitro digestibility

The measurement of in vitro digestibility of the fiber fractions, in particular of NDF, was performed in the laboratory of the Department of Veterinary science and Animal Production, University of Bologna.

The digestibility on the eighteen aromatic plants was estimated after 24 h and after 48 h of fermentation using the method of Tilley and Terry (1963). In particular, a sample of 0.5 g of each plant was weighed and placed in a beaker glass. Subsequently, 10 milliliters of rumen fluid and 40 milliliters of buffer solution were added, so that the pH inside the beaker remained between 6.7 and 6.9. Anaerobic conditions, which are essential to encourage the growth of microorganisms rumen, were obtained through the closure of beakers with rubber stoppers connected to a circuit of tubes in order to guarantee a continuous flow of carbon dioxide inside the container. Subsequently, beakers were kept in a water bath, at 39 ° C, during the fermentation time (24 h or 48 h). The rumen fluid used in this analysis was extracted from the rumen of three cows with the use of a gastro-esophageal probe. Before the extraction of the liquid, the cows fasted for 12 hours, so the ruminal fluid extract was sufficiently uniform and similar in composition in the three cows. Once it was extracted, the ruminal fluid was initially filtered through four layers of gauze, and then stored in a thermos until it was used for the fermentations. After completing the microbial fermentation, the entire contents of the beaker glass was used to determine the NDF residual fiber with the apparatus Fibertec ™ M6 (FOSS Italia S.p.A, Padova, Italy).

RESULTS AND DISCUSSION

The chemical composition of aromatic plants showed a wide variability in all measured components (Table 1), especially for CP, NDF and EE. In particular, CP varied from 9.1% (on DM basis) in *Achillea millefolium* to 24.9% in *Ocimum basilicum*. The content of NDF ranged from 35.1% in *Satureja montana* to 71.1% in *Coriandrum sativum*. The EE content ranged from 1.1 in *Melissa officinalis* to 12.7% in *Coriandrum sativum*. The ADL content was high in all plants, ranging from 9.8% *Chamaemelum nobile* to 23.9% in *Pimpinella anisum*, being higher than that normally found in conventional forages (Van Soest, 1994).

The content of ash was very high in four plants, i.e. 16.8% of DM in *Ocimum basilicum*, 16.1% in *Chamaemelum noble*, 13.9% in *Salvia officinalis* and 13.6% in *Satureja montana*. In other studies, ash content of *Ocimum basilicum* ranged from 8.7% to 8.8% DM (Bihari et al., 2011) and ash content of *Salvia officinalis ranged* from 11.91% DM to 12.67 DM % (Amr and Đorđević, 2000). The reason for the high ash content found in some plants (*Chamaemelum nobile, Ocimum basilicum Salvia officinalis, Satureja montana*) in our study could be contamination of the plant samples with soil at the moment of harvest.

The content of NFC was very high, exceeding 40% of DM, in the following species: *Origanum vulgare* (45.6% of DM), *Rosmarinus officinalis* (42.3%), *Thymus vulgaris* (41.8%) and *Satureja montana* (40.6% DM). The ADL/NDF ratio varied from 19.2 in *Melissa officinalis* to 42.3 in *Rosmarinus officinalis*. The ADL/NDF ratio of several species (e.g. *Rosmarinus officinalis* and *Pimpinella anisum)* was imbalanced in comparison to that usually observed in animal feeds (Van Soest, 1994).

The content of protein fractions of the aromatic plants is presented in Table 2. Fraction A ranged from 4.0% of CP in *Carum carvi* to 22.8% in *Lavandula angustifolia* (vegetative parts). Fraction B1 varied from 2.0% in *Pinus silvestris* to 15.4% in *Satureja montana*. Fraction B2 ranged from 16.7% in *Anethum graveolens* to 58.6% in *Salvia officinalis*. Fraction B3 varied from 11.6% in *Satureja Montana* to 56.3% in *Anethum graveolens*. Fraction C ranged from 1.1% in *Melissa officinalis* to 44.0% in *Origanum vulgare*. In the species *Ocimum basilicum, Satureja montana, Thymus vulgaris* and *Pinus silvestris,* the fraction C was higher than 14%, whereas in the case of *Origanum vulgare*, it exceeded 44% of the total crude protein.

In brief, most of the plants did not have a high content of soluble nitrogen (fractions A and B1), and were rich in fractions with medium and low degradability (fractions B2 and B3). This distribution of the nitrogen fractions is positive, because it favours the normal degradation of CP in the rumen. On the other hand, the high content of the totally indigestible protein fraction (fraction C) of some of the evaluated plants is a negative characteristic because it makes them highly indigestible.

These high values of fraction C are certainly anomalous values, not found on the same products in the fresh form. This probably occurred because of the drying procedures used, i.e. air-drying after collection of the plants. The drying processes probably lasted for too long, thus favoring the development of oxidative stress and Maillard reactions, which are known to favor the increase of poorly degradable protein fractions, especially the fraction C (Van Soest, 1994)

The *in vitro* NDF digestibility and the estimation of energy value of the plants is showed in Table 3. The determination of NDF digestibility in vitro at 24 h and at 48 h

after the start of fermentation reflects the retention times of the fiber in the rumen of very productive animals (24 h) and of dry animals (48 h). In both fermentation times, a high variability of *in vitro* NDF digestibility was observed (Table 3). In particular, the NDF digestibility at 24 h was very high in some species (*Anethum graveolens* 70.7% of NDF; *Carum carvi* 68.7%; *Pimpinella anisum* 61.4%) and very low in *Rosmarinus officinalis* 5.8%. The NDF digestibility at 48 h was very high in some species (*Anethum graveolens* 83.8% of NDF; *Carum carvi* 79.5%; *Pimpinella anisum* 77.5%) and very low in others (*Rosmarinus officinalis* 6.8%, *Pinus silvestris* 21.6%; *Melissa officinalis* 27.1%). It was not possible to determine the NDF of *Ocimum basilicum* after 24 h of fermentation, due to difficulties in the laboratory.

The difference of digestibility between 48 and 24 hours of fermentation was very high in some cases (Figure 1). In particular, this difference was above 25% in *Salvia officinalis* and greater than 15% in *Origanum vulgaris* and *Achillea millefolium.* In contrast, this difference was very low in *Thymus vulgaris,* perhaps because of the low percentage of NDF of this feed (Figure 1).

In conventional forages, NDF digestibility is usually negatively correlated with the ADL/NDF ratio in plants; this probably happens because the ADL, which is totally indigestible, can be influenced by the presence of lignin in the walls of the bag used for NDF determination (Van Soest, 1994). However, in the aromatic plants studied in this experiment there was no association between the NDF digestibility (Table 3) and the ADL/NDF ratio (Table 1 and Figure 2). This result can be explained by the particular physical characteristics of the samples of aromatic species used, which were composed of seeds or leaves and small woody branches. Therefore, the concentration

of ADL was probably higher in the branches and did not affect so much the NDF digestibility of seeds and leaves. Another possible cause of the lack of relationship between ADL and NDF is that the aromatic compounds present in plants interfered with the NDF fermentation *in vitro*, thereby modifying the relationship which normally occurs between lignin and NDF digestibility. Therefore, despite the high content of ADL found in many plants, very high energy values were observed (Table 3), in some plants, i.e. *Carum carvi* 1.87 Mcal/kg DM of NEL*, Anethum graveolens* 1.61*, Pimpinella anisum* 1.56*, Salvia officinalis* 1.54*, Ocimum basilicum* 1.31 and *Chamaemelum nobile* 1.35. These values were almost equal to the energy levels of concentrates (Van Soest, 1994). On the other hand, in some plants very low energy values was found *(Melissa officinalis* 0.88 Mcal/kg DM of NEL*; Pinus silvestris* 0.92 Mcal/kg DM of NEL) (Table 3).

CONCLUSIONS

In conclusion, the chemical composition of the eighteen aromatic plants studied was not homogeneous, with relevant differences especially in protein and fibrous contents and in NDF digestibility. This could be attributed not only to intrinsic species characteristics but also to the type of anatomical parts (e.g. seeds, fruits, leaves, branches, whole plants) forming the samples of each species. The high variability among species in fibrous content, especially that of NDF and ADL, lead to a high variability in ruminal digestibility, with some plants having very low digestibility (e.g. *Rosmarinus officinalis, Pinus Silvestris* and *Melissa officinalis*) and a much higher ADL/NDF ratio in comparison to traditional feeds.

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Table 1. Chemical composition of eighteen aromatic plants.

Scientific Name	DM %	CP % SS	EE % SS	Ash % SS	NDF % SS	ADF % SS	ADL % SS	NFC [1] % SS	ADL/NDF %
Achillea millefolium	88.0	9.1	1.8	7.7	56.2	43.8	11.4	27.0	20.28
Anethum graveolens	89.3	17.6	4.9	8.9	68.5	41.5	21.7	11.8	31.68
Carum carvi	88.6	24.9	10.8	7.1	52.5	31.9	15.4	15.2	29.33
Chamaemelum nobile	90.1	9.6	5.7	16.1	36.9	34.3	9.8	34.7	26.56
Coriandrum sativum	88.6	14.4	12.7	6.4	71.1	52.2	18.5	4.3	26.02
Hyssopus officinalis	87.1	14.1	3.8	9.2	48.3	37.4	10.9	27.6	22.57
Lavandula angustifolia (flowers)	86.8	13.2	2.2	11.0	51.3	39.6	15.2	27.1	29.63
Lavandula angustifolia (herb)	85.5	13.9	2.5	9.5	47.7	36.0	13.0	30.5	27.25
Melissa officinalis	88.3	11.2	1.1	9.9	56.7	44.8	10.9	27.0	19.22
Ocimum basilicum	88.3	24.0	1.8	16.8	35.5	24.5	10.5	30.8	29.58
Origanum vulgare	89.8	9.8	2.2	8.2	40.4	31.8	15.6	45.6	38.61
Pimpinella anisum	89.4	22.4	3.6	6.4	62.9	40.7	23.9	17.8	40.00
Pinus Silvestris	86.7	13.0	3.1	3.0	62.4	43.1	18.3	24.9	29.33
Rosmarinus officinalis	89.1	6.7	5.9	7.2	40.2	34.2	17.0	42.3	42.29
Salvia officinalis	88.9	12.5	12.1	13.9	37.5	26.0	10.7	27.3	28.53
Satureja montana	88.8	12.3	2.2	13.6	35.1	34.9	11.1	40.6	31.62
Thymus serpyllum	87.8	9.3	2.1	9.1	50.2	40.8	11.1	31.3	22.11
Thymus vulgaris	89.1	10.8	2.2	12.4	35.7	30.2	11.3	41.8	31.65

[1]Non-fibrous carbohydrates, calculated as: 100 - (NDF - NDIP) - PG - EE-Ash, where NDF is Neutral detergent fiber, PG = EE = ether extract, crude protein, NDIP = neutral detergent insoluble protein; ADL =acid detergent lignin.

Table 2. Crude protein (CP), true protein and insoluble protein, neutral detergent insoluble protein (NDIP) and acid detergent insoluble protein (ADIP) content and protein fractions of eighteen aromatic herbs.

Scientific Name	CP % SS	True Protein % DM	Insoluble Protein % DM	NDIP % DM	ADIP % DM	A % CP	B1 % CP	B2 % CP	B3 % CP	C % CP	TOTAL % CP
Achillea millefolium	9.1	7.1	5.7	1.8	0.7	22.4	15.0	43.0	12.1	7.5	100.0
Anethum graveolens	17.6	16.3	14.7	11.8	1.9	7.4	8.9	16.7	56.3	10.7	100.0
Carum carvi	24.9	23.9	20.3	10.4	0.7	4.0	14.5	39.5	39.3	2.7	100.0
Chamaemelum nobile	9.6	8.2	7.1	3.0	1.2	14.9	11.6	42.5	18.8	12.2	100.0
Coriandrum sativum	14.4	13.6	12.3	8.9	1.4	6.1	8.9	23.2	52.1	9.7	100.0
Hyssopus officinalis	14.1	11.7	10.9	3.1	1.0	16.9	5.6	55.9	14.7	7.0	100.0
Lavandula ang. (flowers)	13.2	10.8	10.3	4.8	1.4	18.0	4.4	40.9	25.9	10.7	100.0
Lavandula ang. (herb)	13.9	10.7	10.2	4.1	1.7	22.8	3.9	43.7	17.7	11.9	100.0
Melissa officinalis	11.2	9.8	9.0	5.9	0.1	12.8	6.8	28.0	51.3	1.1	100.0
Ocimum basilicum	24.0	18.7	16.4	8.9	4.3	22.0	9.5	31.5	19.1	17.8	100.0
Origanum vulgare	9.8	9.1	8.4	6.0	4.3	7.1	7.4	23.7	17.8	44.0	100.0
Pimpinella anisum	22.4	21.0	18.6	13.2	1.7	6.3	10.9	23.9	51.3	7.6	100.0
Pinus Silvestris	13.0	11.4	11.1	6.4	2.4	12.8	2.0	35.9	30.7	18.6	100.0
Rosmarinus officinalis	6.7	6.0	5.2	2.3	0.9	11.1	12.2	41.9	21.3	13.4	100.0
Salvia officinalis	12.5	11.6	10.6	3.4	0.5	7.1	7.5	58.6	22.9	4.0	100.0
Satureja montana	12.3	10.9	9.0	3.9	2.5	11.1	15.4	41.9	11.6	19.9	100.0
Thymus serpyllum	9.3	7.5	6.6	2.0	0.9	19.3	10.1	48.7	12.4	9.5	100.0
Thymus vulgaris	10.8	9.7	8.5	2.9	1.6	9.7	11.8	51.8	12.2	14.5	100.0

[1]Fraction protein: A = non-protein nitrogen, B1 = true soluble protein, B2 = insoluble protein - insoluble protein in neutral detergent, B3 = neutral detergent insoluble protein (NDIP) - acid detergent insoluble protein (ADIP), and C = insoluble protein in acid detergent.

Table 3. *In vitro* NDF digestibility at 48 h of fermentation and estimation of the energy value of the plants**.**

Scientific Name	Dig. NDF 24 h % NDF	Dig. NDF 48 h % NDF	Dig NDF 48h % DM	Dig NDIP % DM	Dig cell content % DM	DM [1] diger. % DM	App. DM [1] digest. % DM	TDN[1,3] %DM	NEL [2] Mcal/kg DM	UFL [1, 4] kg DM
Achillea millefolium	22.53	34.08	18.5	0.4	42.9	61.9	50.91	47.5	1.04	0.66
Anethum graveolens	70.65	83.77	47.5	4.0	30.8	82.3	71.34	70.4	1.61	1.02
Carum carvi	68.64	79.51	33.4	3.9	46.5	83.9	72.90	81.2	1.87	1.19
Chamaemelum nobile	29.72	45.33	15.4	0.7	61.8	77.9	66.93	59.9	1.35	0.86
Coriandrum sativum	36.08	41.93	26.1	3.0	28.3	57.4	46.42	57.8	1.30	0.82
Hyssopus officinalis	21.38	28.51	12.9	0.8	50.7	64.4	53.39	50.8	1.12	0.71
Lavandula ang. (flowers)	23.38	32.91	15.3	1.4	47.7	64.4	53.39	47.1	1.03	0.66
Lavandula ang. (herb)	27.91	37.23	16.2	1.0	51.2	68.4	57.45	52.9	1.18	0.75
Melissa officinalis	19.46	27.06	13.7	2.3	42.5	58.5	47.50	40.9	0.88	0.56
Ocimum basilicum	-	64.56	17.2	1.8	63.2	82.2	71.23	58.5	1.31	0.83
Origanum vulgare	-	29.43	10.1	0.7	58.4	69.2	58.24	54.7	1.22	0.77
Pimpinella anisum	61.39	77.51	38.5	4.6	36.3	79.5	68.46	68.5	1.56	0.99
Pinus Silvestris	14.17	21.57	12.1	1.6	36.9	50.5	39.53	42.3	0.92	0.58
Rosmarinus officinalis	5.82	6.79	2.6	0.6	58.6	61.7	50.75	52.8	1.17	0.75
Salvia officinalis	11.80	39.29	13.4	1.1	61.2	75.8	64.78	67.9	1.54	0.98
Satureja montana	31.29	42.47	13.3	0.6	63.6	77.4	66.41	57.4	1.29	0.82
Thymus serpyllum	26.21	35.07	16.9	0.5	48.8	66.1	55.13	50.6	1.12	0.71
Thymus vulgaris	28.95	38.23	12.5	0.5	63.1	76.1	65.12	57.4	1.29	0.82

[1] Calculated at maintenance feeding level.
[2] NEL = net energy of lactation, calculated as 3 times the maintenance level of nutrition.
[3] TDN = the totally digestible nutrients.
[4] UFL = Milk Forage Unit. 1 UFL = 1700 kcal of NEL.

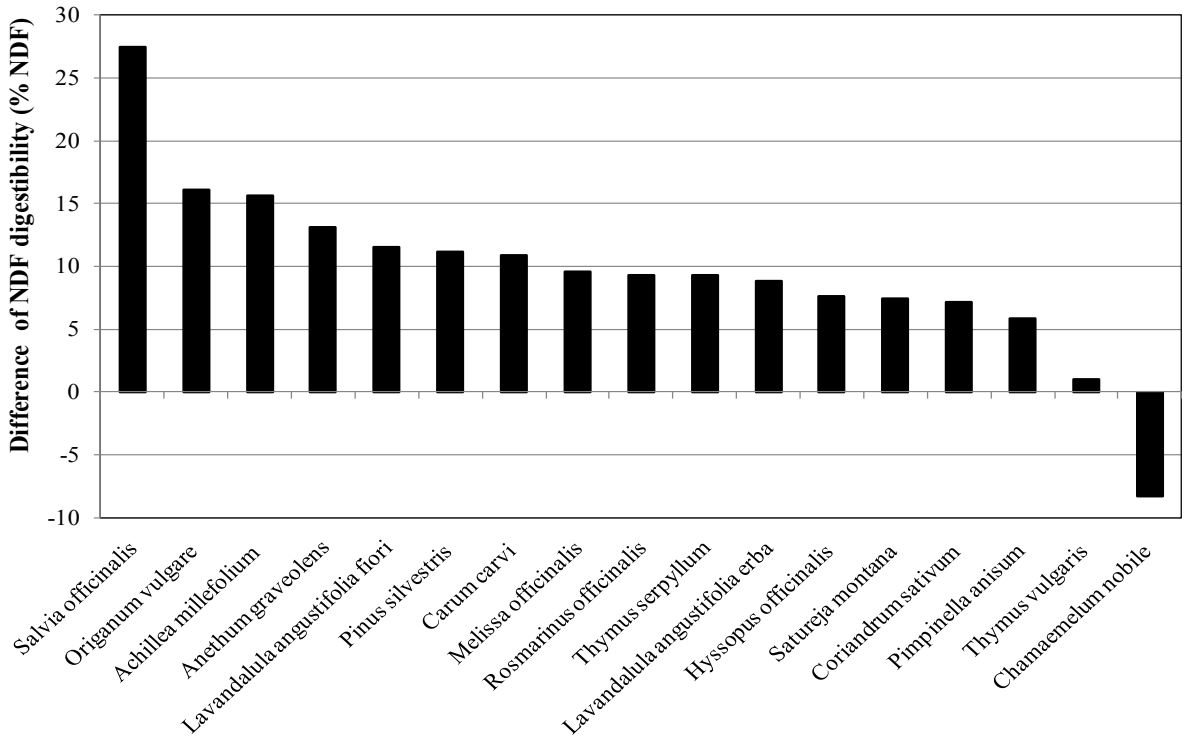

Figure 1. Difference in in vitro digestibility between 48 h and 24 h of fermentation of fiber NDF of 18 aromatic plants.

