UNIVERSITÀ DEGLI STUDI DI SASSARI

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Ciclo XXVI

Effect of the utilization of aromatic plants on diet utilization, milk production, parasitic load,and health status of dairy ewes

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A Dissertation

Presented to the Faculty of Animal Science of University of Sassari – Italy

In Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

by

Oscar Boaventura Neto

November 2013

Boaventura Neto, O. 2013.

Fundings

The results of this dissertation were obtained thanks to fundings of the European Union`s Seventh Framework Programme, managed by REA-Research Executive Agency, assigned to the project “TECHeese. Solving the technological problems of sheep cheese production”, grant agreement n° FP7-SME-2008-2-243638. The author wishes to thank the entire Project Consortiuma and all its components for their support and collaboration.

The information regarding the plant species, the part of the plants and the dosage of aromatic plants supplied to the animals could not be disclosed, because of the secrecy agreements that regulates this EU project. This information will be reported in future publications in scientific journals once the period of secrecy will expire.

BIOGRAPHICAL SKETCH

OSCAR BOAVENTURA NETO – I am son of João Rubens Boaventura and Rosa Maria Morais de Almeida Mesquita Boaventura and I was born on June 17, 1983 in Aracaju, Sergipe, Brazil. In August 2003 I enrolled in the Faculty Pio Décimo (Aracaju - SE), and after 5 years I graduated (Laurea Magistrale) in Veterinary Medicine (August 2008). From September 2008 to February 2009 I had a fellowship from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) to work with goat nutrition in the Department of Animal Science of the Universidade Estadual Paulista (UNESP – Campus Jaboticabal). In March 2009 I started a Master in Animal Science at UNESP – Campus Jaboticabal, with the guidance of Izabelle A.M.A. Teixeira, and concluded it in February 2011 (Thesis: Protein requirements for growth in male and female Saanen goat kids). After that, in March 2011 I started a Ph.D. program in Animal Science at the University of Sassari – Italy, with the guidance of Antonello Cannas, and in November 2013 I finished my dissertation.

Dedico aos meus amados pais,
com muito amor e carinho!

RINGRAZIAMENTI

Innanzitutto vorrei ringraziare Dio per la vita, i miei amatissimi genitori, che mi hanno sempre appoggiato sin dall'inizio, i miei nonni, fratelli, zii, cugini, figliocci, nipote, amici brasiliani che da lontano mi hanno sempre dato forza per andare avanti.

Ringrazio anchela mia amata fidanzata Greicy, che da più di 6 anni mi sta appoggiando stimolando a vincere, soprattutto amandomi, grazie di tutto, amore.

Ringrazio i professori brasiliani, Izabelle Teixeira e Kleber Resende per il coraggio di avermi mandato a Sassari per portare avanti il dottorato.

Ringrazio gli amici brasiliani (Ligia Maria, Simone Silva e Tadeu Oliveira) che per alcuni mesi a Sassari mi hanno avvicinato il Brasile.

Ringrazio a tutti gli amici che mi hanno insegnato la loro lingua madre (Italiano).

Un affettuoso ringraziamento va a tutta la squadra di pallacanestro LiPunti Basket per i bellissimi momenti che abbiamo vissuto sia dentro che fuori campo, soprattutto nelle diverse cene insieme.

Un ringraziamento va alla famiglia Bagedda Mangatia compreso il suo personale (Angelo Canu, Sara, Teta, Kinga, Angela Masia, Lucrezia Clateo nota come piombo, Fabrizio, Andrea, Nicolino) per la accoglienza eppure per i momenti divertenti.

Un ringraziamento vaal personale della scuola di dottorato ed anche della sezione di Scienze Zootecniche: Rita Marras, Marina Pischedda, Emilio Usai, Gavino Biddau per l'aiuto nelle pratiche amministrative.

Ringrazio tutti gli altri amici e amiche del dottorato: Matteo Sini, Alberto Stanislao Atzori, Giustino Gaspa (lo statistico), Claudio Carzedda, Gianfranco Gaias, Elia Pintus, Roberto Steri, Fabio Correddu, Marco Pinna, Gabrielle Marras, Francesco Fancello, Elisa Ortu, Maria Annunziata Pintus, Sara Saba, Maria Grazia Manca, Gabriella Serra, Rossella Manca, Roberta Boe, Osman Karimi, Maura Lovicu, Alessandra Marzano e Natalia Castañares Castro per l'aiuto, la simpatia, l'amicizia e il supporto forniti nel corso di questi tre anni.