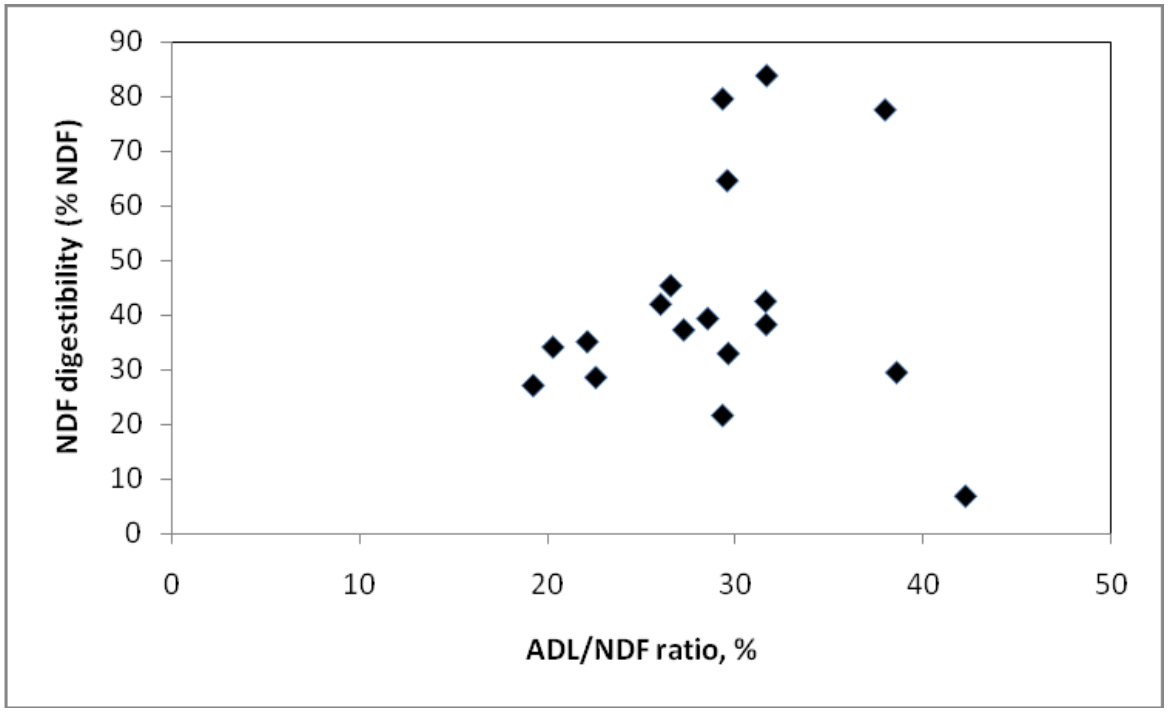

Figure 2. Relationship between concentration of lignin in the NDF and digestibility in vitro at 48 hours of the NDF.

CHAPTER 2

Preliminary test of palatability of seven aromatic plants fed to lambs

INTRODUCTION

The utilization of novel feeds, such as the aromatic plants, in sheep feeding requires the assessment of their palatability, so that possible beneficial effects of the plants are paired with sufficient voluntary intake.

The concept of palatability of feeds has been discussed for a long time and no all definitions are univocal (Canans et al., 2009). Palatability includes in a single word several features attributable to the feed and to the animal to which it is supplied.

Many authors have tried to explain this concept in different ways. According to Greenhalgh and Reid (1971), the term includes the features of feeds and the dietary conditions that stimulate a selective response by animals. Forbes (1986, 1995), instead, argued that palatability cannot be regarded only as a quality of feeds, because it also depends on the experience and the metabolic state of the animal considered. Mertens (1996) further clarified this concept and described palatability as a characteristic of the feed which, in association with the gustatory, olfactory and visual perception, determines the choice by the animal. In this case, even if palatability is a characteristic of the feed, it is also partially the result of a learned behavior of the animal.

The opinion of Rolls (1986) and Provenza (1995, 1996) seems to be the most comprehensive, because these authors describe palatability as the interrelationship between taste and the effects that result from intake, as influenced by the chemical

characteristics of the feed, the nutritional status of the animal and the past experiences at the time of feed supply.

Based on these findings, it is clear that the assessment of palatability of feeds needs to consider the previous experience and the nutrient requirements of the animal used. This need is reinforced by the results of Mereu (2009) and Mereu et al. (2009), who measured the palatability of several concentrate feeds on Sarda lambs and adult mature ewes. The DMI of the lambs during the tests varied from high to low values in a continuum, as if the novelty of the feedstuffs under study pushed them to explore most of feed options but also to refuse those feeds that induced negative, possibly innate, sensorial perceptions. In contrast, the ewes had a marked preference for the feeds often supplied as single ingredients and low intake or complete rejection of the remaining feeds, including several commonly used in sheep feed mixes but rarely supplied alone. This suggests that previous feeding experience had a major role in their sensorial perceptions and evoked a conservative behaviour. In other words the ewes were not prone to eat novel feeds, even those generally considered very palatable.

Based on these results, the assessment of the palatability of new feeds should be done on young animals with little previous experience, in a way that most choices are based on feed components. Indeed, Rapisarda et al. (2012) suggested that for several feeds the short-term choices of the animals could be associated to specific chemical families as long as the animal did not have much previous experience.

Thus, this study aimed to determine the palatability of aromatic plants by using ewe-lambs, i.e animals with limited previous feeding experience.

MATERIALS AND METHODS

The aromatic plants utilized in the palatability tests were caraway (*Carum carvi*), basil (*Ocimum basilicum*), lavender (*Lavandula angustifolia*) (vegetative parts), melissa (*Melissa officinalis*), coriander (*Coriandrum sativum*), winter savory (*Satureja montana*), and common thyme (*Thymus vulgaris*). These plants were selected based on a previous study of the chemical and nutritional composition of eighteen aromatic plants (Chapter 1) and on the analysis of their antibacterial and antimicrobial properties performed by the Techeese research group of the Spanish University of Castilla-La Mancha.

The palatability test was conducted in a Sarda dairy sheep farm located in Torralba (Sardinia), Italy. Eight female ewe lambs of similar age (nine-month old) and body weight (24.6 ± 1.14 kg, mean ± s.d.) were selected from a group of eighty female Sarda lambs.

The lambs had an adaptation period of ten days, during which the following routine was adopted: at 10:00 h lambs entered in the milking parlor, where they were catched and given access to 200 grams per head of whole peas for 10 minutes (normal time for milking). Then, they were released and left for about 45 minutes in a box without feed available. At 11:00 h hay was given *ad libitum* and at 16:00 h concentrate (a mixture of corn grain and whole pea) was supplied at 200 grams per head. From 11:00 h to 16:00h the lambs could move in a fenced area around the box. At 19:00 h all lambs were placed inside the box again, with water ad libitum and no feed available, so that the lambs could fast throughout the night. The adaptation period was essential to avoid

reduce subsequent stress to the animals and to favor the optimal execution of the subsequent tests.

The adaptation period was followed by the experimental period which lasted eight days. During the experimental phase, feed distribution was done according to a Latin square scheme (8 animals x 8 days) and the same daily routine of the adaptation period was applied, with the difference that this time 7 different plant species and the control (again whole peas) were fed following the Latin square design. Each treated animal received for 8 consecutive days a different plant or the control. For the first 4 days of testing, an amount of 100 g per day per head of each aromatic plant was offered to the lambs, while the control was supplied in amount of 250 g per head. Because the intake of plants was very low and the refusal were high, in the last 4 days the amount of plants supplied was reduced to 50 g per head per day. For each lamb, the intake during the palatability test was calculated as the difference between the weight of the feed supplied and the weight of the orts left after 10 min.

RESULTS AND DISCUSSION

The palatability test showed that the aromatic plants tested were not appreciated by the animals (Table 1). The only exception was the intake of 86% of the total amount of *Thymus vulgaris L* supplied at the last day of the trial. The data on intake might have been influenced by the lamb n°1 (Table 2), which was one of the most curious animal when the aromatic plants were supplied. These feeds are very different from those normally utilized in the sheep rations, and this probably lead to a strong animal effect. The lamb n ° 1 did not eat the feed only in the fourth day of trial, refusing *Lavandula angustifolia*. Among the eight plants given, this species was the least palatable for all lambs, as evidenced by the fact that it was the only one to be eaten exclusively by one animal (22% of the total supplied intake) (Table 1). The whole pea (control feed) was very attractive to the lambs, which consumed it in at significantly higher ($P < 0.001$) quantities compared with the aromatic plants. It was totally consumed every day (Table 2), except for the third day (97.2% of intake). This day was characterized by the lowest the total intake of the animals (13%), probably due to adverse meteorological conditions. The difference in intake between the feed control, already consumed by the lambs before the experimental period, and the aromatic plants, never tasted by lambs, could be explained by the fact that animals in general are suspicious of unfamiliar feeds. In particular, according to Provenza (1995), highly gregarious animals, like sheep, tend to acquire a preference for feed with their own experience, the behavior of their similar and the teachings of the mother, which teaches the lamb which are the dangerous feeds, to be discarded, and which ones can

be eaten safely. In the present study lambs were eleven- month old at the beginning of the trial and thus had already acquired same feed experience. In a previous study on palatability of concentrates in newly weaned lambs and adult ewes, Mereu (2009) observed a different behavior between the two categories of sheep. In fact, the lambs with limited previous experience were more inclined to taste new feeds than adult sheep, which selected accurately the concentrate feeds normally used as a single ingredients in the farm (barley, beet pulp and corn and peas), and refused those normally present in pelleted concentrates (soybean, sunflower and rapeseed meals) rarely supplied alone. So the experience brought the adult animals to be wary of what they did not know, whereas the lambs showed "unconsciousness" (Mereu et al., 2009). In the present experiment, the lambs behaved in a similar way to the adult ewes of the study of Mereu (2009), eating large quantities of the known feed (peas) and rejecting almost completely the unknown ones (aromatic plants).

CONCLUSIONS

In conclusion, the palatability tests showed that the aromatic plants supplied alone are little appreciated by the animals. The problem could be solved with the supply of the plants mixed with other feeds more accepted and appreciated by the animals, such as peas, used as a control feed during the test. The aromatic plants could also be pelleted together with the usual ingredients that make up a normal pellet; however, the high temperatures used for the pelleting process may damage the active ingredients in plants, and make them inactive.

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Table 1. Mean intake of the 8 plant species studied. Results expressed as percent of the amount supplied (100 g per head for the aromatic plants, 250 g per head for the pea grains).

Scientific Name	Mean Intake	Standard Deviation	Minimum Intake	Maximum Intake
Carum carvi	2.2 [B]	3.2	0	10
Coriandrum sativum	1.2 [B]	2.1	0	6
Lavandula angustifolia	2.7 [B]	7.8	0	22
Melissa officinalis	6.2 [B]	9.4	0	28
Ocimum basilicum	6.1 [B]	7.8	0	20
Peas	99.7 [A]	1.0	97.2	100
Satureja montana	7.1 [B]	10.5	0	24
Thymus vulgaris	14.7 [B]	29.6	0	86

A, B = P<0.001

Table 2. Mean intake of the 8 lambs used and in each day of the test. Results expressed as percent of the amount supplied (100 g per head for the aromatic plants, 250 g per head for the pea grains).

Lamb	Mean Intake	Standard Deviation	Day	Mean Intake	Standard Deviation
1	32.1	39.2	**1**	13.4	35.0
2	13.1	35.1	**2**	12.9	35.2
3	15.0	34.5	**3**	13.0	34.0
4	13.9	34.9	**4**	15.9	34.6
5	18.2	34.3	**5**	18.3	34.3
6	14.4	34.7	**6**	14.5	35.0
7	16.6	33.3	**7**	18.0	34.0
8	16.7	34.6	**8**	34.0	37.4

CHAPTER 3

Palatability test on aromatic plants and feeding behavior of lambs during the tests

INTRODUCTION

Many factors related to animals affect the selection of feeds and their palatability. The selection of a feed by animals is a particular physiological activity of extraordinary complexity, where factors related to feeds, animals and to the external environment markedly contribute to affect the behavior that the animal will take towards the feed (Cannas et al., 2009).

The "taste" of a feed, is the set of a complex sensory perceptions, which include the combination of taste, odor, appearance, texture, temperature, sensitivity, and subjective experience direct or inherited by the individual animal (Dal Maso, 2004).

It is also very important the volatile component of the feeds (odor emanated), which quickly affects the animal behavior. Certain compounds, such as those containing sulfur and terpenes, are responsible for unpleasant odors that can induce the rejection of the meal, while others, such as aldehydes, can increase palatability (Mereu, 2009; Rapisarda et al., 2012).

When feeds are unpalatable one technique commonly used to increase their palatability is to mix them with palatable feeds. Mereu (2009) and Rapisarda et al. (2012) listed a series of concentrate feeds with high palatability.

Thus, this research had the main goal of finding mixtures of aromatic plants and concentrates that would allow adequate intake of aromatic plants by sheep. Peas were used as ingredient to be mixed with aromatic plants on the basis of their high palatability both by lambs and adult ewes (Mereu, 2009; Rapisarda et al., 2012).

The specific objectives were to: i) compare the palatability of seven aromatic plants mixed with pea grains, ii) determine the percentage of aromatic plant in the mixture which allows the maximum intake of the aromatic plant, and iii) assess the animal behavior during the palatability tests.

MATERIALS AND METHODS

Experimental Design

This experiment was conducted in a dairy sheep farm located in Torralba (Sardegna), Italy. The following aromatic plants were tested: caraway (*Carum carvi*), basil (*Ocimum basilicum*), lavender (*Lavandula angustifolia*) (vegetative parts), melissa (*Melissa officinalis*), coriander (*Coriandrum sativum*), winter savory (*Satureja montana*), and common thyme (*Thymus vulgaris*). Thirty-two lambs of Sarda breed, with 10 months of age and an average body weight of 25 kg were used.

The trial was divided into two main phases.

Adaption period

The adaptation phase, which lasted 8 days, had the purpose of accustoming the animals to go quietly into the milking parlor to eat, in order to reduce the stress factors that could affect the outcome of the experiment. During this period, the animals were fed a basal diet consisting of grass hay *ad libitum* and pea grain supplied at an amount high enough to satisfy lamb requirements for maintenance and growth, because the lambs were confined in the stable, having no access to other feed sources. The lambs fasted from 19:00 h until 10:30 h of the following day, i.e. the time at which the routine procedures of the test started. All lambs were conducted simultaneously in the milking parlor, where 150 g/head of ground pea were supplied. The feed was available to the animal for 10 minutes, after which the orts were weighed. The time interval of 10 minutes was chosen because it is very similar to the average time of

occupancy of the milking parlor by a sheep, and also because it allows to evaluate the level of intake of a greatly appreciated feedstuff in a specific time.

Experimental period

The second phase, i.e. the experimental phase, lasted 16 days, from 26 October 2010 to 10 November 2010. In this period, the 32 lambs were divided into two groups of 16 animals, in order to determine 2 parallel Latin square schemes. The scheme of the Latin square consisted of the supply to each animal of a meal represented by a different mixture of an aromatic plant and a coarsely ground pea, at each of the 16 days of the experimental period. Each aromatic plant was offered to the lambs at increasing doses (12.5%, 25%, 37.5% and 50% of the mixture): 4 plants at 4 doses for 16 days (Table 1). *Thymus vulgaris* was supplied to both groups of 16 animals, in order to evaluate the preference raking of this essence for all animals. Every day, the single mixtures, composed of different plant type and percentage, were prepared for each lamb.

During the first week, the meals were composed of 200 grams/head, which were then increased to 250 grams/head in the second week of treatment, because some animals had not left any residues. This change was necessary to evaluate the effective capacity of intake of the animals. Some of the parameters and procedures adopted in the adaptation phase were not modified in the experimental phase, i.e. the time available to consume the feed (10 minutes), the basal diet (hay and pea) and the period of fasting (from 19:00 h to 10:30 h of the following morning).

Feeding behavior during the test

Throughout the experiment, the behavior of the animal was evaluated, from the moment in which the feed was offered, trying to identify the sensory component that stimulated the lamb (taste or smell). In fact, to understand if the discriminating factor was the smell (feed refusal) or the taste (delayed beginning of intake of the meal or its early termination), 4 lambs were randomly selected inside each group and their behavior was recorded with a digital camera (model DCR-SR32E, Sony, Tokyo, Japan). During the recording, the camera was positioned on a perch inside the milking facility in a way that that the 4 subjects could be filmed together. So it was possible to film in a clear and reliable way the animal behavior in relation to the feed, in order to establish the different reactions towards the various mixtures. On the back of each of the 4 animals recorded per group, a label which reported the number of the subject was applied, so that they could be easily identified when watching movies. After the trial, the videos were analyzed to verify the behavior of each lamb of each subgroup examined through determination of the following parameters:

- time interval from the supply of the feed until the beginning of the first meal;
- duration of the first meal;
- time interval between the first and second meal.

The camera was activated shortly before the distribution of the ration to the animals, and turned off at the end of the meal, which lasted 10 min. During the trial, 16 lambs (i.e. one group at a time) were simultaneously introduced in the milking parlor and, after the feed supply, ate undisturbed during the time established. The residue of each animal was then weighed, and the residues of the 8 animals filmed was sampled for

subsequent chemical analyses. In addition, every day, a small quantity of the ingredients used for the preparation of the mixtures was taken, simultaneously to the phases of dosage, in ordr to have a representative sample for chemical analysis.

Statistical Analysis

The intake of the concentrate mixtures and the single aromatic plants used during the test of palatability were subjected to statistical analysis according to an experimental model that considered two separate Latin squares 16 (4 species with 4 doses) x 16 (animals) for a total of 32 lambs, with a duration of one day of each rotation.

Data on sheep subjected to behavioral observations and those relating to their residues were subjected to ANOVA with 3 factors (feed, dose, and sheep).

The statistical analysis it was performed using the software Minitab® (available for Windows ® 95).

RESULTS AND DISCUSSION

The palatability test was designed to compare the different species between them and to study the effect of the dosage of these plants in a mixture containing also coarsely ground pea grains (Table 1).

Latin Square N°1

The results of the first Latin square showed that the average daily intake of the mixture containing aromatic plants was significantly higher ($P < 0.05$) in *Carum carvi* (131.4%) than in *Ocimum basilicum* and *Lavandula angustifolia*(109.7%; 76.5%),whereas it was intermediate in *Thymus vulgaris*, (116.7%) (Table 2). In particular, the intake of the mixture containing *Carum carvi* was double that containing *Lavandula angustifolia*.

The concentration of aromatic species significantly influenced the dietary intake of the aromatic plants-pea mixture. In fact, for all tested species, the intake was the highest with the concentration of 25% and the lowest with that of 50% of aromatic plant in the mixtures ($P < 0.01$; Table 2). The lower intake at the dose of 12.5% than at 25% suggests that the presence of aromatic plants has, at certain doses, the ability to stimulate the intake of the supplement, even if it was formed by protein pea, known to be a highly palatable feed (Mereu, 2009).

Considering the ingestion of the aromatic plants supplied alone, during the 10 minutes of testing, the dose of 12.5% resulted in a much lower average daily intake (14.1 g, $P < 0.01$) compared to the higher doses (34.6 g with dose 25.0%, 41.3 g with the dose

37.5%, 37 g with the dose 50%), with a numerically higher intake of plant at the dose of 37.5% (Table 3).Only for *Carum carvi,* there was a significantly higher intake at the dose 37.5% (P<0.01) than at the other doses, reaching the highest value of the Latin square (48.7g).

Latin Square N°2

The results of the second Latin square showed that intake of the mixture containing the aromatic plants was significantly higher (P <0.05) in the *Thymus vulgaris* (114.7 g) compared to the *Coriandrum sativum* (87.8 g), with *Melissa officinalis* (95.3 g) and *Satureja montana* (92.3 g) being in the intermediate position. (Table 2). The concentration of the herbs significantly influenced the dietary intake of the aromatic plant-pea mixture. Particularly, as in the Latin square N°1, the intake was the highest with the concentration of 25% and the lowest with the dose of 50%. In reality, the numerical difference between 12.5% (113.5 g) and 25.0% (122.9 g) was not significant, whereas that between these two doses and the one at 37.5% (86.4 g) was significant, the later dose being in turn higher than the dose of 50% (67.4 g) (P <0.05; Table 2). Therefore, the second Latin Square confirmed what has been said for the previous Latin square, i.e. that the aromatic plants, at certain doses, have the capacity to stimulate the intake of pea protein, a very palatable feed (Mereu, 2009). Considering instead the intake of the aromatic plants supplied alone , in the 10 minutes of testing, the dosage of 12.5% resulted in an average daily intake much lower (14.0 g, P <0.01) of plants compared to the higher doses (30.8 g with the dose 25.0%, 32.3 g with the

dose 37.5%, 33.8 g with the dose 50%), with a numerically greater intake of plant at a dose of 50% (Table 3).

This probably happened because the lambs were attracted by smells and flavors emanated from the mixture only at the higher doses of the plants, influencing the selection of feed by the animals (Provenza et al., 1995).

Comparison between two Latin squares

The comparison between the results of the two Latin squares showed that Thymus was eaten similarly , by the lambs of the two Latin squares (116.7 g and 114.7 g in the first and the second Latin squares, respectively) (Table 3).

Based on these results, we can assume that the results of two Latin squares are comparable. Therefore, the intake of the aromatic plant-pea mixture during the test of palatability was in descending order of overall preference: *Carum carvi> Thymus vulgaris> Ocimum basilicum> Melissa officinalis> Satureja montana> Coriandrum sativum> Lavandula angustifolia* (Table 2).

Considering, instead, the intake of the plants alone (no pea), the intake was significantly greater in *Carum carvi* than in *Lavandula angustifolia*, *Coriandrum sativum, Melissa officinalis* and *Satureja montana*. In numerical terms the intake of herbs decreased with the following sequence: *Carum carvi> Thymus vulgaris> Ocimum basilicum> Melissa officinalis> Coriandrum sativum> Satureja montana> Lavandula angustifolia* (Table 3).

As you can see from the two lists, the addition of pea protein to the diet did not cause major changes in preference, except for *Satureja montana* and *Coriandrum sativum*

that exchanged their ranking position according to the presence or absence of the peas in the diet. This can be explained by the fact that in the first list the pea protein masks flavors and odors, making the meal more pleasant, whereas in the second list, where is the plant was supplied alone,, it is likely that its physical form, flavors and odors caused the rejection of the feed by the animal. In fact, the *Coriandrum sativum,* which is supplied in the form of small seeds, was more eaten than *Satureja montana*, which instead is given in the form of dry plant.

Feeding behavior of suckling lambs during the supply of aromatic plants

The feeding behavior of eight lambs (4 for each Latin square),out of 32 lambs, during the test of palatability was studied by viewing of the movies to quantify the duration of the first meal of the animals, the interval between the 1st and 2nd meal and the feeding rate (i.e. the intake quantity divided by the duration of meals). It was not possible to identify the sensory factors that drived the animals trough the feed choice The effects of the feed, the dose and the lamb on these behavioral parameters are presented in Tables 4-13.

Duration of the first meal

In the first Latin square the botanical species and the used dose did not significantly influence the duration of the first meal (Table 4). The average duration of the first meal was very long because many lambs ate during all the available time. This suggests that the intake of the mixtures was in part limited by the time available (10 min) to eat them. However, significant differences between the animals were observed

(Table 4). In fact, lamb N° 1 spent an average of 519 seconds to consume the first meal, whereas lamb N° 2 spent only 309 seconds (Table 4). This highlights a marked animal effect and, therefore, the necessity to have sufficiently numerous experimental groups. The animal effect could be because each individual reacts differently depending on the different stimulation that the feed organoleptic characteristics generates at the neurological level (Antongiovanni, 2004).

In the second Latin square, the duration of the first meal differed significantly among the different botanical species used in the mixtures. In particular, the *Satureja montana* was eaten in 562 seconds whereas *Coriandrum sativum* in 338 seconds only ($P < 0.05$), with the other species being in intermediate positions. This difference was probably due to the physical form of the two species (Antongiovanni, 2004), in fact the seeds of *Coriandrum sativum* were quite large and, thus, easy to ingest. On the contrary, the other species were constituted by the vegetative parts, very hard and fibrous, which probably increased the chewing time and so the intaketime. In this Latin square, there were no significant differences due to the dose and to the animal (Table 5).

Interval between 1st and 2nd meal

The interval between 1st and 2nd meal did not vary significantly inside each Latin square (Tables 6 and 7). The comparison between the values observed in the two Latin squares did not suggest differences between them. Because the films showed that about 80% of the lambs did not interrupt their meal, this parameter was calculated for a small number of animals.

Intake rate

Similarly to what observed for the interval between 1st and 2nd meal, Speed of intake did not vary significantly inside each Latin square (Tables 8 and 9). The feeding rate ranged between 0.35g/s in *Ocimum basilicum* to and 0.58 g/s in *Lavandula angustifolia* in the first Latin square. The variation range was much higher in the second Latin square than in the first, especially for the high speed of intake of *Coriandrum sativum*, which was 8 times greater, even if not significantly, than that of the other species. This might have happened because the *Coriandrum sativum* was supplied in seed form. In the first Latin square, feed intake tended to decrease as the dose of aromatic plants in the mixture increased. This seems to indicate a decrease of mixtures intakeas the proportion of peas in the mixtures decreased .

Feed selection

The feed selection made by the lambs during the test of palatability was estimated considering the difference between the quantity of eaten protein (protein supplied minus crude protein in residues), and the expected amount of eaten protein in the absence of feed selection, i.e. with an equal CP concentration in the mixtures and residues. According to this criterion, the positive values for these differences indicate that the amount of eaten CP was higher than that estimated on the basis of the composition of the mixtures and the amount of residues. Negative values, instead indicate intakes of CP lower than those expected.

The evaluations showed that in the first Latin square lambs selected in favor of CP in a numerically consistent way (intake of CP observed = 9.83% of the expected), whereas differences were statistically significant (P <0.01) between *Lavandula angustifolia* and both *Carum carvi* and *Ocimum basilicum,* but not compared to *Thymus vulgaris* (Table 10). The later three species had, in reality, values of the differences between CP eaten and expected near zero, taking into account normal sampling errors. *Lavandula angustifolia* was the least appreciated plant among the 4 tested in this Latin square (Table 11). Therefore, the high level of selection suggests that the lambs tried to discard the *Lavandula angustifolia* of the mixture in favor of pea grains, which were richer in protein and thus, increased the difference between intake of CP observed and expected.

In the first Latin square, the dose effect was strong at the lowest dose (12.5%) of aromatic plants at (intake of CP observed = 9.87% of the expected), with a selection intensity significantly greater (P <0.01) than at the other 3 doses, at which there was not selection (Table 10). It is probable that the low percentage of inclusion of aromatic plants in the mixtures at 12.5% facilitated the selection in favor of grain pea by lambs, whereas when the inclusion of plants in the mixture was greater this selection could not happen. Differently, the animal effect on the difference between intake of CP observed and expected was not significant and the numerical differences were limited (Table 11).

The second Latin square confirmed the trends observed in the first Latin square. The selective action was very evident for *Melissa officinalis* (intake of CP observed = 26.22% of the expected) (Table 12), which was the less eaten plant among those tested

in this Latin square (Table 13). The selection index was significantly greater for *Melissa officinalis* than that for the other species ($P < 0.01$) and *Satureja montana* also had a numerically high, but not statistically significant, selection index (Table 12).

As the dose effect observed in the first Latin square, in the second Latin square the lambs demonstrated greater selectivity at the lowest dose of aromatic plant in favor of the pea grain (Table 12). No significant differences between animals were observed in this Latin square (Table 12)

CONCLUSIONS

The experiment highlighted several differences among the aromatic plants supplied at various doses to lambs.

Carum carvi was the most consumed plant, whereas *Lavandula angustifolia* was the least appreciated by the lambs. The consumption of *Thymus vulgaris* was almost identical in both groups of lambs, indicating the absence of animal effect.

Animals had the highest intake of mixtures with the lowest dose of aromatic plants, i.e. in the proportion of 25% of the mixture. The intake of aromatic plants alone was greater at the dosages of 37.5% and 50% in the first Latin square, and at the three doses tested in the second Latin square.

The intake during the test of palatability of the aromatic plant-pea mixture was, in descending order of overall preference, as follows: *Carum carvi, Thymus vulgaris, Ocimum basilicum, Melissa officinalis, Satureja montana, Coriandrum sativum and Lavandula angustifolia.*

The palatability tests highlighted substantial differences in palatability among the plants studied. Even if it was not possible to identify the sensory factors that drived the animals through the feed choice, the palatability test gave important information for the use of aromatic plants to feed sheep. These findings could be useful also for their possible use in other animal species.

The experiment showed several differences in the feeding behavior of the lambs. *Lavandula angustifolia* and *Melissa officinalis* were the most discarded aromatic plant, whereas the *Thymus vulgaris* had a high consumption, very similar in both Latin

squares. This suggests that consumption by the animals was influenced by the characteristics of the mixtures and not by the animal effect.

In both Latin squares, the dose with 25% of aromatic plant in the mixture caused the highest intake in lambs. The duration of the first meal was not related with the aromatic species or the dose used. In the case of *Coriandrum sativum,* the average duration of the first meal tended to be shorter, probably because it was supplied in the form of large seeds that facilitated the intake. The rate of intake was not significantly modified by the botanical species or by the dose used. However, the *Coriandrum sativum* had a feeding rate numerically much higher than that of other species. The data on feed selection supplied in the tests were particularly interesting. In particular, the two least eaten species alone (*Lavandula angustifolia* and *Melissa officinalis*) were also those that stimulated a greater selection in favor of protein pea in the mixture and thus determined a higher intake of CP than that of the other aromatic plants supplied with the mixture. Feed selection was also relevant at the lowest dose (12.5%) of aromatic plants, at which the lambs selected intensively, by ingesting a quantity of CP exceeding that expected on the basis of the average composition of the mixtures.

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Table 1. Concentration of the aromatic plants in the feed mixtures used in the two Latin squares .

	Dose (% of plants, DM basis, in the mix)			
	A	**B**	**C**	**D**
Latin Square 1				
Carum carvi	12.5	25.0	37.5	50.0
Lavandula ang. (fiore)	12.5	25.0	37.5	50.0
Ocimum basilicum	12.5	25.0	37.5	50.0
Thymus vulgaris	12.5	25.0	37.5	50.0
Latin Square 2				
Coriandrum sativum	12.5	25.0	37.5	50.0
Melissa officinalis	12.5	25.0	37.5	50.0
Satureja montana	12.5	25.0	37.5	50.0
Thymus vulgaris	12.5	25.0	37.5	50.0

Table 2 - Intake of the mixes (g as fed) in 10 minutes. The values include the pea grains of the mix and are expressed on as fed basis.

Species	Dose (% of plant in the mix)				
	12.5%	**25.0%**	**37.5%**	**50.0%**	**MEAN**
Latin Square 1					
Carum carvi	148.6^{A}	172.1^{A}	129.1^{A}	75.9^{B}	131.4^{x}
Lavandula angustifolia	73.1	90.1	87.1	55.5	76.5^{z}
Ocimum basilicum	102.9^{AB}	140.2^{A}	114.1^{AB}	81.5^{B}	109.7^{y}
Thymus vulgaris	119.7^{AB}	153.7^{A}	111.5^{AB}	80.4^{B}	116.7^{xy}
MEAN	111.1 ^{B}	139.0 ^{A}	110.4 ^{B}	73.3 ^{C}	
Latin Square 2					
Coriandrum sativum	89.6 ^{ab}	115.4 ^{a}	77.8 ^{ab}	68.5 ^{b}	87.8^{y}
Melissa officinalis	105.7^{ab}	113.8^{a}	91.6^{ab}	70.0^{b}	95.3^{xy}
Satureja montana	119.4^{a}	117.1^{a}	70.9^{b}	62.0^{b}	92.3^{xy}
Thymus	139.4^{A}	145.1^{A}	105.2^{AB}	69.2^{B}	114.7^{x}
MEAN	113.5 $^{A\,a}$	122.9 $^{A\,a}$	86.4 $^{B\,b}$	67.4 $^{B\,c}$	

$^{a, b}$ in the same line = P<0.05 $^{A, B}$ in the same line = P<0.01 $^{x, y,z}$ in the same column within square = P<0.05

Table 3 - Mean intake (g per head) of the plants (without the other ingredients of the mixture) in 10 minutes in each Latin square and for each dose. The values are expressed on as fed basis.

Species	Dose (% of plants in the mix)				
	12.5%	**25.0%**	**37.5%**	**50.0%**	**AVERAGE**
Latin Square 1					
Carum carvi	18.5 ^{D}	42.7 ^{B}	48.7 ^{A}	38.3 ^{C}	37.7^{x}
Lavandula angustifolia (fiore)	9.0 ^{B}	22.4 $^{A B}$	32.7 ^{A}	28.7 ^{A}	23.2^{z}
Ocimum basilicum	12.7 ^{B}	34.9 ^{A}	42.3 ^{A}	41.0 ^{A}	32.6^{xy}
Thymus vulgaris	15.6 ^{B}	38.2 ^{A}	41.6 ^{A}	40.0 ^{A}	33.3^{xy}
AVERAGE	14.1 $^{B c}$	34.6 $^{A b}$	41.3 $^{A a}$	37.0 $^{A ab}$	
Latin Square 2					
Coriandrum sativum	10.9 ^{B}	28.9 ^{A}	29.0 ^{A}	34.3 ^{A}	25.9^{yz}
Melissa officinalis	13.0 ^{b}	28.6 ^{a}	34.2 ^{a}	35.1 ^{a}	27.7^{yz}
Satureja montana	14.5 ^{b}	29.5 ^{a}	26.6^{ab}	31.4 ^{a}	25.5^{yz}
Thymus vulgaris	17.7 ^{B}	36.1 ^{A}	39.3 ^{A}	34.4 ^{A}	31.5^{xy}
AVERAGE	14.0 ^{B}	30.8 ^{A}	32.3 ^{A}	33.8 ^{A}	

$^{a, b}$ in the same line = P<0.05 $^{A, B}$ in the same line = P<0.01 $^{x, y,z}$ in the same column, including the data of both the Latin square = P<0.05

Table 4 - Duration of the first meal in the animals subjected to behavioral observations. **Latin square N°1**.

PLANTS	**Duration of 1st meal (s)**	**DOSE**	**Duration of 1st meal (s)**	**LAMB**	**Duration of 1st meal (s)**
Carum carvi	444.6	**12.5**	351.8	**1**	519.7[a]
Lavandula angust,	359.7	**25.0**	508.2	**2**	309.4[b]
Ocimum basilicum	507.6	**37.5**	502.0	**3**	457.7[ab]
Thymus vulgaris	515,2	**50.0**	465.1	**4**	540.3[a]
SEM	22.47	SEM	22.47	SEM	22.47
P <	0.075	P <	0.064	P <	0.003

[a, b] in the same column = P<0.05 [A, B] in the same column = P<0.01

Table 5 - Duration of the first meal in the animals subjected to behavioral observations. **Latin square N ° 2.**

PLANTS	**Duration of 1st meal (s)**	**DOSE**	**Duration of 1st meal (s)**	**LAMB**	**Duration of 1st meal (s)**
Coriandrum sativum	338.1[b]	**12.5**	502.0	**5**	538.1
Melissa officinalis	505.6[ab]	**25.0**	471.8	**6**	351.8
Satureja montana	562.0[a]	**37.5**	487.5	**7**	508.1
Thymus vulgaris	480.9[ab]	**50.0**	425.3	**8**	488.7
SEM	24.87	SEM	24.87	SEM	24.87
P <	0.016	P <	NS	P <	0,056

[a, b] in the same column= P<0.05 [A, B] in the same column = P<0.01

Table 6. Time interval between 1st e 2nd meal of animals filmed to study their behavior. **Latin square N 1.**

PLANTS	**Time interval between 1st and 2nd meal (s)**	**DOSE**	**Time interval between 1st and 2nd meal (s)**	**LAMB**	**Time interval between 1st and 2nd meal (s)**
Carum carvi	33.1	**12.5**	12.8	**1**	35.9
Lavandula angustifolia	24.6	**25.0**	31.2	**2**	17.2
Ocimum basilicum	21.2	**37.5**	29.6	**3**	19.2
Thymus vulgaris	4.8	**50.0**	10.1	**4**	11.3
SEM	5.02	SEM	5.02	SEM	5.02
P <	NS	P <	NS	P <	NS

[a, b] in the same column = P<0.05 [A, B] in the same column = P<0.01

Table 7. Time interval between 1st e 2nd meal of animals filmed to study their behavior. **Latin square N 2.**

PLANTS	Time interval between 1st e 2nd meal (s)	DOSE	Time interval between 1st e 2nd meal (s)	LAMB	Time interval between 1st e 2nd meal (s)
Coriandrum sativum	18.1	**12.5**	3.7	**5**	11.6
Melissa officinalis	10.1	**25.0**	21.3	**6**	12.2
Satureja montana	6.8	**37.5**	9.2	**7**	13.0
Thymus vulgaris	5.1	**50.0**	6.1	**8**	3.4
SEM	2.77	SEM	2.77	SEM	2.77
P <	NS	P <	NS	P <	NS

[a, b] in the same coloumn = P<0.05 [A, B] in the same coloumn = P<0.01

Table 8 – Rate of intake of animals filmed to study their behavior. **Latin square N° 1.**

PLANTS	Rate of intake g/s	DOSE	Rate of intake g/s	LAMB	Rate of intake g/s
Carum carvi	0.56	**12.5**	0.80	**1**	0.51
Lavandula angustifolia	0.58	**25.0**	0.58	**2**	0.39
Ocimum basilicum	0.35	**37.5**	0.33	**3**	0.72
Thymus vulgaris	0.41	**50.0**	0.19	**4**	0.28
SEM	0.1	SEM	0.1	SEM	0.1
P <	NS	P <	NS	P <	NS

Table 9. Rate of intake of animals filmed to study the behavior. **Latin square N ° 2.**

PLANTS	**Rate of intake g / s**	**DOSE**	**Rate of intake g / s**	**LAMB**	**Rate of intake g / s**
Coriandrum sativum	1.64	**12.5**	0.25	**5**	1.5
Melissa officinalis	0.25	**25.0**	0.50	**6**	021
Satureja montana	0.16	**37.5**	0.40	**7**	0.26
Thymus vulgaris	0.26	**50.0**	0.20	**8**	0.36
SEM	9.4	SEM	9.4	SEM	9.4
P <	NS	P <	NS	P <	NS

Table 10 – Differences between observed and expected protein intake based on the composition mixture. Positive values indicate that the quantity of eaten CP was higher than that expected on the basis of the CP of the supplied; negative values indicate that the quantity of eaten CP was lower than that expected. **Latin square N ° 1.**

PLANTS	**Observed - expected CP intake (% expected CP intake)**	**DOSE**	**Observed - expected CP intake (% expected CP intake)**	**LAMB**	**Observed - expected CP intake (% expected CP intake)**
Carum carvi	-2.52 ^{B}	**12.5**	9.87 ^{A}	**1**	2.38
Lavandula angustifolia	9.83 ^{A}	**25.0**	-0.70 ^{B}	**2**	2.00
Ocimum basilicum	0.13 ^{B}	**37.5**	0.27 ^{B}	**3**	4.73
Thymus vulgaris	1.88 ^{AB}	**50.0**	-0.11 ^{B}	**4**	0.26
SEM	1.08	SEM	1.08	SEM	1.08
P <	0.002	P <	0.003	P <	NS

$^{A,\ B}$ in the same column = P<0.01

Table 11 - Mixture of herbs and pea grains eaten by the animals subjected to behavioral observations. **Latin square N ° 1.**

PLANTS	**Intake, g/d**	**DOSE**	**Intake, g/d**	**LAMB**	**Intake, g/d**
Carum carvi	136.3^{A}	**12.5**	121.1^{ab}	**1**	135.6^{ab}
Lavandula angust.	69.3^{B}	**25.0**	167.9^{a}	**2**	88.2^{b}
Ocimum basilicum	144.4^{A}	**37.5**	117.4^{b}	**3**	123.3^{ab}
Thymus vulgaris	137.1^{A}	**50.0**	80.7^{b}	**4**	140^{a}
SEM	6.4	SEM	6.4	SEM	6.4
P <	0.001	P <	0.001	P <	0.026

$^{a, b}$ in the same column = P<0.05 $^{A, B}$ in the same column= P<0.01

Table 12 - Differences between observed and expected protein intake based on the composition mixture. Positive values indicate that the quantity of eaten CP was higher than that expected on the basis of the CP of the supplied; negative values indicate that the quantity of eaten CP was lower than that expected. **Latin square N ° 2.**

PLANTS	**Observed - expected CP intake (% expected CP intake)**	**DOSE**	**Observed - expected CP intake (% expected CP intake)**	**LAMB**	**Observed - expected CP intake (% expected CP intake)**
Coriandrum sativum	-4.08 ^{B}	**12,5**	17.28 ^{a}	**5**	9.20
Melissa officinalis	26.22 ^{A}	**25,0**	1.78 ^{b}	**6**	11.28
Satureja montana	7.59 ^{B}	**37,5**	3.80 ^{b}	**7**	3.6
Thymus vulgaris	3.03 ^{B}	**50,0**	9.90 ^{ab}	**8**	8.68
SEM	1.49	SEM	1.49	SEM	1.49
P <	0.001	P <	0.002	P <	NS

$^{a, b}$ in the same column = P<0.05 $^{A, B}$ in the same column= P<0.01

Table 13 - Mixture of herbs and pea grains eaten by the animals subjected to behavioral observations. **Latin square 2.**

PLANTS	**Intake, g/d**	**DOSE**	**Intake, g/d**	**LAMB**	**Intake, g/d**
Coriandrum sativum	85.8^{ab}	**12.5**	115.8^{ab}	**5**	90.1^{ab}
Melissa officinalis	68.6^{b}	**25.0**	121.1^{a}	**6**	97.4^{ab}
Satureja montana	106.3^{a}	**37.5**	93.4^{ab}	**7**	126.6^{a}
Thymus vulgaris	136.3^{a}	**50.0**	66.7^{b}	**8**	82.9^{b}
SEM	4.53	SEM	4.53	SEM	4.53
P <	0.001	P <	0.001	P <	0.007

$^{a, b}$ in the same column = P<0.05 $^{A, B}$ in the same column = P<0.01

CHAPTER 4

Effects of the supply of six aromatic plant species to Sarda dairy ewes on their milk yield and composition and rumen fluid characteristics

INTRODUCTION

One of the most important aspects of the utilization of aromatic plants in dairy sheep feeding is, beside the study of the carry-over of antimicrobial compounds to the milk, to assess if they cause any modification on milk production and composition and on the rumen function.