Ringrazio i miei tesisti: Antonio Piras, Gianluigi Delrio, Giuseppe Bassu, Miquèu Segalas, Francesca Demelas e Jessica Serdino ed anche la amica Gabriella Serra per l'importantissimo aiuto nello svolgimento delle prove sperimentale.

Ringrazio i miei coinquilini: Mauro Russu, Giovanni Carbini, Angelo Tiloca e Gabrielle Marras per le belle risate che abbiamo fatto.

Ringrazio ancora i miei amici e primi maestri nella produzione animale: Lisiane Dorneles de Lima, Herymá Giovane de Oliveira Silva e Samuel Figueiredo de Souza per i primi insegnamenti nella produzione e nutrizione dei ruminanti.

Un ringraziamento va all'Università degli Studi di Sassari per la borsa di studio durante i tre anni di dottorato e ai professori della sezione di Scienze Zootecniche: Anna Nudda, Ana Helena Dias Francesconi, Salvatore Pier Giacomo Rassu, Giuseppe Pulina, Gianni Battacone, Antonio Pais, Nicolò P.P. Macciotta, Corrado Dimauro per le lezioni, chiacchierate, supporto scientifico.

Infine vorrei ringraziare in modo speciale il prof. Antonello Cannas, mio docente guida, che mi ha dato l'opportunità e la fiducia necessarie per la mia crescita scientifica.

Soggu Tattaresu,accudiddu però!!!

Siete meravigliosi!!!

GRAZIE MILLE A TUTTI QUANTI!!!

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List of Abbreviations

ALB	Albumin
ALP	Alkaline Phosphatase
AH	Anthelmintic
AI	Atherogenic index
ADG	Average daily gain
aNDF	Ash corrected Neutral detergent fiber
ADL	Acid detergent lignin
ADF	Acid detergent fiber
BASOS	Basophils Cells
BCS	Body condition score
BT	Total Bilirubine
BW	Body weight
CBC	Complete blood count
CH_4	Methane
CLA	Conjugated linoleic acid
CME	Crude methanolic extract
CP	Crude protein
CP	Crude powder
CTR	Control
CT	Condensed tannins
CRE	Creatinine
DPA	Docosapentaenoic acid
DGGE	Denaturating gradient gel eletrophoresis
DHA	Docosahexaenoic acid
DIM	Days in milking
DM	Dry matter
DMI	Dry matter intake
EAE	Echium amoenum extract
EDTA	Ethylenediaminetetraacetic acid
EE	Extract ether
EPA	Eicosapentaenoic acid
EPG	Eggs per gram of feces
EO	Essential oil
EOBC	Essential oil and bioactive compounds
EOS	Eosinophils Cells
FA	Fatty acid
FAME	Fatty acid methyl esters
FCM	Fat-corrected milk
FEC	Faecal egg count
FECR	Faecal egg count reduction

continued

FPCM	Fat-protein-corrected milk
g	Gram
g/d	Gram per day
GE	Gross energy
GGT	Gamma Glutamil Transpeptidase
GOT	Glutamic Oxaloacetic Transaminase
GPT	Glutamic Pyruvic Transaminase
h	Hours
Hct	Hematocrit
HGB	Hemoglobin
h/H	hypocholesterolemic/hypercholesterolemic ratio
Kg	Kilogram
LCFA	Long-chain fatty acid
LYMPHS	Lymphocytes Cells
M	Mole
MCV	Mean Corpuscular Volume of Red Blood Cells
MCHC	Mean Corpuscular Hemoglobin Concentration
MO	Monensin
MONOS	Monocytes Cells
MUFA	Monounsaturated fatty acid
N	Nitrogen
NDF	Neutral detergent fiber
NEUTS	Neutrophils Cells
NFC	Non fiber carbohydrate
NH_3	Ammonia
OBCFA	Odd- and branched-chain fatty acid
OM	Organic matter
P	Probability
PCA	Principal component analysis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PLT	Total Platelets
PROT	Total Protein
PSM	Plant secondary metabolites
PT	Post-treatment
PUFA	Polyunsaturated fatty acid
RBC	Red blood cells
SCC	Somatic cell count
SCFA	Short-chain fatty acid
S.D	Standard deviation
SED	Standard error of the difference
SFA	Saturated fatty acid
TFA	Trans fatty acid
TI	Thrombogenic index
TTGE	Temporal Temperature Gradient Electrophoresis

VFA	Volatile fatty acid
WBCB	White Blood Cells

General abstract

This dissertation studied the effects of aromatic plants on diet utilization, milk production, parasitic load, and health of Sarda dairy ewes. The first chapter reviewed the effects of aromatic plants and their extracts on ruminants in *in vivo* studies. The second chapter reported a long-term feeding trial on the effects of *Carum* sp., *Coriandrum* sp. and *Satureja* sp. at three doses on milk production, feed intake, blood and ruminal parameters and digestibility of lactating Sarda dairy ewes. All mixtures were eaten by the ewes without harming animal health; most milk parameters did not change; milk fatty acids were modified by the plants, especially at the highest dose; rumen pH was affected by plant and dose; NDF digestibility increased with the plants and *in vivo* digestibility of most nutrients increased with Satureja. The third chapter demonstrated anthelmintic effects (reduction of fecal egge count) of *Satureja* sp. alone or blended with *Carum* sp., and *Coriandrum* sp. in non-lactating pregnant Sarda ewes naturally infested by gastro-intestinal parasites. The fourth chapter tested effects of blends of the same plants on milk production, rumen function and health of lactating Sarda ewes. Milk production was not affected by plants; rumen pH tended to be positively affected, but rumen volatile fatty acids were not affected by the plants; bacteria communities in the ruminal liquid were affected by the plants, and archaea rumen population were not clearly affected.

CHAPTER 1

Effects of essential oils on rumen fermentation, milk and meat production and gastro-intestinal parasite control

Introduction

Oscar Boaventura Neto

Effects of essential oils on rumen fermentation, milk and meat production and gastro-intestinal parasite control

Abstract: This review gives an overview of several experiments conducted *in vivo* on the effects of plants rich in essential oils (**EO**) or of EO extracts on rumen fermentation, ruminant production performance and anthelmintic effects. Effects of these compounds and additives on rumen pH and volatile fatty acids, methane, ammonia, microbial population, *in vivo* feed digestibility, milk productionand composition, and average daily gain were considered.

Based on *in vivo* studies on the effects of EO or whole plants rich in EOin ruminants, it was possible to conclude that: i) in cattle the effects on rumen pH and VFA are very limited, whereas in sheep and goats several studies reported an increase in total VFA and a decrease in the acetate to propionate ratio; ii) methane *in vivo* production was reduced in most studies in cattle, sheep and goats; iii) ammonia production was reduced in some cases but not affected in many others; iv) rumen microorganisms were often affected but no clear patterns could be observed; v) NDF digestibility was often reduced, but in many cases it was not affected or increased; vi) milk yield was often positively affected after the first part of the lactation, in long-term studies, at high dosages of EO, and in sheep and goats rather than in dairy cows; vii) milk composition was marginally affected, except for milk fatty acid composition, for which all studies on lactating goats and ewes, but none of those on cattle, observed increases on the unsaturated FA and concentrations of CLA, with possible reductions of the biohydrogenation process; and viii) in some cases average daily gain (**ADG**) was improved, but too few studies are available on growing animals to understand which conditions might favour this effect; and ix) anthelmintic effects were reported in various experiments carried out on small ruminants fed indoors, whereas few data are available on grazing ruminants.

In general, it appears that small ruminants are more responsive to the action of EO, possibly because they have a higher rumen feed and liquid passage rate than cattle and buffaloes.

Keywords: essential oils, plant extracts, milk production, rumen, parasites

1.Introduction

For a long time nutritionists have worked on the modification of rumen environment as a way of improving feed efficiency (Benchaar et al., 2008a). Among the various compounds and additives studied to modify rumen environment and improve feed utilization efficiency, plant extracts have gained widespread interest. They have been also considered the only alternative to antibiotics (Wallace, 2004). This Introduction will consider, in particular, the effects of plants rich in essential oils (**EO**) and of their extracts on rumen metabolism, milk production and composition, feed utilization and anthelmintic effectsin ruminants.