Unfortunately, no studies report the utilization of aromatic plants on milk production and other productive performances on ruminants. The few studies available on lactating animals are based on the utilization of essential oil rich extracts of aromatic plants and none has been carried ut on lactating ewes.

Benchaar et al. (2007) tested the supply in Holstein lactating cows of 750 mg/d of a Milk production (normalized to 4% of fat) was not influenced by the addition of essential oils, whereas the concentration of lactose was higher in cows fed the diet supplemented with essential oils than in the control group (4.78% vs. 4.58% of lactose in the milk). Milk fat, protein and urea were no significantly different between the two groups. Milk fatty acid profile was not significantly influenced, suggesting that the essential oils added to the ration did not influence lipid metabolism in the rumen.

Malecky et al. (2009), instead, tested the effect of the addition to the ration of a mixture of monoterpenes at two different doses (0.043 and 0.43 g/kg of dry matter eaten) on the milk production and composition in Alpine and Saanen goats. The intake

of dry matter and the production of milk were not affected by the addition of the mixture of monoterpenes. Milk protein was lower in the group fed the highest dose of monoterpenes, but this effect was attributed to a greater milk production. The somatic cell count was slightly higher in the group fed the highest dose, whereas the milk fat content did not differ between groups.

More research has been carried out on the effects of essential oils on rumen function but no studies used whole aromatic plants of plant parts (Benchaar et al., 2008).

Most of the studies showed evident effects on rumen metabolism, volatile fatty acid production and microbial population. It is not possible to generalize the results, because they are dependent on the plant species used to make the extracts or on he specific molecules used (Benchaar et al., 2008). However, most of the effects were transient, suggesting that microbial population can adapt to the effects of essential oils and reduce their effects (Benchaar et al., 2008).

Based on the limited amount of research available on ruminants, the goals of this research were to: i) assess any possible effect of the utilization of aromatic plants on intake, milk production, on other productive performances and on the health status of lactating dairy ewes; ii) test different dosages, to find up to which level aromatic plants can eb used without adverse effects on the animals and on their performances; iii) study possible modifications at rumen level caused by aromatic plants.

The study used 6 plants selected on the basis of their in vitro antimicrobial activity, nutritive values and palatability (Chapters 1, 2, and 3).

MATERIALS AND METHODS

Experimental protocol

The experiment was conducted during 56 days, from 28 March 2011 until 01 June 2011, in the experimental farm of the Department for Research in Animal Production (DiRPA) of the Regional Agency for Research in Agriculture (AGRIS), located in Bonassai (Olmedo, Sassari, Sardegna), Italy.

On the basis of previous experiments, the following six aromatic species (and relative plant parts) were selected for the study: *Carum carvi* (seeds), *Coriandrum sativum* (seeds), *Melissa officinalis* (vegetative part), *Ocimum basilicum* (vegetative part), *Satureja montana* (vegetative part) and *Thymus vulgaris* (vegetative part). The experimental trial started with a preliminary adaptation period, which lasted 10 days, during which 55 lactating Sarda ewes were group fed a diet consisting of dried and coarsely chopped dehydrated alfalfa (1.70 kg/d as fed), beet pulps (0.50 kg/d as fed), and ground corn and pea grains (0.50 kg/d as fed).

At the end of the preliminary period, all ewes were controlled for their milk production, weight, body condition score (BCS) and blood composition.

According to the results of these measurements and analysis, 48 ewes were chosen for the trial. These were divided into 6 groups homogeneous for milk production, milk fat and protein concentration, body weight and BCS. Each group was then assigned an aromatic plant to be tested with 3 different dosages and a control treatment (without aromatic plant), according to 4 x 4 Latin squares with 14 rotations and two replicates per treatment (8 animals for each square). In total, 6 groups of sheep were formed,

each consisting of 8 animals, for a total of 6 different and independent Latin squares (one per plant) carried out at the same time.

All groups received daily chopped alfalfa at a lower dose than that of the preliminary period (1.20 kg/d as fed) and beet pulps at the same dose of the previous period (0.50 kg/d as fed). In addition, the ewes of each Latin square were divided into pairs (i.e. one pair for each dose), and were offered, at a 14-day cycle, the same mixture (0.50 kg/d as fed , 0.25 kg per each of the two daily milkings) containing corn grain, ground pea and the aromatic plant in various proportions. The daily doses of aromatic plant present in the mixtures being tested were 200 g/d (A), 125 g/d (B), 50 g/d (C) and 0 g/d (D). The *Satureja* dosage was reduced (180 g/d for group A, 110 g/d for group B), and 40 g/d fro group C) because there were difficulties to obtain adequate quantities of this plant by the suppliers.

During the preparation of the mixtures, finely ground carob at the concentration of 10 g/0.25 kg of mixture was added, in order to make the ration more palatable.

The mixtures were prepared daily in the laboratory of the Department of Animal Science of the University of Sassari, by weighing with a precision scale the different quantities of corn, peas and aromatic plant. Every day, during the preparation of the ration, the aromatic plants, peas and corn grain were sampled for analysis in order to have a daily average composition of the ration.

The rations were placed in plastic bags and tagged with the name of the aromatic plant, the letter corresponding to the dose and the identification number of sheep (e.g. Carum C 238: Carum species, dose C, sheep N ° 238). For this reason, medals with the sheep number were tied to one leg and on the neck to each sheep.

The mixtures containing different doses of the aromatic plants were supplied during the two daily machine milkings (at 8:00h and at 15:00h), remaining available to the animal for 20 minutes. In the milking parlor, for each rack of 24 places, only 12 ewes were captured, keeping one empty place between ewes (Figure 1), so that one ewe could not eat the rations of the others (Figures 1 and 2). After 20 minutes, orts were collected and weighed.

Chemical analysis of feeds

The aromatic plants were coarsely ground with a mill having a mesh size of 1 mm. Chemical analysis were carried out to asses their content of dry matter (DM), crude protein (CP), ether extract (EE), ash, neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL).

The percentage of DM was determined by oven drying the plants at 100°C for 24 h. The concentration of NDF and ADL were determined with the method of Van Soest et al. (1991). In accordance with the method of Van Soest et al. (1991), the samples of plants with EE higher than 4% were submitted to a pre-treatment with ethanol before NDF analysis, in order to solubilize the lipids of the same plant and to prevent interference with the NDF analysis. Ether extract was determined according to AOAC (1990a). Ash and CP were determined following the AOAC (1990b).

Measurements on sheep

Total milk production of the flock was measured daily, whereas individual milk production was measured in three different days for each 14 day cycle. The milk produced by each ewe in the two daily milkings was collected in a brick and weighed with a precision scale, after which a milk sample of approximately 33 ml was taken for analysis. In the control ewes, at the end of each cycle three samples of milk per ewe were collected, to make subsequent standard qualitative analysis. At the end of each cycle of 14 day, in addition to milk production measurement and milk sampling for composition analysis, the following activities were performed: blood sampling, body weight and BCS measurement, and rumen fluid sampling.

The blood samples were taken before the meal, in the morning, from the jugular vein using a needle gauge of 18, a one-use holder and two different tubes (vacuteiners with purple or red cap), depending on the examination required. Blood samples were analyzed for complete blood count (CBC) and biochemical profile. For the CBC test, 3 ml vacutainer tubes with purple cap, containing EDTA, to prevent the coagulation of the blood sample, were used. Instead, for the analysis of the biochemical profile, 5 ml vacutainer tubes with red cap, without anticoagulant substances, were used. Once collected, the sample tube for the CBC was shaken gently for about 10 times, to make sure the blood was mixed with EDTA, and then kept upright in the dark in a refrigerator (4-8 °C) until being delivered, within 24 hours from collection, to the laboratory of the Istituto Zooprofilattico Sperimentale della Sardegna (Sassari, Sardinia, Italy), where analysis were performed. In a total of 48 samples from 48 ewes per cycle of experimentation, CBC was determined using an automated cell counter

(MI OCD/07 rev.01) and biochemical profile was determined with an automated spectrophotometer (MI OCD/01 rev.02).

AT the same day of blood sampling, the BCS was measured at the end of the morning milking, when sheep were still captured, by four different experienced operators, who gave the scores using the classical scale from 0 to 5, the means and the quarter-point. At the end of the BCS measurement, sheep were conducted in a corral and weighed on with a electronic scale.

Throughout the experiment, at the end of each cycle the rumen fluid was taken 2 hours after the meal in the morning milking. The liquid was collected from 24 ewes, i.e. one ewe per each of the 4 pairs of each Latin square that received plants at 4 different dosages. Samples of rumen fluid were taken using esophageal probes.

The first rumen fluid collected was discarded to avoid saliva contamination, which could alter the subsequent detection of the pH. For this purpose, two different plastic bottles were used for each sample, the first collecting the first liquid (at least 50 ml), which was discarded, and the second containing the sample for analysis. Soon after sampling, the pH was measured with a pH meter and the ruminal fluid was immediately filtered, collected in plastic tubes with snap cap and frozen, initially at a temperature of -18°C and after few days at -80 °C, until analyses.

Chemical analysis of milk

The analyses of milk were performed in the laboratory of the DiRPA (AGRIS, Olmedo, Sardinia, Italy). Milk fat, protein and lactose were determined with a Milkoscan 400 and somatic cell with a Fossomatic 5000, using the integrated milk

testing instrument. The calibration of Milkoscan and Fossomatic was made with sheep milk samples analyzed by wet chemetry. The standard methods for calibration of the various parameters were as follows: fat with the Rose-Gottlieb method (Standard FIL/IDF 1D: 1996), total protein Nx6.38 with the Kjeldahl method (IDF 20B: 1993), lactose with the differential pH-metry and somatic cells with cell-flow fluoroptometric method (Standard FIL / IDF 148 method 1995 C).

The analysis of urea was performed with an automatic infra-red device, after calibration with enzyme-colorimetric method.

Analysis of the fatty acid profile of the lipid component of milk

Extraction of fat - The lipid fraction was separated according to the Rose-Gottlieb method (AOAC, 1990) with modifications described by Nudda et al. (2005). A sample of 1 g of milk was added with 0.4 ml of 25% ammonia, 2 ml of 95% ethanol and 5 ml of hexane. The samples were then centrifuged at 3,000 rpm for 15 minutes and the upper layer was collected in a Pyrex flask previously weighed. The extraction was repeated a second time with 1 ml of 95% ethanol and 5 ml of hexane, the samples were then centrifuged at 3,000 rpm for 15 minutes and the upper layer was collected in the Pyrex flask.

A third extraction was repeated with 5 ml of hexane, the samples were then centrifuged at 3,000 rpm and the upper layer was added to the previous extract.

Esterification of extract lipid - Fatty acid methyl esters (FAME) were obtained by basic transmethylation performed in accordance with the standard procedure FIL-IDF (1999). Approximately 25 mg of lipid extract were added to 0.1 ml of a solution of

sodium methoxide in methanol, being vortexed for 2 minutes, and then added with 1 ml of hexane containing the internal standard (0.5 mg / ml of C19: 0) and then vortexed for 1 minute. The supernatant fraction was used for gas chromatographic analysis.

Analysis of fatty acids of aromatic plants and rumen fluid

Analysis of fatty acids of aromatic plants and rumen fluid was performed according to the method described by Kramer et al. (1997). Briefly, lipids were extracted from 1g of feed and added with 1 mL exhane and 2 mL of sodium methoxide (0.5 M in methanol) solution. Samples were then vortexed lightly and incubated in water bath at a 50°C for 10 min. After that, samples were removed from the water bath, in order to allow cooling for 5 min. Samples were then added with 3 mL of methanolic HCl, vortexed, incubated in the water bath at 50°C for 10 min. After water bath, samples cooled down for 7 min and then added with 3 mL of exhane and 7.5 mL of K2CO3 (6%), vortexed and finally centrifuged to allow the separation of the two phases.

Analysis of fatty acid methyl esters

The fatty acid methyl esters (FAME) were separated in a capillary column (CP-select CB for Fame; 100 m × 0.32 mm i.d., 0.25-μm film thickness, Varian Inc., Palo Alto, CA, USA) and quantified using nonadecanoic acid (C19:0) methyl ester (Sigma Chemical Co., St. Louis, MO, USA). The injector and FID temperatures were 255 °C. For all samples the temperature program was as follows: 75 °C for 1 min, increased at 8 °C/min to 165 °C, held for 35 min, increased at 5.5 °C/min to 210 °C, held for 1 min,

and finally increased at 15 °C/min to 240 °C held for 15 min. The split ratio was 1:40 and He was the carrier gas with a pressure of 37 psi. Individual FAME were identified by comparing retention retention times of peaks with those of methyl ester standards (Matreya Inc. Pleasant Gap, Pa., USA) and published isomeric profile were also used to identify C18:1 isomers. The relative amount of each fatty acid (% of total FAME) is reported as a percentage of total peak area for all fatty acids.

Identification of single chromatographic peaks

The identification of the chromatographic peaks was performed in milk, aromatic plants and rumen fluid, through the comparison with the retention times of standard mixtures of 37 FAME (Supelco, Bellefonte, PA). To identify the polyunsaturated fatty acids standards PUFA-2, a mixture of isomers non-conjugated 18:2, the standard individual cis-5, 8, 11, 14, 17 C20: 5, cis-4, 7, 10 , 13, 16, 19 C22: 6 (Supelco), cis-6, 9, 12 C18: 3, and cis-9, 12, 15 C18: 3 were used (Matreya Inc., Pleasant Gap, PA, USA). To identify the isomers of CLA, pure standards of cis-9, trans-11 and trans-10, cis-12 CLA were used (Matreya Inc.). To identify the peaks of CLA in the chromatogram, a mixture of isomers of CLA (Sigma Chemical Co) and isomeric profiles already published (Kramer et al., 2004) were used. To identify the trans isomers C18, individual standards trans-9 C18:1, trans-11 C18:1, trans-12C18:1, trans-13 C18:1 (Supelco) and isomeric profile of trans C18:1 already published (Griinari et al., 1998) were used. The content of each fatty acid was expressed as percentage of total FAME present.

Analysis of ammonia

Ammonia was analyzed with the phenol:alkaline hypochlorite technique of Chaney and Marbach (1962), with the difference that salicylate was used instead of phenol . In particular, immediately after sampling, rumen fluid samples were acidified with 200µl of 50% sulfuric acid and preserved in a freezer (-80 °C) until analysis. At the time of analysis, the samples were thawed, centrifuged at 10000 rpm for 10 min and neutralized with sodium hydroxide. An amount of 100 uL of supernatant was sampled and placed in a volumetric flask of 25 ml containing approximately 10 ml of distilled water. Then, 1 ml of the EDTA solution was added to the flask and shaked. Then 4 ml of solution of salicylate-nitroprusside were added and well mixed. After that, 2 ml of buffered solution of sodium hypochlorite were added and the solution was immediately brought to a volume of 25 ml with distilled water.

A blank was prepared into a flask of 25 ml by adding the same quantities of reagents of the previously described mixture, except that the rumen sample was absent, and brought to volume with water. After 30 min at room temperature, the absorbance was measured at 625 nm with a Cary 50 scan UV-vis spectrophotometer (Varian Inc., Palo Alto, CA, USA). The ammonia values were calculated using an external standard technique from a standard curve (from 0.1 to 2.0 mg/l; $y = 0.56434\ x - 0.01813$; $R^2 = 0.999$) of ammonium chloride (NH_4Cl).

Statistical analysis

Statistical analysis of intake and milk production data was carried out considering a Latin square model with the following factors: the botanical species (6 levels) x dose (4 levels) x period (4 levels) x sheep within the botanical species (8 levels).

For the analysis of milk and rumen fluid, the experimental data were subjected to ANOVA with the use of MINITAB® statistical package with the following model:

$Y_{ijrk} = \mu + plant_i + periodo_j * dose_k (plant)_{ij} + \varepsilon_{ijk}$

where:Y = dependent variable (fatty acid), μ = general average, $Plant_i$ = effect of the plant supplied (i = 6), $Period_j$ = effect of the sampling period (j = 4), $dose_k$ = effect of the supplied dose within the plant (k = 4), and ε_{ijk} = error.

RESULTS AND DISCUSSION

Chemical composition of diets

The chemical composition of the single ingredients of the diets and the aromatic plants are presented in Table 1.

The chemical composition of the dehydrated alfalfa and beet pulp used in the basic ration of the ewes (Table 1) was within the normal range of these ingredients (Van Soest, 1994).

Aromatic plants differed noticeably for all parameters considered. In particular, protein concentration varied from 11.04% in *Satureja montana* to 25.58% in *Carum carvi*, fiber NDF from 30.26% in *Ocimum basilicum* to 75.72% in *Coriandrum sativum*, ether extract from 1.04% in *Melissa officinalis* to 9.69% in *Coriandrum sativum*. The ADL content was very high in all studied species, with particularly high values for *Coriandrum sativum, Thymus vulgaris* and *Carum carvi* (Table 1).

The mixtures were isoproteic (18.05% of PG) and had, instead, a variable content of NDF, lipids and energy (data not reported).