Essential oils are secondary metabolites of very diverse composition, usually isolated by stem distillation or solvent extraction, made up mainly by volatile terpenoids and phenyolpropanoids (Calsamiglia et al., 2007; Benchaar et al., 2008a; Patra, 2011). The composition and amount of EO of plant extracts vary with plant parts (e.g. leaf, root, stem, fruit peel or pulp, flower or seed) (Dorman and Deans, 2000) and plant species (Bezic et al., 2005) and they are strongly affected by genetics, age and environmental factors (Cosentino et al., 1999). Various properties and modes of action have been associated to EO (Calsamiglia et al., 2007): i) antioxidant action, with scavenging of free radicals, inhibition of peroxidation of membrane lipids, stimulation of antioxidants enzymes, ii) activity against bacteria, mainly gram +, because EO occupy space in their hydrophobic cell membrane, fluidizing it and allowing leakage of ions, thus forcing bacteria to spend energy for ionic pumps and decreasing the energy available for their growth. In some cases (e.g. EO carvacrol and thymol), the hydrophilic external cell membrane of gram – bacteria can be disrupted, iii) denaturation and coagulation of cell protein constituents, and iv) inactivation of nucleic acids and proteins.

Essential oils has been fed to animals as: i) whole or part of plants containing EO. In this case nutrients other than EO are also supplied, content of EO is variable and rumen utilization and fermentation is slower than when oil extracts are used, ii) oil extracts from specific plants, with variable content of EO and potentially high interaction with rumen microorganisms, iii) specific mixes of selected EO, normally sold as commercial products. They have constant and specific content of EO, with potentially high interaction with rumen

microorganisms, and iv) single compounds, with known concentration and potentially high interaction with rumen microorganisms.

Many studies have been published on the use of EO, supplied in the various forms described above, on rumen fermentation and production performance, but most of them regard *in vitro* trials (Cardozo et al., 2005, 2006; Castillejos et al., 2005; Busquet et al., 2006; Benchaar et al., 2007a; Castillejos et al., 2008; Patra et al., 2010; Bhatta et al., 2012). On this regard, extensive reviews have been published in the last years (e.g. Calsamiglia et al., 2007; Benchaar et al., 2008a; Patra, 2011). Therefore, the main objective of this review is to give an updated overview of experiments conducted *in vivo* on the effects EO on rumen fermentation, production performance and anthelmintic effects, with a special emphasis on possible differences among ruminant species. The results of this review are synthesized in Tables 1 to 10.

2. Effects of essential oils on rumen pH and volatile fatty acids

Rumen pH is a very important variable in ruminant nutrition. When rumen pH is reduced beyond certain values, appetite (Britton and Stock, 1987) and fiber digestion are reduced (Mould et al., 1983), and health problems become more likely (Slyter, 1976). In addition, rumen pH is closely related to the production of volatile fatty acids (**VFA**) (Russell and Dombrowski, 1980), which are the end products of rumen fermentation and are very important for the energy metabolism of ruminants. In the last years, several studies have been conducted trying to find out if EO can change rumen pH and improve ruminal VFA metabolism in cattle, sheep and goats.

Dairy cattle

In lactating Holstein cows, Tager and Krause (2011) did not find any effect of three EO (cinnamaldehyde, eugenol and capsicum) supplied at different doses and combinations on rumen pH (mean 5.72), total VFA (mean 129.1 mmol/L), and individual VFA concentration (Table 1).

In a study on lactating cows carried out by Benchaar et al. (2007b), a diet which included alfalfa silage plus 750 mg/day of a blend of EO (thymol, eugenol, vanillin, guaiacol, and limonene) tended (P=0.09) to increase the total VFA in the rumen, whereas when corn silage substituted alfalfa total in EO supplemented diets VFA concentration tended to decrease, suggesting that the effects of EO mixture on total VFA concentration may depend on the composition of the diet (Benchaar et al., 2008a; Table 1). No effects of EO on individual VFA or rumen pH were observed.

Three studies used leaves of plants rich in EO. In a study on multiparous and primiparous lactating Holstein dairy cows, Tekippe et al. (2011) found that *Origanum vulgare* L. leaves fed at a dosage of 500 g/d did not influence rumen pH, and total and individual VFA concentration (Table 1). In a recent study of the same research group on multiparous and primiparous lactating Holstein dairy cows, Hristov et al. (2013) found that *Origanum vulgare* L. leaves fed at different doses (0, 250, 500 and 750 g/head/d) did not influence rumen pH (mean 6.21), total VFA concentration (mean 135.6 m*M*), acetate and propionate concentrations (mean 83.2 and 29.9 m*M*) but decreased butyrate concentration (from 18.3

m*M* in the control group to 17.0 m*M* in the three groups supplemented with *Origanum*; P<0.035) (Table 1).

In Holstein Friesian non-lactating crossbred dairy cows, Manh et al. (2012) found that eucalyptus (*Eucalyptus camaldulensis*) leaf meal powder, fed at different doses (0, 100 and 200 g/head/d), did not have any effect of rumen pH (mean 6.77) but reduced (P<0.05) total VFA production (from 120.7 in the control group to 97.9 mmol/L as mean of the other treatments) and acetate concentration (from 67.7 mol/100 mol in the control group to 65.8 mol/100 mol in the group supplemented with the highest dose) (Table 1). There was a significant but small increase in propionate concentration (from 20.7 mol/100 mol in the control group to 22.2 mol/100mol in the group supplemented with the highest dose, P<0.05), whereas butyrate concentration did not change. A significant reduction in the acetate to propionate ratio (3.3 for the control group vs. 3.0 for that supplemented with the highest dose, P<0.05) also occurred.

Beef cattle

In beef cattle heifers, Yang et al. (2010a) reported that increasing doses (0, 400, 800 and 1600 mg/head/d) of eugenol linearly reduced (P=0.05) the concentration of acetate (from 80.1 mmol/L in the control group to 71.6 in the group fed the highest dose) but did not influence rumen pH (mean 6.2) and the concentration of propionate (mean 21.6 mmol/L), butyrate (mean 16.3 mmol/L) and total VFA (mean 118.5 mmol/L) (Table 1). In addition, the molar proportion of propionate tended (P=0.09) to increase (from 17.3 to 20.9 mol/100 mol) and the acetate to propionate ratio tended (P=0.10) to decrease (from 4.3 to 3.2 mol/100 mol) as doses increased from 0 to 1600 mg/head/d.

Geraci et al. (2012) supplemented beef cattle with monensin (**MO**) or with a mixture of plant extracts (cinnamaldehyde, eugenol and capsicum oleoresin) at the following doses: 1) MO at 46.7 mg/kg dietary dry matter (**DM**), and 2) a plant extract mixture composed of 266 mg/steer/d of an extract containing cinnamaldehyde (at 170 g/kg), eugenol (at 280 g/kg) and 133 mg/steer/d of a capsicum oleoresin extract containing 12 g/kg of capsaicin. The authors found that the various doses of plant extract did not influence rumen pH (mean 5.8), and total (mean 70.4 m*M*) and individual VFA concentrations (Table 1). Yang et al. (2010b)

supplied three doses of cinnamaldehyde to beef heifers and did not observe any effect at rumen level.

Devant et al. (2007) supplied an EO complex made from artichokes, ginseng and fenugreek to young bulls fed a diet rich in concentrate and found a marked reduction in rumen pH (from 6.52 to 6.12; P<0.001), which was observed in cattle only in this study, and a decrease of the acetate to propionate ratio (from 1.20 to 0.84; P<0.05).

Buffaloes

Hassan and Abdel-Raheem (2013) supplemented male buffalo calves with caraway (*Carum carvi* L.) seed powder (CS) at 2 g/kg diet, dried garlic powder (DG) at 2g/kg diet, and a mixture of CS plus DG (CS+DG) at 2g/kg diet each (Table 1). The control diet was based on a concentrate mixture, wheat straw and green berseem clover (*Trifolium alexandrinum* L.). The treated groups did not differ from the control group for rumen pH values, but groups fed CS and CS+DG had an increase in total VFA concentrations 6 hours after supplementation (from 11.05 and 11.40 mEq./100 ml in the control and DG groups, respectively, to 12.61 and 16.12 mEq/100 ml in the CS and CS+DG groups, respectively; P=0.001).