Intake of aromatic plants

Average daily intake of the aromatic plants was very close to the planned values (Table 2). Only *Melissa officinalis* showed, at the highest dose of 200g/d (Dose A), an average intake lower than expected, because during all the experimental periods, the ewes did not eat completely the mixtures containing this species. This limited intake might have been due to the fact that this plant was very bulky and quite leathery to the touch. Another reason for it could be a direct effect of the secondary compounds of this plant on feed palatability. The lower intake of *Satureja* at all doses, instead, occurred because its amount in the mixture was lower than that of the other species. The control mixture, which did not include any aromatic plant, was completely eaten by the ewes at all meals.

Production and composition of milk

Comparison between different aromatic herbs

Milk yield and composition are presented in Table 3. Milk yield was the highest for *Satureja montana* and *Carum carvi* (1467 g/d and 1442 g/d, respectively, $P < 0.01$), intermediate for *Ocimum basilicum* (1393 g/d) and significantly lower for *Melissa officinalis, Thymus vulgaris* and *Coriandrum sativum* (1339, 1329 and 1311 g/d, respectively, $P < 0.01$) (Table 3). These substantial differences in milk yield among aromatic plants could be due in part to the different nutritional values of the studied species and in part to interactions between the active substances of the plants and the ruminal microorganisms. In the specific case of Melissa, the low milk yield might

have been caused by its lower intake at the highest dose compared to the intake of the other species (Table 2).

Milk fat concentration was the lowest ($P < 0.01$) in sheep fed *Ocimum basilicum* and *Thymus vulgaris*, intermediate for those fed *Satureja montana* and significantly higher ($P < 0.01$) for ewes fed the other four species (Table 3). The high lipid concentration in the group fed *Coriandrum sativum* (6.23%) might have happened because the percentage of fat in milk is normally positively correlated with the concentration of fiber in the ration (Nudda et al., 2001). Indeed, the chemical analyzes on the composition of the aromatic plants (Table 1) showed that *Coriandrum sativum* contained a much higher percentage of NDF (75.7%) than the other plants. In addition, this species also had a high ether extract content, which may have contributed to the observed increased milk fat concentration. The very low percentage of milk fat for *Thymus vulgaris* and *Ocimum basilicum*, instead, suggests a negative interaction, at the rumen or mammary gland level, between these herbs and the synthesis of milk fat.

The daily production of fat was significantly greater ($P <0.05$) for *Satureja montana* and *Carum carvi* than for the other four species, due to the combination of milk yield and milk fat percentage (Table 3).

Milk protein concentration was significantly higher ($P < 0.01$) for *Ocimum basilicum*, *Satureja montana* and *Melissa officinalis* than for the other three species, with *Carum carvi* and *Thymus vulgaris* having particularly low protein concentrations (5.01 and 4.97%) (Table 3). The low milk protein concentration for *Carum carvi* could be, at least in part, due to the high milk yield. Differently, the low protein concentration for *Thymus vulgaris* might have been caused by a negative effect of this plant, probably at

the rumen level, as confirmed by the low milk yield and the low milk fat concentration in ewes fed this species.

The daily production of protein was the highest (P <0.05) for *Satureja montana*, intermediate for *Ocimum basilicum, Carum carvi* and *Melissa officinalis*, and the lowest for *Coriandrum sativum* and *Thymus vulgaris* (Table 5), probably due to the combination of milk yield and milk protein concentration (Table 3).

Milk lactose concentration was significantly lower (P < 0.01) for *Satureja montana* and *Thymus vulgaris* (both with 4.46%) than for the other four species (with values ranging from 4.56 to 4.62%) (Table 3).

Milk urea content was quite high for all species (above 38 mg/dl) (Table 3) but in accordance with the values suggested by Cannas et al. (1998) for ewes fed diets with similar concentrations of CP. *Carum carvi*, however, determined values of urea in milk significantly higher (46.3 mg/dl, P <0.01) than those determined by the other five species (Table 3). *Carum carvi* was the species with the highest crude protein content (25.6% of DM) among the studied plants. As a consequence, the rest of the ration containing this species was composed almost exclusively by corn grain. Therefore, it is possible that this high value of urea was due to the fact that the corn starch, having a low rate of rumen degradation, lead to a high escape and thus an insufficient availability of the energy required for the full utilization of the proteins of *Carum carvi* by rumen bacteria.

The somatic cells in milk were significantly (P <0.01) lower for the treatments with *Melissa officinalis* and *Carum carvi* than for the other treatments (Table 3).

Effect of used different doses

Milk production and milk composition-were significantly (P <0.001 for both) affected by the effect of the plant and the period, but not significantly affected by dose.

This is difficult to explain because it may be due to many factors such as the following : i) limitations of the used statistical model, even if the numerical differences associated to doses were very limited, ii) the fact that the maximum effect was reached with the lower dose and that the rotation of 2 weeks was too short to cause a relevant reduction in milk yield during the control period, and iii) the fact that the positive effect of some herbs might have been only due to their nutritional value, comparable to that of mixture of control and therefore did not determine nutritional differences varying the doses.

Evaluation of the effects of individual aromatic species

The effects of the use of the single aromatic species on milk yield and composition are shown in Tables 4-9. The effects due to the dosages were not significant in most of cases and, therefore, will be described in brief.

In the case of *Carum carvi*, dose had a significant(P<0.05) effect only on milk protein concentration, which decreased with increasing doses of inclusion of this species (Table 4). This result suggests an interference of secondary compounds of this species in the ruminal microbial activity and a probable lower synthesis of bacterial proteins, which are known to be the main factor responsible for the protein content of milk. It is also possible, however, that the replacement of pea (rich in proteins but also in fermentable starch) with *Carum carvi* (similar protein content but with a much lower

starch content) has led to a reduced availability of energy substrates in the rumen. This may also explain the fact that ewes fed this plant had a significantly higher concentration of urea in milk than ewes fed the other species. A third hypothesis is that the low milk protein content may be due to the high fat content of *Carum*, which exceeded 8%. In fact, as demonstrated by Nudda et al. (2001), dietary fat interferes with the microbial activity reducing their synthesis of microbial proteins.

With regard to the *Coriandrum sativum*, milk fat concentration increased ($P< 0.15$) progressively with the increase of dosage of this herb, changing from 6.03% in the control group to 6.50% at the highest dose (Table 5).This effect was certainly due to the rather high concentration of ether extract (9.7% DM) of the *Coriandrum sativum* and its likely high escape, caused by the physical form of this seed (spherical form, rather small and lignified). The contribution of intestinal lipids, in fact, often leads to an increase in the fat content of the milk (Nudda et al., 2001). Milk yield and composition of *Melissa officinalis, Ocimum basilicum* and *Satureja montana* were not affected by dose (Table 6-8).

Dosage had a significant effect ($P<0.05$) on milk protein and urea concentration of *Thymus vulgaris* (Table 9). Milk protein concentration, was the highest at the maximum dose (200 g/d), intermediate at second (125 g/d) and similar between the low dose (50 g/d) and the control. Milk urea concentration, instead was the highest in the low dose and in the control groups. These results seem to indicate a greater ability of the ewes fed high doses of *Thymus vulgaris* to use the protein fermented in the rumen. Currently the reasons of this phenomenon are unclear.

Hemogram and blood serum biochemistry

In the preliminary period, the ewes assigned to the 6 experimental groups were not significantly different for their biochemical blood parameters (Table 10), blood count parameters (Table 11) and leukocyte counts (Table 12). All values were within or very close to the reference ranges, suggesting that the ewes were in a good health status before the application of the experimental feeding treatments. Red blood cells were slightly lower than the reference values (Table 11), suggesting a possible, even though limited, iron deficiency.

During the experiment, among the blood biochemical parameters analysed (Table 13), albumin, alkaline phosphatase, gamma glutamiltranspeptidase, glutamic oxaloacetic transaminase, total proteins ($P<0.01$) and urea ($P<0.02$) were significantly influenced by the different aromatic plants. Of some interest are the urea values, which were in agreement with the values observed in milk (Table 3), being significantly higher in the *Carum carvi* (46.3 and 48.1 mg/dl in milk and blood, respectively), lowest for the *Thymus vulgaris* (38.3 mg/dl and 41.7 mg/dl in milk and blood, respectively) and intermediate for the other species. A possible explanation of this pattern was already given describing the results on milk urea content, i.e. a possible greater ability of the ewes fed high doses of *Thymus vulgaris* to use the protein fermented in the rumen. Another interesting finding is the pattern of creatinine, alkaline phosphatase and gamma-glutamiltranspeptidas, which, together with total bilirubine, decreased ($P<0.001$) as the experiment proceeded. This suggests a depletion of these enzymes, probably due to the needs of the animals to deal with the new compounds (aromatic compounds) provided by the experimental plants. Differently, albumin and glutamic

oxaloacetic transaminase reached the highest (P<0.001) value at the last period of the study. Dose of aromatic plants did not influence significantly theblood biochemical parameters.

Regarding the hemogram (Table 14), aromatic plants caused significant effects on various parameters (RBC, HGB, MCV, MCH and PLT), but differences were numerically small and values of all treatments were within the normal reference range, except for red blood cells which were , as in the preliminary period (Table 11), slightly lower than the reference values. The different doses did not influence the hemogram parameters, whereas period affected MCH, MCHC and PLT.

Total and differential white blood cells (Table 15) were significantly affected by the plant but not by the doses. *Carum carvi* and *Melissa officinalis* had the highest values of white blood cell counts and *Thymus vulgaris* the lowest (Table 15). These cells were also significantly reduced as the experiment proceeded. This was mainly due to a decrease in neutrophil and eosinophil cells. Commentare altri parametri significativi…

In summary, the effects of aromatic plants during the experimental period on the hematogram and blood serum biochemistry were significant for several values, even though in biological terms the differences were small and the values were within the reference normal values, except for the red blood cells, with values slightly below the minimum reference values. No effects associated to the dose of aromatic plants on blood parameters could be observed.

Fatty acid profile of plants

The fatty acid profile of the plants used are shown in Table 16. Total saturated fatty acids (SFA, as percentage of total fatty acids) ranged from 7.82% in *Coriandrum sativum* to 32.46% in *Melissa officinalis*. Among the SFA, the most abundant is the palmitic acid (C16:0) which represents approximately the 67% of the total in all analyzed plants, followed by stearic acid (C18:0) that is present in lesser amounts.

The monounsaturated fatty acids (MUFA) ranged from 13.54% in *Satureja Montana* to 70.61% in *Coriandrum sativum*, being the most represented by the oleic acid (C18:1 cis-9), whose percentage is generally higher in seeds of plants than in vegetative parts such as flowers and leaves. Among the analyzed plants, the percentage of C18:1 cis-9 was considerably higher in *Carum carvi* and in *Coriandrum sativum* probably because the seeds of these plants were used. It is important to note, however, that such high value of oleic acid in *Coriandrum sativum* could be due to a coelution of other isomers of C18: 1 with oleic acid in our chromatographic conditions.

The proportion of polyunsaturated fatty acids (PUFA) ranged from 21.58% in *Coriandrum sativum* to 58.18% in *Satureja montana*, being represented mainly by linoleic acid (C18: 2 n-6) and by acid α-linolenic (C18: 3 n-3). Generally, the C18: 2 n-6 was higher in plants of which seeds have been used *(Carum carvi* and *Coriandrum sativum*) while the C18: 3 n-3 was higher in plants of which the vegetative part was used, especially in *Satureja montana* and in *Thymus vulgaris* (43% and 40% of the total fatty acids, respectively).

The composition and fatty acid profile of sheep milk

The effect of type of plant supplied, the dose and the sampling period on the fatty acid composition of sheep milk are summarized in Tables 17-21. The type of supplied plant and the sampling period influenced significantly (P <0.001) all groups of considered fatty acids, whereas the dose had significant effect only for SFA, MUFA and TFA.

Saturated Fatty Acids

The effects of the type of plant, of the period and the dose of aromatic plant on the proportions of the individual SFA and on total SFA in milk are presented in Table 17. The most represented SFA is the C16:0, which constitutes about 30% of the total fatty acids, followed by C14:0 (average 12%). Considering the effect of the plant, *Ocimum basilicum* (76.42 g/100g FAME), *Satureja montana* (76.79 g/100g FAME), *Thymus vulgaris* (75.79 g/100g FAME) and *Melissa officinalis* (77, 45 g/100g FAME) had higher values (P< 0.05) of SFA in milk than those of *Carum carvi* and *Coriandrum sativum* (70.25 and 69.64 g/100g of FAME, respectively).The lower proportion of SFA in milk from ewes fed *Carum carvi* and *Coriandrum sativum* was mainly due to a decrease of the percentage for C14:0, C16:0 and C12:0 (Table 17).

Monounsaturated Fatty Acids

In contrast to what observed for SFA, the proportion of monounsaturated fatty acids (MUFA) was higher in the milk of sheep fed *Carum carvi* and *Coriandrum sativum* (24.75 and 25.82 g/100g of FAME, respectively), whereas the other plants showed

significantly lower content (P <0.001) of these fatty acids (18.15, 18.94, 18.84 and 19.17 g/100g of FAME, respectively, for *Melissa officinalis, Ocimum basilicum, Satureja montana* and *Thymus vulgaris*) (Table 18).

The increase of MUFA with the supply of *Carum carvi* and *Coriandrum sativum* was mainly due to an increase of the C18: 1 cis-9, which represents about 80% of the total MUFA and, as already mentioned, to the predominance of these fatty acids in the seeds of these plants (Table 16).

Branched Fatty Acids

The effect of the type of plant, the dose and the period on the individual and total branched fatty acids (BCFA) are reported in Table 19. It is important to highlight that these fatty acids are considered interesting parameters for the study of the ruminal activity because they originate from the rumen bacteria (Vlaeminck et al., 2006). Also in this case, *Carum carvi* and *Coriandrum sativum* caused a reduction of the content of BCFA, probably due to a decrease in ruminal activity. This was particularly evident for *Carum carvi*, with the milk of sheep fed this plant showing the lowest content of BCFA (2.16 g/100g of FAME). Regarding the other plants, also in this case *Ocimum basilicum*, *Satureja montana, Thymus vulgaris* and *Melissa officinalis* showed a similar effect on the proportion of BCFA in milk.

Polyunsaturated Fatty Acids

The influence of plant type, dose and period on the contents of polyunsaturated fatty acids (PUFA) is reported in Table 20. In this case, *Carum carvi* and *Coriandrum sativum* did not behave in a similar way. In fact, highest (P <0.001) values of PUFA n-6 (2.38 and 2.42 g/100g of FAME, respectively) occurred with the supply of *Carum carvi* and *Thymus vulgaris*, whereas the highest proportions of n-3 PUFAs were observed with *Carum carvi, Ocimum basilicum* and *Thymus vulgaris* (0.94; 0.98 and 1.04 g/100g of FAME, respectively). The concentration of rumenic acid (RA, cis-9, trans-11 CLA), which was on average equal to 60% of the total CLA, was the highest in the milk of sheep fed *Carum carvi* (0.62 g/100g of FAME) and the lowest for those fed *Satureja montana* and *Melissa officinalis* (0.51and 0.53 g/100g of FAME, respectively). It is important to note that the observed concentrations of cis-9, trans-11 CLA are lower than previous observations on sheep milk (Addis et al., 2005; Nudda et al., 2005). The concentration of eicosapentaenoic acid (EPA, C20: 5 n-3) and docosahexaenoic acid (DHA, C22: 6 n-3) resulted, as expected, very low with all used plants. In fact, the milk of ruminants generally has a very limited content of these fatty acids due to the poor transfer capability of these from the feed to the milk (less than 3-5%). This is due to both the high biohydrogenation to which they are subjected during the rumen fermentation (Chikunya et al., 2004b) and the low activity of elongation and desaturation of fatty acids to 18 and 20 carbon atoms in the mammary gland (Gulati et al., 2003).

Trans Fatty Acids

The effect of the type of plant, the dose and the period on individual and total trans fatty acids (TFA) are shown in Table 21. Also in this case, a similar behavior for the *Carum carvi* and the *Coriandrum sativum* was found. In fact, with the use of these plants, , the highest proportion (P <0.001) of TFA (1.89 and 2.19 g/100g of FAME, respectively) was observed, mainly due to a higher concentration of vaccenic acid (VA, C18: 1 trans-11) that represents about 70% of total TFA.

Also in the case of this group of fatty acids, the other species used had similar values of BCFA (1.26, 1.11, 1.10 and 1.18 g/100g of FAME, respectively, for *Melissa officinalis, Ocimum basilicum , Satureja montana* and *Thymus vulgaris*). As already observed for the CLA also in this case the values of VA were lower than those previously observed for ovine milk (Nudda et al., 2005).

The dose, of aromatic plants significantly influenced (P <0.001) only the percentage of SFA, MUFA and TFA (Tables 17-21). In all cases, the higher doses (200 g/d and 125 g/d) determined an alteration of rumen processes leading to a reduction of the SFA, and then a greater "escape" in the milk of PUFA of the series n-3 (P <0.001) and of the series n-6, although in this case the observed increase was not statistically significant.

Composition and fatty acid profile of sheep rumen fluid

Saturated fatty acids

The effects of the type of plant, the period and the dose of plant on the proportions of the individual SFA and their sum in the rumen fluid are presented in Table 22. Among the saturated fatty acids, the C18:0 is the predominant, representing c.a. 30% of total SFA, followed by C16:0, constituting c.a. 20% of SFA.

Considering the effect of the plant, the concentration of SFA present in *Coriandrum sativum* (78.22 g/100g of FAME), *Carum carvi* (76.02 g/100g of FAME), *Satureja montana* (76.59 g/100g of FAME), *Thymus vulgaris* (75.38 g/100g FAME); *Ocimum basilicum (*74.47 g/100g of FAME), *Melissa officinalis* (74.10 g/100g FAME) had values within a narrow range, varying from 74.10 to 78.22 g/100 g of FAME. The concentrations of SFA in ruminal fluid were higher (P <0.05) in *Coriandrum sativum*, *Satureja montana* and *Carum carvi* (78.22, 76.59 and 76.02 g/100g of FAME; respectively) and lower in ruminal fluid of *Melissa officinalis, Ocimum basilicum* and *Thymus vulgaris* (74.10, 74.47 and 75.38 g/100g of FAME, respectively).

Branched fatty acids

The effects of type of plant, dose and period of supply on the main branched fatty acids (OBCFA) and their sum are in Table 23. The most abundant OBCFA in the rumen fluid of ewes fed aromatic plants were C15:0 and anteiso C15:0, which represented about 20% of the OBCFA. It is important to note that these fatty acids are interesting parameters for the study of the ruminal activity because they originate from

the ruminal bacteria (Vlaeminck et al., 2006). Similarly to what occurred in milk (Tabella 20), both *Carum carvi* and *Coriandrum sativum* caused a reduction of the content of OBCFA, probably due to a decrease in ruminal activity. This is particularly evident in the case of *Coriandrum sativum*, because the ruminal fluid of sheep fed with this plant showed the lowest content of OBCFA (6.97 g/100 g of FAME).

Monounsaturated fatty acids

Contrary to what was observed for the SFA (Table 22), the proportion of monounsaturated fatty acids *(*MUFA) (Table 24), there were not important significance except for fatty acids C18:1 trans 6-8; C18: 1 trans 9; C18:1trans10, it was result an increase (P <0.001) in the rumen fluid of sheep fed with *Carum carvi* and *Coriandrum sativum* (0.38, 0.23; 1.08; 0.25; 0.26; 1:36; g/100g of FAME, respectively) in contrast to what was observed for other species *Melissa officinalis, Ocimum basilicum, Satureja montana* and *Thymus vulgaris* (Table 24). These data reflect the values of MUFA encountered during the analysis of fatty acids in the milk of sheep fed this group of plants (Table 18).

The high presence of C18: 1 cis-9 in the rumen fluid (Table 24) is mainly due to its abundance in the seeds of *Carum carvi* and *Coriandrum sativum*, in which it was represented for approximately 80% of the total of MUFA (Table 16).

Polyunsaturated fatty acids

As regards the content of Polyunsaturated Fatty Acids (PUFA) in rumen fluid, *Carum carvi* and *Coriandrum sativum* behaved in a similar way, causing a decrease of PUFA (Table 25), contrary to what happened for the milk.