Sheep and goats

In Mehraban growing male lambs fed increasing doses of *Echium amoenum* extract (EAE) (0, 0.3, 1.5 and 3 ml of EAE/kg diet DM) for 70 days, Nooriyan Soroor et al. (2013) found that pH values did not differ among treatments (mean 6.3, P=0.6) on day 35 but tended (P=0.061) to decrease linearly as dose increased (6.35, 6.16, 6.15, 6.02 at doses of 0, 0.3, 1.5 and 3 ml EAE, respectively) on day 70 (Table 2). Total VFA concentration linearly increased (P=0.027) as dose increased on days 35 and 70. Regarding VFA, on day 35 acetate was significantly (P=0.026) higher in the control group (55.1 mol/100mol) than in the treated groups (mean about 51.3 mol/100mol), propionate showed a linear increase with dose (from 34.1 to 45.4 mol/100mol as doses increased from 0 to 3 ml of EAE/kg diet DM; P=0.023), whereas butyrate did not change (mean 28.9 mol/100mol; P=0.402). The acetate to propionate ratio decreased linearly with dose (from 1.66 to 1.16; P<0.05). On day 70, the proportion of acetate decreased linearly with dose (from 58.4 to 50.6 mol/100mol; P=0.001), the proportion of propionate was lower in the control group (37.8 mol/100mol) than in the treated groups (mean 43.3 mol/100mol; P=0.002), butyrate did not change (mean 24.4

mol/100mol; P=0.165), and the acetate to propionate ratio linearly decreased with dose (from 1.57 to 1.23; P<0.05).

Lin et al. (2013) supplemented sheep with the following diets (Table 2): 1) diet C = a basal diet added with 25 g/day of monosodium fumarate, 2) diet EOC = diet C plus 1 g/day of EO combination (a blend composed of oil from clove, oregano, cinnamon and lemon in an equal ratio), 3) diet LEOAC = diet C plus 0.5 g/day of EO active components combination (a blend of eugenol, carvacrol, citral and cinnamaldehyde in an equal ratio), and 4) diet HEOAC = diet C plus 1.0 g/day of active components combination (i.e., the same blend of diet LEOAC but at a higher concentration). The Authors did not find effects of treatments on rumen pH (mean 6.76; P=0.095). Total VFA did not differ between diet C and diet LEOAC, with mean of about 63.7 mM, but it differed from EOC and HEOAC (mean 59.8 Mm; P=0.002). Acetate proportion was not influenced by the addition of EO combination or by EO active components combination, whereas propionate proportion was lower in diet C (17.7 mol/100mol) than in diet EOC (20.1 mol/100mol; P=0.016) and the acetate:propionate ratio was higher in diet C (4.29) than in the three treated groups (mean 3.73; P=0.018).

In Canadian Arcott lambs, Chaves et al. (2011) did not find effects of cinnamaldehyde at various doses (0, 102, 210 and 457 mg/kg of DMI) on rumen pH (mean about 6.7), and total (mean 103.3 mM/L) and individual VFA production (Table 2).

In mature ewes, Newbold et al. (2004) found that the addition of a blend of EO (thymol, guajacol and limonene) to the diet did not influence rumen pH (mean about 6.2), and acetate, propionate and butyrate proportions (619.3; 179.0; 142.8 mmol/mol total VFA respectively) but tended to increase total VFA concentration (from 85 to 106 mmol/l, P<0.079) (Table 2).

Likewise, Anassori et al. (2011) carried out three experiments to test the effects of garlic and monensin supplementation on Iranian Makoui male sheep, using a resting interval of 4 weeks between trials. In the first trial, the doses used were: control diet (basal total mixed ration with no additive = CTR), control diet with raw garlic (75 g/kg DM = GAR75), control diet with garlic oil (500 mg/kg DM = GO500). In the second trial, the diets were similar to those of trial 1, with the difference that the dose of GAR and GO were increased to 100 g/kg DM (GAR100) and 750 mg/kg DM (GO750), respectively. In the third trial, each of the four sheep was fed the basal diet with GAR 75 g/kg DM, GAR 100 g/kg DM, GO 500 mg/kg DM

and GO 750 mg/kg DM. In all trials, the authors did not find any effect (P>0.05) of garlic supplementation on pH values (mean about 6.43, 6.28, 6.68 in trials 1, 2 and 3, respectively) and total VFA production (mean about 97.04, 95.18, 96.02 mmol/L, respectively) (Table 2). In general, garlic supplementation reduced acetate and increased propionate concentration, causing a reduction of the acetate to propionate ratio.