Instead, the supply of *Melissa officinalis, Ocimum basilicum* and *Thymus vulgaris* caused higher ($P < 0.001$) values of C18: 2 n-6 (7.44, 7.21, 7.14 g/100 g FAME, respectively), and PUFA (11.31, 11.46, 11.04 g/100 g FAME, respectively), whereas in the the supply *of Thymus vulgaris* and *Ocimum basilicum* caused the highest proportions of C18:3 n-3 (1.64 and 1.99 g/100 g FAME, respectively) and PUFA (11.04 and 11.46 g/100 g of FAME, respectively). The concentration of rumenic acid (RA, cis-9, trans-11 CLA) in rumen fluid did not differ significantly among the aromatic plants (Table 25). It is important to note that the concentrations of cis-9, trans-11 CLA were much lower than previous in vitro observations in rumen fluid (32.0 mg/l, Wallace et al., 2007).

Effect of different doses used

The effect of the doses used was particularly evident for SFA (Table 22), and PUFA (Table 25). The highest dose (200 g/d) of aromatic plant caused the highest SFA and the lowest PUFA contents in rumen fluid . The highest dose increased PUFA of the series n-3 ($P < 0.001$) and decreased those of the n-6 series in the ruminal fluid. The cis-9, trans-11 (CLA) did not vary significantly, even if the highest dose of plant decreased PUFA. This might suggest a slowing of the process of ruminal biohydrogenation, because the CLA, in the rumen, is an intermediate product of this

process. This explanation is supported by the data obtained in milk, where the cis-9, trans-11 CLA increased (P <0.001) from 0.50 g/100 g of FAME in the control group to 0.6 g/100 g of FAME in the group with the highest dose (Table 20).

Rumen ammonia

The ammonia concentration in rumen fluid was not significantly influenced by plant and dose (Table 26), contrary to what found by Hristov et al. (2007),who assessed the variation of the ammonia in the rumen fluid *in vitro* after inoculation of essential oils, including the oil derived from *Carum carvi,* which was the only one to cause a reduction of ammonia by 8%.

Probably *in vivo* bacterial microflora can adapt to changes in the diet which can be easily detected, whereas *in vitro* incubation times can be too short to ensure the adaptation of the ruminal microflora (Teferedegne, 2000; Castillejos et al., 2007).

Ammonia concentration was influenced only by period of supply being the lower in the thirt period (14.89 mg/100ml) than in the other periods (18.37 mg/100ml in the first period and 19.26 mg/100ml in the second and fourth periods; Table 26).

Rumen pH

Rumen pH was fairly high for all the plants, probably because the fairly high crude protein content of the diets induced high ammonia production and thus an increase in rumen pH (Table 27).

There were significant differences among plants, with the highest value for *Thymus vulgaris* and the lowest for *Coriandrum sativum* (Table 27).

Regarding the dose, rumen pH was lowest for the Control diet and increased as the content of the aromatic plants increased. This suggests an interference of aromatic plants on volatile fatty acids production.

CONCLUSIONS

The research carried out showed that:

a) *Melissa officinalis* had the lowest palatability and intake among the plants studied, especially at the highest dosages;
b) Milk production persistency was good for all plants and treatments and did not differ from the control for all plants tested; in the two months of the experimental period milk production decreased only 200 g/d per ewe.
c) Carum carvi and *Satureja montana* induced the highest average milk yield, while *Melissa officinalis*, *Thymus vulgaris* and *Coriandrum sativum* the lowest;
d) Milk composition was markedly affected by the plant species; milk fat concentration was highest for *Carum carvi*. *Coriandrum sativum* and *Melissa officinalis* and lowest for *Thymus vulgaris* and *Ocimum basilicum*. Milk protein concentration was highest for *Melissa officinalis*, *Ocimum basilicum* and *Satureja Montana* and lowest for the other 3 plants;
e) The correlation between milk yield and milk fat and protein content was very low, suggesting a direct effects of the plants, unrelated to the dilution effect, to the chemical composition of the milk;
f) The Dose used within each plant did not affect milk production, while for some of the plants affected milk composition;
g) The utilization of aromatic plants did not cause any noticeable effect on their metabolic profiles and their health status.

h) Milk fatty acids composition was strongly influenced by the effect botanical species. In particular, *Carum carvi* and *Coriandrum sativum* changed to a similar extent the proportion of fatty acids, decreasing BCFA and SFA and increasing MUFA and TFA. *Thymus vulgaris* and *Carum carvi* increased PUFA content. The Dose significantly affected the percentage of SFA, MUFA and TFA.

i) the data related to rumen fluid fatty acids are in agreement with the findings described for milk fatty acids. In particular, in both cases *Carum carvi* and *Coriandrum sativum* caused a increase of SFA and a decrease of PUFA and OBCFA. Rumen ammonia was not affected by the plant species, while was highest with *Thymus vulgaris* and lowest with *Coriandrum sativum*.

Overall, it appeared that plant species affected many productive and rumen function variables. These results are somehow in contrast with most of the literature, which reported limited effects of the supply essential oils in vivo. The mechanism by which plant extracts resulted more effective that extracts of essential oils needs to be elucidates.

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Table 1. Chemical composition of the feeds used in the experiment.

Feed	Chemical composition							
	DM, % as fed	**Ash, % DM**	**CP, % DM**	**E.E., % DM**	**NDF, % DM**	**ADF, % DM**	**ADL, % DM**	**NEL [1] (Mcal/kg DM)**
Dehydrated alfalfa	88.31	8.89	16.2	2.40 *	41.97	29.86	6.62	1.33 *
Beet pulps	88.68	5.96	11.68	0.60 *	51.47	31.94	10.34	1.79 *
Corn grains	87.32	1.95	8.55	4.30 *	18.09	5.53	1.53	1.98 *
Pea grains	87.25	3.42	24.99	1.10 *	25.88	12.33	0.54	2.01 *
Carum carvi	87.85	8.14	25.58	8.24	53.88	31.73	11.46	1.87
Coriandrum sativum	87.50	6.35	12.80	9.69	75.72	53.01	17.11	1.30
Melissa officinalis	86.65	13.03	13.26	1.04	43.49	34.16	7.93	0.88
Ocimum basilicum	86.66	17.75	22.63	2.46	30.26	23.22	9.03	1.31
Satureja montana	85.60	9.11	11.04	1.70	34.14	36.82	9.47	1.29
Thymus vulgaris	90.07	11.51	16.88	3.11	39.40	27.86	10.93	1.29

* estimates based on the archive of the Small Ruminant Nutrition System.
[1] NEL = net energy of lactation, calculated at a feeding level of 3 times that of maintenance (from Chapter 1)

Species	Dose H			Dose M			Dose L		
	Supplied, g/d	Eaten, g/d	S.D.	Supplied, g/d	Eaten, g/d	S.D.	Supplied, g/d	Eaten, g/d	S.D.
Carum	228	228	± 10.5	143	143	± 0.0	57	57	± 0.1
Coriandrum	229	228	± 1.2	144	144	± 1.6	58	58	± 0.2
Melissa	231	145	± 76.9	144	137	± 12.8	58	58	± 0.7
Ocimum	231	216	± 11.1	146	146	± 0.7	58	58	± 0.0
Satureja	210	203	± 11.0	122	122	± 0.0	44	44	± 0.0
Thymus	222	199	± 32.1	142	138	± 5.9	57	57	± 0.4

Table 2. Average intake (g/d as fed) of the aromatic plants fed at milking time.

H = high; M = medium; L = Low.

Table 3. Body weight, body condition score, and milk yield and composition. Comparison among plant species.

Species	Milk production								Body weight (kg)	Body condition score (scale 0-5)
	Yield (g/d)	Fat content (%)	Fat yield (g/d)	Protein content (%)	Protein yield (g/d)	Lactose (%)	Urea (mg/dl)	SCC (logSCC)		
Carum carvi	1442 ^{A}	6.16 ^{A}	87.9 ^{a}	5.01 ^{B}	71.4 ^{b}	4.62 ^{A}	46.3 ^{A}	5.083 ^{B}	47.27 ^{A}	2.68
Coriandrum sativum	1311 ^{B}	6.23 ^{A}	80.6 ^{b}	5.10 ^{B}	66.4 ^{c}	4.61 ^{A}	40.8 ^{B}	5.609 ^{A}	46.44 ^{BC}	2.67
Melissa officinalis	1339 ^{B}	6.20 ^{A}	81.8 ^{b}	5.13 ^{A}	68.2 ^{bc}	4.56 ^{A}	41.8 ^{B}	5.292 ^{B}	46.96 ^{AB}	2.69
Ocimum basilicum	1393 ^{AB}	5.86 ^{B}	80.9 ^{b}	5.20 ^{A}	72.0 ^{ab}	4.56 ^{A}	40.3 ^{B}	5.659 ^{A}	46.89 ^{AB}	2.67
Satureja montana	1467 ^{A}	6.08 ^{AB}	88.3 ^{a}	5.17 ^{A}	75.4 ^{a}	4.46 ^{B}	40.3 ^{B}	5.609 ^{A}	46.14 ^{C}	2.63
Thymus vulgaris	1329 ^{B}	5.71 ^{B}	75.5 ^{b}	4.97 ^{B}	65.5 ^{c}	4.46 ^{B}	38.3 ^{B}	5.559 ^{A}	47.17 ^{A}	2.63
SEM	21.7	0.05	1.24	0.03	0.97	0.01	0.53	0.037	0.36	0.01
P for PERIOD factor	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
P for DOSE factor	NS	NS	NS	NS	0.13	NS	NS	NS	NS	NS
P for EWE factor	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

$^{A, B}$ P <0.01 $^{a, b, c}$ P <0.05 NS = P > 0.15; SCC = somatic cell count.

Table 4. Milk yield and composition for the Latin square based on **Carum carvi.**

	Milk yield and composition							
Dose (g/d of DM of the aromatic plant)	**Yield (g/d)**	**Fat content (%)**	**Fat yield (g/d)**	**Protein content (%)**	**Protein yield (g/d)**	**Lactose (%)**	**Urea (mg/dl)**	**SCC (logSCC)**
H (200 g/d)	1424	6.19	88.08	4.93 [b]	69.80	4.66	47.2	5.125
M (125 g/d)	1476	6.22	91.87	4.99 [ab]	72.91	4.65	48.2	5.115
L (50 g/d)	1436	6.19	86.55	5.01 [ab]	70.85	4.60	45.4	5.060
C (0 g/d)	1432	6.04	85.25	5.11 [a]	72.06	4.57	44.6	5.031
SEM	19.7	0.07	1.67	0.02	0.96	0.01	0.74	0.03
P for PERIOD factor	<0.001	<0.1	<0.15	<0.1	<0.001	NS	NS	<0.05
P for DOSE factor	NS	NS	NS	<0.05	NS	NS	NS	NS
P for EWE factor	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.05

[a, b] in the same row= P<0.05 H = high; M = medium; L = Low; C = control (no aromatic plant)

Table 5. Milk yield and composition for the Latin square based on **Coriandrum sativum.**

	Milk yield and composition							
Dose (g/d of DM of the aromatic plant)	**Yield (g/d)**	**Fat content (%)**	**Fat yield (g/d)**	**Protein content (%)**	**Protein yield (g/d)**	**Lactose (%)**	**Urea (mg/dl)**	**SCC (logSCC)**
H (200 g/d)	1281	6.50 ^{x}	81.45	5.02	63.72	4.62	42.97	5.544
M (125 g/d)	1263	6.25 $^{x\ y}$	77.86	5.09	63.72	4.62	42.56	5.541
L (50 g/d)	1370	6.15 ^{xy}	83.78	5.13	70.00	4.64	39.73	5.616
C (0 g/d)	1329	6.03 ^{y}	79.33	5.15	68.04	4.56	38.00	5.736
SEM	22	0.06	1.20	0.026	1.12	0.01	0.81	0.06
P for PERIOD factor	<0.01	NS	<0.05	NS	NS	NS	<0.1	NS
P for DOSE factor	NS	<0.10	NS	NS	<0.01	NS	<0.15	NS
P for EWE factor	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

$^{a, b}$ in the same row = P<0.05, $^{x, y}$ in the same row = P<0.1 H = high; M = medium; L = Low; C = control (no aromatic plant)

Table 6. Milk yield and composition for the Latin square based on **Melissa officinalis.**

	Milk yield and composition							
Dose (g/d of DM of the aromatic plant)	**Yield (g/d)**	**Fat content (%)**	**Fat yield (g/d)**	**Protein content (%)**	**Protein yield (g/d)**	**Lactose (%)**	**Urea (mg/dl)**	**SCC (logSCC)**
H (200 g/d)	1324	6.20	80.59	5.15	67.87	4.56	39.36	5.32
M (125 g/d)	1344	6.30	83.44	5.09	67.99	4.55	41.05	5.38
L (50 g/d)	1319	6.08	79.08	5.13	67.17	4.52	44.14	5.16
C (0 g/d)	1369	6.21	84.24	5.16	69.92	4.62	42.58	5.30
SEM	20	0.07671	1.22	0.03	0.93	0.02	0.82	0.04
P for PERIOD factor	<0.001	<0.05	<0.001	<0.1	<0.001	NS	<0.1	NS
P for DOSE factor	NS	NS	NS	NS	NS	NS	NS	NS
P for EWE factor	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	NS	NS

H = high; M = medium; L = Low; C = control (no aromatic plant)

Table 7. Milk yield and composition for the Latin square based on **Ocimum basilicum.**

	Milk yield and composition							
Dose (g/d of DM of the aromatic plant)	**Yield (g/d)**	**Fat content (%)**	**Fat yield (g/d)**	**Protein content (%)**	**Protein yield (g/d)**	**Lactose (%)**	**Urea (mg/dl)**	**SCC (logSCC)**
H (200 g/d)	1399	5.85	81.17	5.17	71.92	4.56	40.46	5.570
M (125 g/d)	1359	5.86	78.87	5.13	69.29	4.57	38.49	5.580
L (50 g/d)	1402	5.80	81.30	5.27	73.56	4.52	40.02	5.861
C (0 g/d)	1412	5.90	82.19	5.23	73.23	4.56	42.37	5.624
SEM	27	0.05	1.53	0.02	1.23	0.01	1.00	0.08
P for PERIOD factor	<0.1	<0.001	NS	<0.05	<0.05	NS	<0.15	NS
P for DOSE factor	NS	NS	NS	NS	NS	NS	NS	NS
P for EWE factor	<0.001	<0.001	<0.001	<0.001	<0.001	<0.005	NS	<0.05

H = high; M = medium; L = Low; C = control (no aromatic plant)

Table 8. Milk yield and composition for the Latin square based on **Satureja montana.**

	Milk yield and composition							
Dose (g/d of DM of the aromatic plant)	**Yield (g/d)**	**Fat content (%)**	**Fat yield (g/d)**	**Protein content (%)**	**Protein yield (g/d)**	**Lactose (%)**	**Urea (mg/dl)**	**SCC (logSCC)**
H (180 g/d)	1465	6.04	87.63	5.19	75.49	4.45	38.98	5.60
M (110 g/d)	1424	6.00	84.73	5.13	72.62	4.47	43.28	5.64
L (40 g/d)	1507	6.22	92.84	5.22	78.08	4.44	37.54	5.59
C (0 g/d)	1473	6.03	88.04	5.14	75.21	4.49	41.20	5.60
SEM	23	0.06	2.02	0.02	1.09	0.01	0.80	0.04
P for PERIOD factor	<0.005	<0.005	NS	<0.005	<0.01	<0.15	<0.05	NS
P for DOSE factor	NS	NS	NS	NS	NS	NS	<0.1	NS
P for EWE factor	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

H = high; M = medium; L = Low; C = control (no aromatic plant)

Table 9. Milk yield and composition for the Latin square based on **Thymus vulgaris.**

	Milk yield and composition							
Dose (g/d of DM of the aromatic plant)	**Yield (g/d)**	**Fat content (%)**	**Fat yield (g/d)**	**Protein content (%)**	**Protein yield (g/d)**	**Lactose (%)**	**Urea (mg/dl)**	**SCC (logSCC)**
H (200 g/d)	1347	5.83	78.37	4.97 [ab]	66.69	4.47	36.62 [ab]	5.65
M (125 g/d)	1263	5.84	73.31	5.06 [a]	63.50	4.44	34.98 [b]	5.60
L (50 g/d)	1398	5.61	76.55	4.92 [b]	66.76	4.44	40.12 [a]	5.53
C (0 g/d)	1339	5.54	73.76	4.93 [b]	65.22	4.49	41.29 [a]	5.45
SEM	26	0.04	1.55	0.01	1.27	0.01	0.66	0.05
P for PERIOD factor	<0.001	<0.15	<0.05	<0.005	<0.005	<0.15	<0.01	NS
P for DOSE factor	NS	<0.15	NS	<0.05	NS	NS	<0.05	NS
P for EWE factor	<0.001	<0.001	<0.001	<0.001	<0.001	NS	NS	<0.001

[a, b] in the same row = P<0.05 H = high; M = medium; L = Low; C = control (no aromatic plant).

Table 10. Blood serum chemistry in the preliminary period for the ewes later assigned to different plant Latin squares.

PLANTS	ALB (g/dl)	ALP (U/L)	BT (mg/dl)	CRE (mg/dl)	GGT (U/L)	GOT (U/L)	GPT (U/L)	PROT (g/dl)	UREA (mg/dl)
				Normal	**range**				
	2.0-3.5	**45-250**	**0.15-0.65**	**0.3-0.9**	**60-120**	**70-200**	**15-45**	**6-8.5**	**25-60**
Carum	2.66	130.30	0.18	0.51	95.86	146.3	34.71	7.54	54.14
Coriandrum	2.70	81.50	0.10	0.30	103.50	127.5	33.00	8.10	51.00
Melissa	2.60	94.75	0.17	0.50	77.50	116.0	28.75	7.12	53.75
Ocimum	2.63	183.68	0.20	0.50	92.50	142.3	26.33	7.20	50.00
Satureja	2.63	110.50	0.15	0.51	97.17	148.7	29.33	7.70	48.00
Thymus	2.45	100.00	0.24	0.35	106.50	122.0	29.00	6.90	40.00
SEM	0.019	4279	0.005	0.001	142.1	790.2	122.8	0.206	117.3
P PLANT	NS	NS	NS	NS	NS	NS	NS	NS	NS

ALB = Albumin; ALP = Alkaline Phosphatase; BT = Total Bilirubine; CRE = Creatinine; GGT = Gammaglutamiltranspeptidas; GOT = Glutamic Oxaloacetic Transaminase; GPT= Glutamic Pyruvic Transaminase; PROT = Total Protein; UREA= Urea.

Table 11. Hemogram values in the preliminary period for the ewes later assigned to different plant Latin squares.

	RBC ($x10^6$ cells/µL)	HGB (g/dL)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	PLT ($x10^3$ cells/µL)
Normal range	8.8-16.0	8.9-15.4	25.8-44.0	21.6-34.9	8.3-12.3	32.7-37.3	247.3-764.8
Carum	8.50	8.57	26.35	32.80	10.67	32.55	669.5
Coriandrum	8.33	8.70	26.30	31.71	10.49	33.06	659.5
Melissa	8.24	8.63	26.24	37.86	1052	33.00	786.4
Ocimum	8.25	8.51	26.25	31.82	10.31	32.39	600.6
Satureja	8.38	9.00	27.46	32.74	10.75	32.86	733.0
Thymus	8.07	8.41	25.54	31.76	10.50	33.10	838.2
SEM	0.10	0.09	0.32	0.27	0.09	0.13	35.4
P PLANT	NS	NS	NS	NS	NS	NS	NS

WBCB = White Blood Cells; RBC = Red Blood Cells; HGB = Hemoglobin; HTC = Hematocrit; MCV = Mean Corpuscular Volume of Red Blood Cells; MCHC = Mean Corpuscolar Haemoglobin Concentration; PLT= Total Platelets.

Table 12. Total and differential white blood cells count in the preliminary period for the ewes later assigned to different plant Latin squares.

	WBCB ($x10^3$ cells/µL)	NEUTS ($x10^3$ cells/µl)	LYMPHS ($x10^3$ cells/µL)	MONOS ($x10^3$ cells/µL)	EOS ($x10^3$ cells/µL)	BASOS ($x10^3$ cells/µL)
Normal range	4.0-13.0	1.4-6.0	2.0-9.5	0-0.9	0-1.3	0-0.2
Carum	9.83	3.52	4.43	0.51	1.25	0.6
Coriandrum	10.18	4.44	4.34	0.42	0.86	0.5
Melissa	10.03	3.65	4.97	0.37	0.91	0.5
Ocimum	10.25	4.23	4.22	0.37	1.28	0.7
Satureja	9.12	3.64	3.85	0.45	1.07	0.6
Thymus	7.59	2.62	3.76	0.24	0.87	0.6
SEM	0.37	0.23	0.15	0.04	0.10	0.001
P PLANT	NS	NS	NS	NS	NS	NS

NEUTS = Neutrophil Cells; LYMPHS = Lymphocytes Cells; MONOS = Monocytes Cells; EOS = Eosinophils Cells; BASOS = Basophils Cells.

Table 13. Blood serum chemistry during the experimental period.

Plants	*ALB (g/dl)*	*ALP (U/L)*	*BT (mg/dl)*	*CRE (mg/dl)*	*GGT (U/L)*	*GOT (U/L)*	*GPT (U/L)*	*PROT (g/dl)*	*UREA (mg/dl)*
	2,0-3,5	45-250	0,15-0,65	0,3-0,9	60-120	70-200	15-45	6-8,5	25-60
Carum	2.81^{b}	145.6^{a}	0.13	0.45	89.56^{b}	155.1^{a}	31.47	7.47^{ab}	48.10^{a}
Coriandrum	2.82^{b}	121.8^{b}	0.12	0.48	92.34^{ab}	138.5^{ab}	31.81	7.52^{a}	45.60^{ab}
Melissa	2.89^{a}	119.6^{b}	0.14	0.48	78.81^{c}	119.8^{b}	32.19	7.32^{b}	45.00^{ab}
Ocimum	2.82^{b}	142.7^{a}	0.12	0.44	83.62^{bc}	135.6^{ab}	31.87	7.42^{ab}	43.20^{ab}
Satureja	2.82^{b}	106.4^{b}	0.14	0.47	90.56^{ab}	134.0^{ab}	39.78	7.49^{ab}	42.81^{ab}
Thymus	2.85^{ab}	135.4^{a}	0.12	0.45	98.97^{a}	136.9^{ab}	30.53	7.05^{c}	41.70^{b}
SEM	0.010	4.74	0.07	0.008	1.35	3.31	1.07	0.03	0.73
P PLANT	0.001	0.000	NS	NS	0.000	0.002	NS	0.000	0.019
DOSAGE									
A (200 g/d)	2.83	129.1	0.13	0.45	88.25	134.4	32.06	7.37	44.06
B (125 g/d)	2.83	131.2	0.12	0.47	90.08	139.6	32.79	7.40	44.42
C (50 g/d)	2.84	125.6	0.13	0.45	88.73	138.2	35.42	7.40	44.31
D (0 g/d)	2.85	128.5	0.13	0.48	88.85	134.4	31.50	7.33	44.73
SEM	0.010	4.74	0.07	0.008	1.35	3.31	1.07	0.03	0.73
P DOSAGE	NS	NS	NS	NS	NS	NS	NS	NS	NS
PERIOD									
1	2.72^{b}	143.7^{a}	0.16^{a}	0.51^{a}	90.77^{ab}	129.2^{b}	32.48	7.35	40.60^{b}
2	2.86^{ab}	135.8^{a}	0.17^{a}	0.52^{a}	93.27^{a}	129.7^{b}	32.85	7.43	45.30^{a}
3	2.86^{ab}	115.5^{b}	0.12^{c}	0.44^{c}	84.44^{b}	132.9^{b}	32.46	7.32	46.23^{a}
4	2.91^{a}	119.3^{b}	0.07^{b}	0.37^{b}	87.44^{ab}	154.8^{a}	33.98	7.40	45.42^{a}
SEM	0.010	4.74	0.07	0.008	1.35	3.31	1.07	0.03	0.73
P PERIOD	0.000	0.000	0.000	0.000	0.000	0.000	NS	NS	0.002

$^{a\ b}$ in the same column = P<0.05

ALB = Albumin; ALP = Alkaline Phosphatase; BT = Total Bilirubine; CRE = Creatinine; GGT = Gammaglutamiltranspeptidas; GOT = Glutamic Oxaloacetic Transaminase; GPT= Glutamic Pyruvic Transaminase; PROT = Total Protein; UREA= Urea.

Table 14. Hemogram values during the experimental period.

Plants	RBC (x10^6 cells/µL)	HGB (g/dL)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	PLT (x10^3 cells/µL)
Normal range	8.85 -16.0	8.9- 15.4	25.8-44.0	21.6-34.9	8.3-12.3	32.7-37.3	247-765
Carum	8.07	8.44^{ab}	25.80	32.10^{ab}	10.48^{ab}	32.72	576.0^{b}
Coriandrum	8.37	8.59^{ab}	26.50	31.82^{ab}	10.32^{c}	32.44	554.6^{b}
Melissa	7.97	8.56^{ab}	25.50	31.03^{b}	10.41^{bc}	31.59	667.3^{a}
Ocimum	8.50	8.35^{b}	26.10	30.80^{b}	9.87^{d}	32.08	544.0^{b}
Satureja	7.93	8.36^{b}	25.90	32.72^{a}	10.60^{a}	32.31	579.0^{b}
Thymus	8.39	8.74^{a}	26.95	32.20^{ab}	10.42^{bc}	32.40	702.0^{a}
SEM	1.04	0.78	3.06	2.95	0.58	2.54	181.6
P PLANT	0.012	0.021	NS	0.005	0.000	NS	0.000
DOSAGE							
A (200 g/d)	8.21	8.47	26.11	31.94	10.35	32.44	593.4
B (125 g/d)	8.26	8.50	26.23	31.85	10.31	32.43	627.6
C (50 g/d)	8.14	8.56	25.93	31.30	10.32	31.70	583.0
D (0 g/d)	8.20	8.49	26.20	32.02	10.40	32.50	611.0
SEM	1.04	0.78	3.06	2.95	0.58	2.54	181.6
P DOSAGE	NS	NS	NS	NS	NS	NS	NS
PERIOD							
1	8.17	8.51	26.36	32.33	10.43^{a}	32.30^{ab}	625.0^{ab}
2	8.15	8.39	26.00	32.00	10.34^{b}	32.35^{ab}	584.0^{bc}
3	8.09	8.46	25.84	31.40	10.28^{b}	31.43^{b}	635.0^{a}
4	8.40	8.65	26.30	31.40	10.32^{b}	33.00^{a}	571.5^{c}
SEM	1.04	0.78	3.06	2.95	0.58	2.54	181.6
P PERIOD	NS	NS	NS	NS	0.001	0.031	0.001

$^{a\ b}$ in the same column = P<0.05

WBCB = White Blood Cells; RBC = Red Blood Cells; HGB = Hemoglobin; HTC = Hematocrit; MCV = Mean Corpuscular Volume of Red Blood Cells; MCHC = Mean Corpuscolar Haemoglobin Concentration; PLT= Total Platelets.

Table 15. Total and differential white blood cells count during the experimental period.

PLANT	WBCB (x10^3 cells/µL)	NEUTS (X10^3 cells/µL)	LYMPHS (X10^3 cells/µL)	MONOS (X10^3 cells/µL)	EOS (X10^3 cells/µL)	BASOS (X10^3 cells/µL)
Normal range	4.0-13.0	1.4-6.0	2.0-9.5	0-0.9	0-1.3	0-0.2
Carum	8.85^a	2.93^b	4.61^a	0.27^{ab}	0.93^{ab}	0.05^a
Coriandrum	8.19^{ab}	2.84^b	4.15^{ab}	0.21^b	0.90^{ab}	0.04^b
Melissa	8.83^a	2.73^b	4.60^a	0.29^{ab}	1.11^a	0.05^{ab}
Ocimum	8.51^{ab}	2.90^b	4.14^{ab}	0.44^a	0.92^{ab}	0.05^a
Satureja	8.62^{ab}	3.56^a	4.08^{ab}	0.21^b	0.67^b	0.04^b
Thymus	7.98^b	2.98^b	3.65^b	0.27^{ab}	1.00^a	0.05^b
SEM	1.85	1.07	1.00	0.27	0.63	0.02
P PLANT	0.001	0.000	0.000	0.000	0.006	0.004
DOSE						
A (200 g/d)	8.25	2.76	4.13	0.34	0.91	0.05
B (125 g/d)	8.68	3.14	4.29	0.27	0.90	0.05
C (50 g/d)	8.61	2.79	4.24	0.27	1.00	0.04
D (0 g/d)	8.43	2.55	4.15	0.25	0.91	0.05
SEM	1.85	1.07	1.00	0.27	0.63	0.02
P DOSE	NS	NS	NS	NS	NS	NS
PERIOD						
1	9.15^a	3.21^a	4.43	0.27	1.12^a	0.06^a
2	8.5^b	3.42^a	3.98	0.23	0.84^b	0.04^b
3	8.23^{bc}	2.79^b	4.16	0.33	0.90^{ab}	0.04^b
4	8.05^c	2.55^b	4.25	0.30	0.85^b	0.04^b
SEM	1.85	1.07	1.00	0.27	0.63	0.02
P PERIOD	0.000	0.000	NS	NS	0.004	0.000

$^{a\ b}$ in the same column = $P < 0.05$

NEUTS = Neutrophil Cells; LYMPHS = Lymphocytes Cells; MONOS = Monocytes Cells; EOS = Eosinophils Cells; BASOS = Basophils Cells.

Table 16. Fatty acid composition of the aromatic plants.

	Plants					
Fatty acid (g/100g of FAME)	***Carum carvi***	***Coriandrum sativum***	***Melissa officinalis***	***Ocimum basilicum***	***Satureja montana***	***Thymus vulgaris***
C14:0	0.10	0.09	0.43	0.42	0.26	0.41
C16:0	5.71	4.82	22.40	22.06	20.05	17.33
C16:1 *cis-9*	0.00	0.23	0.00	2.58	2.49	2.54
C18:0	1.80	0.86	3.68	4.83	2.66	3.52
C18:1 *cis-9*	41.32	69.48	7.64	6.00	8.20	9.21
C18:2 *n-6*	35.07	20.47	17.58	12.29	13.76	14.89
C18:3 *n-3*	0.66	0.37	31.63	34.54	43.13	39.65
C22:1 *n-9*	0.38	0.02	0.46	1.12	1.26	3.47
ΣSFA	9.32	7.82	32.46	34.18	28.28	27.14
ΣMUFA	54.66	70.61	18.34	18.45	13.54	17.27
ΣPUFA	36.02	21.58	49.21	47.37	58.18	55.58

FAME = fatty acids methyl-esters; SFA = short chain fatty acids; MUFA = mono-unsaturated fatty acids; PUFA = Poly-unsaturated fatty acids

Table 17. Effects of plant, period and dose on proportions of individual and total saturated fatty acids (SFA; g/100 g of FAME) in milk.

	Fatty Acids (g/100g of FAME)								
PLANT	**C4:0**	**C6:0**	**C8:0**	**C10:0**	**C12:0**	**C14:0**	**C16:0**	**C18:0**	**SFA**
Carum	2.68	1.48^{bc}	1.62^{bc}	5.77^{c}	3.65^{b}	11.49^{b}	29.90^{b}	9.31^{a}	70.25^{b}
Coriandrum	2.64	1.41^{c}	1.53^{c}	5.53^{c}	3.60^{b}	11.60^{b}	28.70^{b}	9.85^{a}	69.64^{b}
Melissa	2.73	1.69^{a}	1.96^{a}	7.50^{a}	4.84^{a}	12.88^{a}	34.03^{a}	6.38^{b}	77.45^{a}
Ocimum	2.59	1.59^{ab}	1.86^{a}	7.14^{ab}	4.81^{a}	13.25^{a}	33.41^{a}	6.34^{b}	76.42^{a}
Satureja	2.61	1.56^{ab}	1.80^{ab}	6.84^{b}	4.54^{a}	13.31^{a}	34.28^{a}	6.26^{b}	76.79^{a}
Thymus	2.66	1.57^{ab}	1.80^{a}	6.87^{b}	4.62^{a}	12.95^{a}	33.68^{a}	6.22^{b}	75.79^{a}
P Plant	NS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
SEM	0.03	0.02	0.03	0.12	0.09	0.14	0.37	0.24	0.47
DOSE									
A (200 g/d)	2.60	1.45^{b}	1.61^{c}	5.97^{b}	3.92^{b}	12.02^{c}	31.32^{c}	8.35^{a}	72.59^{c}
B (125 g/d)	2.68	1.54^{ab}	1.73^{bc}	6.38^{b}	4.16^{b}	12.30^{c}	31.59^{bc}	7.88^{a}	73.43^{c}
C (50 g/d)	2.68	1.59^{a}	1.82^{ab}	6.86^{a}	4.53^{a}	12.77^{b}	32.68^{ab}	7.06^{b}	75.10^{b}
D (0 g/d)	2.64	1.62^{a}	1.88^{a}	7.22^{a}	4.76^{a}	13.24^{a}	33.75^{a}	6.29^{c}	76.43^{a}
P Dose	NS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
SEM	0.03	0.02	0.03	0.12	0.09	0.14	0.37	0.24	0.47
PERIOD									
1	2.69^{b}	1.54^{b}	1.75^{b}	6.39^{b}	4.12^{b}	11.95^{c}	30.73^{c}	7.97^{a}	72.82^{b}
2	2.86^{a}	1.69^{a}	1.94^{a}	7.09^{a}	4.54^{a}	12.40^{b}	31.43^{b}	7.27^{b}	74.33^{a}
3	2.59^{bc}	1.51^{b}	1.70^{b}	6.51^{b}	4.34^{ab}	12.92^{a}	34.11^{a}	6.95^{b}	75.60^{a}
4	2.47^{c}	1.45^{b}	1.66^{b}	6.45^{b}	4.37^{ab}	13.06^{a}	33.06^{a}	7.38^{ab}	74.80^{a}
P Period	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
SEM	0.03	0.02	0.03	0.12	0.09	0.14	0.37	0.24	0.47

$^{a\ b}$ in the same column= P<0.05; FAME = fatty acid methyl esters

Table 18. Effects of plant, period and dose on proportions of individual and total mono-unsaturated fatty acids (MUFA; g/100 g of FAME) in milk.

Fatty Acids (g/100g **of FAME)**							
PLANT	**C14:1**	**C16:1**	**C17:1**	**C18:1 *cis* 9**	**C22:1 *n*9**	**C24:1 *cis* 15**	**MUFA**
Carum	0.25^{bc}	0.98^{c}	0.23^{d}	19.69^{a}	0.00	0.01	24.75^{a}
Coriandrum	0.27^{abc}	1.02^{bc}	0.27^{c}	20.30^{a}	0.00	0.01	25.82^{a}
Melissa	0.23^{c}	1.01^{bc}	0.30^{b}	13.80^{b}	0.00	0.01	18.15^{b}
Ocimum	0.29^{ab}	1.12^{b}	0.32^{ab}	14.58^{b}	0.00	0.01	18.94^{b}
Satureja	0.31^{a}	1.25^{a}	0.34^{a}	14.24^{b}	0.01	0.01	18.84^{b}
Thymus	0.26^{bc}	1.02^{bc}	0.32^{ab}	14.81^{b}	0.00	0.01	19.17^{b}
P Plant	0.000	0.000	0.000	0.000	NS	NS	0.000
SEM	0.001	0.012	0.000	1.508	0.000	0.000	2.015
DOSE							
A (200 g/d)	0.25^{b}	1.03^{b}	0.30	17.47^{a}	0.003	0.008	22.52^{a}
B (125 g/d)	0.26^{b}	1.00^{b}	0.29	17.05^{a}	0.004	0.008	21.83^{a}
C (50 g/d)	0.27^{ab}	1.08^{ab}	0.29	15.82^{b}	0.005	0.009	20.28^{b}
D (0 g/d)	0.30^{a}	1.15^{a}	0.30	14.60^{c}	0.002	0.009	19.14^{c}
P Pianta	0.001	0.000	NS	0.000	NS	NS	0.000
SEM	0.001	0.012	0.000	1.508	0.000	0.000	2.015
PERIOD							
1	0.24^{b}	1.03	0.30^{ab}	17.11^{a}	0.00	0.008^{ab}	22.09^{a}
2	0.26^{b}	1.08	0.31^{a}	16.36^{ab}	0.00	0.006^{b}	20.76^{b}
3	0.28^{ab}	1.08	0.29^{b}	15.44^{b}	0.00	0.007^{ab}	20.12^{b}
4	0.30^{a}	1.08	0.29^{b}	16.04^{b}	0.01	0.010^{a}	20.80^{b}
P Period	0.001	NS	0.001	0.000	NS	0.047	0.000
SEM	0.001	0.012	0.000	1.508	0.000	0.000	2.015

^{ab} in the same column = P<0.05; FAME = fatty acid methyl esters

Table 19. Effects of plant, period and dose on proportions of individual and total branched chain fatty acids (BCFA; g/100 g of FAME)in milk.

	Fatty Acids (g/100g **of FAME**)								
PLANT	**iso C13:0**	**anteiso C13:0**	**iso C14:0**	**iso C15:0**	**anteiso C15:0**	**iso C16:0**	**iso C17:0**	**anteiso C17:0**	**BCFA**
Carum	0.03^{b}	0.05^{cd}	0.13^{c}	0.27^{c}	0.50^{d}	0.30^{d}	0.43^{b}	0.45^{c}	2.16^{c}
Coriandrum	0.03^{b}	0.05^{d}	0.17^{bc}	0.30^{bc}	0.56^{cd}	0.34^{cd}	0.46^{ab}	0.51^{b}	2.41^{b}
Melissa	0.03^{b}	0.06^{bcd}	0.20^{ab}	0.32^{ab}	0.63^{ab}	0.39^{ab}	0.48^{ab}	0.58^{a}	2.71^{a}
Ocimum	0.05^{a}	0.08^{a}	0.19^{ab}	0.32^{ab}	0.64^{ab}	0.38^{bc}	0.47^{ab}	0.55^{ab}	2.68^{a}
Satureja	0.05^{a}	0.07^{abc}	0.22^{a}	0.35^{a}	0.65^{a}	0.43^{a}	0.51^{a}	0.57^{a}	2.86^{a}
Thymus	0.04^{ab}	0.07^{ab}	0.21^{a}	0.34^{a}	0.58^{bc}	0.41^{ab}	0.52^{a}	0.55^{a}	2.72^{a}
P Plant	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
SEM	0.46	0.02	0.03	0.31	0.12	0.30	2.32	0.60	2.77
DOSE									
A (200 g/d)	0.03^{b}	0.04^{c}	0.20	0.32	0.63^{a}	0.40^{a}	0.47	0.54	2.64
B (125 g/d)	0.04^{b}	0.06^{b}	0.19	0.32	0.59^{ab}	0.37^{ab}	0.49	0.52	2.59
C (50 g/d)	0.04^{ab}	0.06^{b}	0.18	0.31	0.58^{b}	0.38^{ab}	0.49	0.54	2.58
D (0 g/d)	0.05^{a}	0.08^{a}	0.18	0.32	0.58^{ab}	0.36^{b}	0.46	0.54	2.56
P Dose	0.001	0.000	NS	NS	0.041	0.036	NS	NS	NS
SEM	0.46	0.02	0.03	0.31	0.12	0.30	2.32	0.60	2.77
PERIOD									
1	0.03^{b}	0.05^{b}	0.24^{a}	0.37^{a}	0.68^{a}	0.45^{a}	0.54^{a}	0.59^{a}	2.96^{a}
2	0.04^{b}	0.06^{b}	0.19^{b}	0.29^{b}	0.60^{b}	0.37^{b}	0.44^{b}	0.53^{b}	2.53^{b}
3	0.04^{ab}	0.06^{b}	0.17^{bc}	0.30^{b}	0.55^{c}	0.35^{b}	0.45^{b}	0.51^{b}	2.41^{b}
4	0.05^{a}	0.08^{a}	0.15^{c}	0.31^{b}	0.55^{c}	0.33^{b}	0.47^{b}	0.51^{b}	2.46^{b}
P Period	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
SEM	0.46	0.02	0.03	0.31	0.12	0.30	2.32	0.60	2.77

$^{a\ b}$ in the same column = P<0.05; FAME = fatty acid methyl esters

Table 20. Effects of plant, period and dose on proportions of individual and total poli-unsaturated fatty acids (PUFA; g/100 g of FAME) in milk.