In whethers supplemented with ropadiar, a compound from an extract from oregano, Wang et al. (2009) observed an increase in total VFA production, but no changes in rumen pH and acetate to propionate ratio. In contrast, when carvacrol or cinnamaldehyde were supplied to lambs fed diets with very high starch content, Chaves et al. (2008a) observed a marked reduction of rumen pH besides the increase in total VFA production.

In lactating dairy Chios ewes, Giannenas et al. (2011) reported that increasing levels (0, 50, 100 and 150 mg/kg of concentrate) of a mixture of EO (thymol, eugenol, vanillin, guaiacol, and limonene) did not alter rumen pH (mean about 6.6), but increased (P<0.04) the molar proportion of propionate (from 19.6 to 24.9 mol/100mol). In addition, increasing doses of the EO mixture tended to increase the total VFA concentration (from 115.2 to 126.2 mM, P=0.078) and tended to decrease the molar proportion of acetate (from 67.4 to 62.7 mol/100 mol, P=0.06), without influencing the molar proportion of butyrate (mean about 9.8 mol/100 mol) (Table 2).

Only one study was based on the supply of plants rich in EO to ewes. In particular, Manca et al. (2012a) supplied the vegetative components (leaves and small twigs) of three plants (*Melissa officinalis, Ocimum basilicum and Thymus vulgaris*) at 3 dosages (50, 125 and 200 g/d, DM basis) to lactating dairy Sarda ewes. The plants were mixed with a different proportion of corn and pea meal, to form isoproteic mixes. The mixes were supplied individually in two doses (225 g/d of DM each) during the two daily milkings. The rest of the diet was made of dehydrated alfalfa and beet pulps and was supplied in collective pens. The results showed that the aromatic plants did not affect rumen pH, ammonia, and total and individual VFA. However, the plants caused significant effects on rumen fatty acids (Table 6). In particular, as the dose increased there was an increase in branched chain fatty acids, which originate from the ruminal bacteria (Vlaeminck et al., 2006), suggesting a positive effect of the plants rich in EO on overall microbial ruminal activity. At the same time, the observed increase in PUFA n3 suggests that the studied plants reduced the rumen

biohydrogenation process. This is confirmed by the modification of milk fatty acids that occurred in the same experiment, described later in this review.

Kholif et al. (2012) supplemented lactating Damascus goats with EO at the following doses: 1) Control = no additive, 2) Control + 2 ml/d per head of garlic oil, 3) Control + 2 ml/d per head of cinnamon oil, and 4) Control + 2 ml/d per head of ginger oil. The EO tested did not affect rumen pH (mean about 5.92) but increased (P=0.001) total VFA production (from 77.0 in the control group to 94.4 mMol/L in the garlic and cinnamon oil supplemented groups), propionate proportion (from 25.4 in the control group to 31.1 mol/dl in the garlic, cinnamon and ginger oil groups, P=0.031) and butyrate proportion (from 9.3 in the control group to 15.7 mol/dl in the ginger oil group, P=0.001) (Table 2). Conversely, the EO decreased acetate proportion (from 69.3 in the control to 52.4 mol/dl in the ginger oil group, P=0.051) and the acetate to propionate ratio (from 2.72 in the control to 1.62 mol/dl in the ginger oil group, P<0.05).

In Saanen and Alpine goats in early lactation, Malecky et al. (2009) did not observe differences in rumen pH (mean about 6.4), and total VFA (mean 83.2 mM) and individual VFA production among three levels (0, 0.043, 0.43 g/kg DMI) of monoterpene blends (Table 2).

Some comments

Considering all the experiments reported, it appears that in cattle the effects of EO or whole plants rich in EO on rumen pH and VFA are very limited.