	Fatty Acids (g/100g **of FAME)**								
PLANT	**C18:2 n6**	**C18:3 n3**	**cis 9, trans11 CLA**	**C20:4 n6**	**C20:5 n3**	**C22:5 n3**	**C22:6 n3**	**PUFA n3**	**PUFA n6**
Carum	2.03^{a}	0.78^{abc}	0.62^{a}	0.13^{b}	0.05^{b}	0.13^{bc}	0.03^{ab}	0.94^{abc}	2.38^{a}
Coriandrum	1.78^{b}	0.70^{cd}	0.55^{ab}	0.13^{b}	0.06^{ab}	0.12^{c}	0.04^{a}	0.86^{c}	2.11^{b}
Melissa	1.67^{b}	0.74^{bcd}	0.53^{b}	0.14^{ab}	0.06^{ab}	0.12^{c}	0.02^{b}	0.89^{bc}	2.06^{b}
Ocimum	1.73^{b}	0.82^{ab}	0.57^{ab}	0.15^{a}	0.07^{ab}	0.13^{abc}	0.03^{ab}	0.98^{ab}	2.11^{b}
Satureja	1.62^{b}	0.67^{d}	0.51^{b}	0.16^{a}	0.07^{ab}	0.14^{ab}	0.03^{ab}	0.84^{c}	2.02^{b}
Thymus	2.01^{a}	0.86^{a}	0.57^{ab}	0.16^{a}	0.08^{a}	0.14^{a}	0.04^{ab}	1.04^{a}	2.42^{a}
P Plant	0.000	0.000	0.004	0.000	0.007	0.000	0,036	0.000	0.000
SEM	0.029	0.01	0.00	0.00	0.00	0.00	0.03	0.01	0.03
DOSE									
A (200 g/d)	1.82	0.82^{a}	0.63^{a}	0.14^{b}	0.06	0.13	0.03	0.99^{a}	2.19
B (125 g/d)	1.83	0.77^{ab}	0.58^{b}	0.14^{ab}	0.06	0.13	0.03	0.94^{ab}	2.20
C (50 g/d)	1.83	0.74^{b}	0.53^{bc}	0.15^{ab}	0.07	0.13	0.03	0.90^{b}	2.21
D (0 g/d)	1.76	0.71^{b}	0.50^{c}	0.15^{a}	0.06	0.13	0.03	0.87^{b}	2.13
P Dose	NS	0.005	0.000	0.014	NS	NS	NS	0.003	NS
SEM	0.029	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.03
PERIOD									
1	2.02^{a}	0.76^{b}	0.69^{a}	0.15	0.07	0.15^{a}	0.04	0.95^{ab}	2.42^{a}
2	1.94^{a}	0.86^{a}	0.59^{b}	0.15	0.06	0.13^{b}	0.04	1.02^{a}	2.31^{a}
3	1.71^{b}	0.67^{c}	0.50^{c}	0.14	0.06	0.12^{b}	0.03	0.82^{c}	2.07^{b}
4	1.56^{c}	0.76^{b}	0.46^{c}	0.14	0.06	0.12^{b}	0.03	0.91^{b}	1.94^{b}
P Period	0.000	0.000	0.000	NS	NS	0.000	NS	0.041	0.000
SEM	0.029	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.03

$^{a\,b}$ in the same column= P<0.05; FAME = fatty acid methyl esters

Table 21. Effect of plants, period and dose on the proportions of the individual and total trans fatty acids (TFA; g/100 g of FAME) in milk.

	Fatty Acids (g/100g **of FAME)**					
PLANT	**C18:1 t 4**	**C18:1 t 6 - 8**	**C18:1 t 9**	**C18:1 t 10**	**C18:1 t11**	**TFA**
Carum	0.06^{ab}	0.22^{a}	0.15^{ab}	0.25^{a}	1.14^{a}	1.89^{a}
Coriandrum	0.09^{a}	0.31^{a}	0.18^{a}	0.24^{ab}	1.37^{a}	2.19^{a}
Melissa	0.04^{b}	0.08^{b}	0.09^{c}	0.19^{abc}	0.86^{b}	1.26^{b}
Ocimum	0.03^{b}	0.07^{b}	0.08^{c}	0.16^{c}	0.77^{c}	1.11^{b}
Satureja	0.04^{b}	0.07^{b}	0.09^{c}	0.17^{bc}	0.73^{c}	1.10^{b}
Thymus	0.02^{b}	0.07^{b}	0.09^{bc}	0.18^{abc}	0.80^{c}	1.18^{b}
P Plant	0.000	0.000	0.000	0.001	0.000	0.000
SEM	0.001	0.008	0.003	0.005	0.035	0.189
DOSE						
A (200 g/d)	0.07^{a}	0.22^{a}	0.14^{a}	0.22	1.15^{a}	1.83^{a}
B (125 g/d)	0.05^{ab}	0.16^{ab}	0.13^{ab}	0.22	1.05^{a}	1.59^{a}
C (50 g/d)	0.04^{b}	0.09^{bc}	0.09^{b}	0.17	0.87^{b}	1.25^{b}
D (0 g/d)	0.04^{ab}	0.08^{c}	0.10^{ab}	0.19	0.72^{c}	1.13^{b}
P Dose	0.030	0.000	0.026	NS	0.000	0.000
SEM	0.001	0.008	0.003	0.005	0.035	0.189
PERIOD						
1	0.06	0.17^{a}	0.13^{a}	0.24^{a}	1.18^{a}	1.79^{a}
2	0.03	0.08^{b}	0.05^{b}	0.13^{b}	0.90^{b}	1.21^{b}
3	0.05	0.15^{ab}	0.12^{a}	0.21^{a}	0.87^{b}	1.43^{b}
4	0.05	0.15^{ab}	0.15^{a}	0.20^{a}	0.83^{b}	1.38^{b}
P Period	NS	0.014	0.000	0.000	0.000	0.000
SEM	0.001	0.008	0.003	0.005	0.035	0.189

$^{a\,b}$ in the same column P<0.05; FAME = fatty acid methyl esters

Table 22. Effects of plant, period and dose on proportions of individual and total saturated fatty acids (SFA; g/100 g of FAME) in rumen fluid.

	Fatty acid (g/100g of FAME)								
	C4:0	**C6:0**	**C8:0**	**C10:0**	**C12:0**	**C14:0**	**C16:0**	**C18:0**	**SFA**
PLANT									
Carum	13.14^{b}	0.86^{b}	0.01	0.01	0.16^{b}	0.54^{b}	16.77^{bc}	34.67^{a}	76.02^{abc}
Coriandrum	13.34^{b}	0.97^{ab}	0.01	0.01	0.14^{b}	0.55^{b}	15.82^{c}	37.66^{a}	78.22^{a}
Melissa	15.60^{ab}	0.91^{b}	0.02	0.03	0.30^{a}	0.76^{a}	20.77^{a}	22.75^{b}	74.10^{c}
Ocimum	15.97^{ab}	0.97^{ab}	0.01	0.02	0.29^{a}	0.75^{a}	20.22^{a}	23.48^{b}	74.47^{bc}
Satureja	15.98^{ab}	1.26^{a}	0.03	0.02	0.27^{a}	0.84^{a}	20.16^{a}	24.72^{b}	76.59^{ab}
Thymus	18.64^{a}	0.95^{b}	0.06	0.02	0.22^{ab}	0.73^{a}	18.78^{ab}	23.50^{b}	75.38^{bc}
P PLANT	0.001	0.005	NS	NS	0.000	0.000	0.000	0.000	0.000
SEM	0.42	0.04	0.01	0.001	0.01	0.02	0.28	0.87	0.27
PERIOD									
1	15.51	1.14^{a}	0.01	0.02	0.16^{b}	0.66	18.99	27.18	75.60
2	14.91	1.08^{ab}	0.01	0.02	0.23^{a}	0.73	19.60	26.75	75.69
3	15.90	0.82^{c}	0.05	0.02	0.27^{a}	0.73	18.30	28.33	76.40
4	15.46	0.90^{bc}	0.02	0.02	0.25^{a}	0.66	18.13	28.93	75.50
P PERIOD	NS	0.001	NS	NS	0.000	NS	NS	NS	NS
SEM	0.42	0.04	0.01	0.001	0.01	0.02	0.28	0.87	0.27
DOSE									
A (200 g/d)	14.57	0.76^{b}	0.04	0.02	0.23	0.72	17.93^{b}	30.36^{a}	76.56^{a}
B (125 g/d)	14.59	0.88^{b}	0.03	0.02	0.22	0.67	18.30^{ab}	29.50^{a}	75.58^{ab}
C (50 g/d)	15.78	1.11^{a}	0.02	0.02	0.23	0.73	19.04^{ab}	27.30^{ab}	76.23^{ab}
D (0 g/d)	16.86	1.19^{a}	0.01	0.02	0.23	0.66	19.74^{a}	24.04^{b}	74.82^{b}
P DOSE	NS	0.000	NS	NS	NS	NS	0.015	0.004	0.039
SEM	0.42	0.04	0.01	0.001	0.01	0.02	0.28	0.87	0.27

$^{a\ b}$ in the same column= P<0.05; FAME = fatty acid methyl esters

Table 23. Effects of plant, period and dose on proportions of individual and total odd branched chain fatty acids (OBCFA; g/100 g of FAME) in rumen fluid.

	Fatty acid (g/100g of FAME)									
	iso **C13:0**	***anteiso*** **C13:0**	***iso*** **C14:0**	***iso*** **C15:0**	***anteiso*** **C15:0**	***iso*** **C17:0**	***anteiso*** **C17:0**	**C15:0**	**C17:0**	**OBCFA**
PLANT										
Carum	0.02	0.06^{bc}	0.22^{c}	0.77^{bc}	0.92^{c}	0.42^{b}	0.66^{c}	1.15^{c}	0.57^{b}	7.18^{b}
Coriandrum	0.02	0.05^{c}	0.27^{bc}	0.66^{c}	1.15^{bc}	0.40^{b}	0.66^{c}	1.31^{c}	0.61^{b}	6.97^{b}
Melissa	0.02	0.08^{abc}	0.35^{ab}	0.99^{ab}	1.40^{ab}	0.49^{ab}	0.92^{a}	1.67^{a}	0.77^{a}	9.75^{a}
Ocimum	0.02	0.07^{ab}	0.32^{ab}	1.07^{a}	1.29^{ab}	0.48^{ab}	0.82^{ab}	1.64^{a}	0.76^{a}	9.37^{a}
Satureja	0.02	0.08^{a}	0.41^{a}	1.07^{a}	1.53^{a}	0.52^{a}	0.87^{ab}	1.85^{a}	0.85^{a}	10.02^{a}
Thymus	0.02	0.08^{a}	0.34^{ab}	0.93^{abc}	1.37^{ab}	0.51^{a}	0.75^{bc}	1.61^{ab}	0.79^{a}	8.93^{a}
P PLANTS	NS	0.002	0.000	0.000	0.000	0.005	0.000	0.000	0.000	0.000
SEM	0.001	0.003	0.001	0.03	0.04	0.01	0.02	0.04	0.02	0.18
Period										
1	0.02	0.06	0.35^{a}	1.00^{a}	1.26^{ab}	0.51	0.81	1.55^{ab}	0.71	8.63^{ab}
2	0.02	0.07	0.35^{a}	1.00^{a}	1.42^{a}	0.47	0.82	1.70^{a}	0.76	9.26^{a}
3	0.02	0.08	0.29^{ab}	0.99^{a}	1.34^{a}	0.47	0.76	1.55^{ab}	0.74	8.83^{ab}
4	0.02	0.07	0.28^{b}	0.67^{b}	1.09^{b}	0.43	0.73	1.35^{b}	0.70	8.10^{b}
P PERIOD	NS	NS	0.010	0.000	0.001	NS	NS	0.001	NS	0.022
SEM	0.001	0.003	0.001	0.03	0.04	0.01	0.02	0.04	0.02	0.18
DOSE										
A (200 g/d)	0.02	0.07	0.36^{a}	0.92	1.33	0.44	0.71^{ab}	1.67^{a}	0.77	8.83
B (125 g/d)	0.02	0.06	0.31^{ab}	0.90	1.20	0.47	0.73^{b}	1.47^{ab}	0.70	8.43
C (50 g/d)	0.02	0.07	0.32^{ab}	0.90	1.32	0.48	0.81^{ab}	1.57^{ab}	0.73	8.82
D (0 g/d)	0.02	0.07	0.28^{b}	0.95	1.26	0.50	0.86^{a}	1.44^{b}	0.70	8.75
P DOSE	NS	NS	0.026	NS	NS	NS	0.002	0.028	NS	NS
SEM	0.001	0.003	0.001	0.03	0.04	0.01	0.02	0.04	0.02	0.18

[a b] in the same column= P<0.05; FAME =fatty acid methyl esters

Table 24. Effects of plant, period and dose on proportions of individual and total mono-unsaturated fatty acids (MUFA; g/100 g of FAME) in rumen fluid.

	Fatty acid (g/100g of FAME)						
	C16:1 *cis* 9	**C18:1 *trans* 6-8**	**C18:1 *trans* 9**	**C18:1 *trans* 10**	**C18:1 *trans* 11**	**C18:1 *cis* 9**	**MUFA**
PLANT							
Carum	0.03	0.38^{a}	0.23^{a}	1.08^{a}	3.47	6.29^{ab}	14.40
Coriandrum	0.03	0.25^{ab}	0.26^{a}	1.36^{a}	3.57	5.14^{b}	13.74
Melissa	0.03	0.18^{b}	0.09^{b}	0.27^{b}	3.75	7.09^{a}	14.56
Ocimum	0.05	0.20^{b}	0.09^{b}	0.25^{b}	3.49	6.55^{a}	14.07
Satureja	0.04	0.14^{b}	0.07^{b}	0.21^{b}	3.39	6.31^{a}	13.28
Thymus	0.03	0.17^{b}	0.07^{b}	0.24^{b}	3.62	6.54^{a}	13.59
P PLANTS	NS	0.000	0.000	0.000	NS	0.000	NS
SEM	0.003	0.02	0.01	0.009	0.07	0.13	0.18
PERIOD							
1	0.01^{b}	0.23	0.16	0.80	3.74^{a}	6.65	14.69^{a}
2	0.04^{a}	0.21	0.15	0.66	3.47^{ab}	6.24	14.02^{ab}
3	0.05^{a}	0.21	0.10	0.43	3.30^{ab}	6.02	13.24^{b}
4	0.04^{a}	0.23	0.13	0.38	3.68^{b}	6.37	13.79^{ab}
P PERIOD	0.000	NS	NS	NS	0.021	NS	0.017
SEM	0.003	0.02	0.01	0.009	0.07	0.13	0.18
DOSE							
A (200 g/d)	0.04^{ab}	0.23	0.17^{a}	0.81^{a}	3.76	5.42^{c}	13.81
B (125 g/d)	0.05^{a}	0.25	0.18^{a}	0.82^{a}	3.41	6.16^{b}	14.11
C (50 g/d)	0.03^{b}	0.23	0.11^{ab}	0.38^{ab}	3.46	6.51^{b}	13.59
D (0 g/d)	0.03^{ab}	0.18	0.09^{b}	0.26^{b}	3.57	7.20^{a}	14.21
P DOSE	0.017	NS	0.009	0.008	NS	0.000	NS
SEM	0.003	0.02	0.01	0.009	0.07	0.13	0.18

$^{a\ b}$ in the same column= P<0.05; FAME =fatty acid methyl esters

Table 25. Effects of plant, period and dose on proportions of individual and total poli-unsaturated fatty acids (PUFA; g/100 g of FAME) in rumen fluid.

	Fatty acid (g/100g of FAME)				
	CLA *cis*9, *trans* 11	**CLA *trans* 10, *cis* 12**	**C18:2 *n*-6**	**C18:3 *n*-3**	**PUFA**
PLANT					
Carum	0.05	0.03	6.53^{ab}	1.21^{c}	9.58^{bc}
Coriandrum	0.05	0.03	5.35^{b}	1.04^{c}	8.03^{c}
Melissa	0.03	0.04	7.44^{a}	1.59^{b}	11.31^{a}
Ocimum	0.04	0.02	7.21^{a}	1.99^{a}	11.46^{a}
Satureja	0.01	0.02	6.67^{a}	1.40^{bc}	10.11^{ab}
Thymus	0.05	0.03	7.14^{a}	1.64^{ab}	11.04^{a}
P PLANT	NS	NS	0.000	0.000	0.000
SEM	0.02	0.003	0.14	0.05	0.19
PERIOD					
1	0.05	0.03^{ab}	6.53	1.34^{b}	9.72
2	0.04	0.01^{b}	6.78	1.43^{b}	10.23
3	0.02	0.03^{ab}	6.65	1.49^{ab}	10.35
4	0.04	0.03^{a}	6.94	1.65^{a}	10.71
P PERIOD	NS	0.026	NS	0.038	NS
SEM	0.02	0.003	0.14	0.05	0.19
DOSE					
A (200 g/d)	0.02	0.02	5.82^{c}	1.65^{a}	9.63^{b}
B (125 g/d)	0.04	0.03	6.68^{b}	1.52^{ab}	10.31^{ab}
C (50 g/d)	0.03	0.03	6.79^{b}	1.38^{ab}	10.10^{ab}
D (0 g/d)	0.05	0.03	7.60^{a}	1.36^{b}	10.97^{a}
P DOSE	NS	NS	0.000	0.035	0.011
SEM	0.02	0.003	0.14	0.05	0.19

$^{a\ b}$ in the same column = P<0.05; FAME = fatty acid methyl esters

Table 26. Ammonia concentration (mg/100 ml) in rumen fluid samples.

	Ammonia (mg/100ml)
PLANT	
Carum carvi	19.01
Coriandrum sativum	18.79
Melissa officinalis	18.35
Ocimum basilicum	17.82
Satureja montana	18.32
Thymus vulgaris	15.37
P PLANT	NS
DOSE	
A (200 g/d)	16.57
B (125 g/d)	18.07
C (50 g/d)	19.18
D (0 g/d)	17.95
P DOSE	NS
PERIOD	
1	18.37[a]
2	19.26[a]
3	14.89[b]
4	19.26[a]
P for PERIOD	0.001

Table 27. Rumen pH as affected by experimental treatments.

PLANT	Rumen pH	PERIOD	Rumen pH	DOSE	Rumen pH
Carum carvi	6.75[ab]	**1**	6.72	**High**	6.82[a]
Coriandrum sativum	6.69[b]	**2**	6.77	**Medium**	6.76[ab]
Melissa officinalis	6.76[ab]	**3**	6.75	**Low**	6.73[b]
Ocimum basilicum	6.72[ab]	**4**	6.76	**Control**	6.69[b]
Satureja montana	6.77[ab]				
Thymus vulgaris	6.81[a]				
SEM	0.009	**SEM**	0.009	**SEM**	0.009
P	0.009	**P**	NS	**P DOSE**	0.001

[a, b] in the same column = P<0.05

Figure 1. Sarda dairy ewes in the rack waiting for the mixture.

Figure 2. Sarda dairy ewes eating the mixture during milking.