In cattle, only one study reported a rumen pH variation (decrease; Devant et al., 2007), two reported a total VFA decrease (Benchaar et al., 2007b; Manh et al., 2012) and one a VFA increase (Benchaar et al., 2007b). The acetate to propionate ratio was never affected in dairy cattle, whereas it decreased, due to increased propionate production, only in two studies in growing beef cattle (Devant et al., 2007; Yang et al., 2010a).

In contrast, in sheep and goats, only one study reported a decrease of rumen pH (Chaves et al. 2008a), whereas several experiments reported an increase in total VFA (Chaves et al., 2008a; Wang et al., 2009; Nooriyan Soroor et al., 2013) and a decreasein the acetate to propionate ratio (Anassori et al., 2011; Giannenas et al., 2011; Kholif et al., 2012; Lin et al., 2013; Nooriyan Soroor et al., 2013).

This difference among species is somehow surprising. One possible explanation is that the doses used per kg of BW appear to be higher in sheep and goats (Table 2) than in cattle (Table 1). The other possible explanation is that the lower rumen liquid and solid passage rate that usually characterize cattle compared to small ruminants (Van Soest, 1994; Cannas et al., 2003) might have allowed a better adaptation of rumen microflora to the addition of EO and thus lower rumen modifications associated to their utilization.

Recently, Khiaosa-ard and Zebeli (2013) used data from the literature of *in vivo* experiments to study with a meta-analysis the influence of essential oils and their bioactive compounds on rumen metabolism and feed efficiency in cattle, sheep and goats. Regarding rumen pH, they did not observe any effect associated to EO. Regarding VFA, they observed a reduction of the acetate to propionate ratio in beef cattle, a almost significant tendency for reduction in small ruminants, and no effects in lactating cows. Thus, this meta-analysis basically confirms what reported above, i.e. that beef cattle and small ruminants are sensitive to EO, whereas dairy cattle are not.

3.Effects of essential oils on methane

Methane (CH_4) is a major greenhouse gas, well-known for its important impact on the earth surface temperature (Moss et al., 2000). Nowadays, livestock produces a considerable amount of CH_4, confirming the prediction that in 2010 the emissions would have been approximately 5.3 Tg CO_2-equivalents higher than those observed in 1991 (Desjardins et al., 2001). As reported by Moss et al. (2000), different strategies have been proposed to reduce enteric CH_4 emissions from ruminants. In the last few years, EO or extract of plants rich in EO have been studied for this purpose, even though the *in vivo* studies are very few (Tables 1 and 2).

Cattle

Manh et al. (2012) observed a reduction ($P<0.05$) of methane production by non-lactating dairy cows from 35.5 in the control group to 26.3 mmol/L in the group supplemented with 200 g/head/d of *E.camaldulensis* leaf meal powder.

In a study on lactating dairy cows, Tekippe et al. (2011) found that *Origanum vulgare* L. fed at a dosage of 500 g/d reduced methane production (Table 1). Recently, Hristov et al. (2013) found that as doses of *Origanum vulgare* L. in the diet of primiparous Holstein lactating dairy cow sincreased, methane production by the cows tended (P=0.08) to decrease (mean 18.2, 16.5, 11.7 and 13.6 g of CH_4/kg of DMI at 0, 250, 500 and 750 g/cow/d, respectively).

In contrast, in Angus beef heifers, Beauchemin and McGinn (2006) observed (data not reported in Table 1) no differences between the control group and a group fed EO mixture based on thymol, eugenol, vanillin, and limonene (1 g/d) on daily methane production (mean about 26.5 g/kg of DMI).

Sheep and goats

In a study conducted by Abdalla et al. (2012) on Santa Ines sheep, methane production (data not reported in Table 1) was not influenced by supplementation with eucalyptus oil (0, 10 and 20 ml/d per head), even if a numerical decrease was observed between the two supplemented groups and the control group (from 29 to 39 L/d, respectively). More recently, Heidarian Miri et al. (2013) reported that cumin (*Cuminum cyminum*) seed extract at two doses (12.7 and 25.3 g/kg DMI) reduced (P>0.006) methane emission by crossbred (Alpine × Beetal) early lactating goats from 14.72 g/kg DMI in the control group to 12.64 g/kg DMI in the supplemented groups.

Out of six studies which measured methane *in vivo*, five reported a reduction of its production when EO were used. This is a very interesting result, in light of the supposed importance of methane production from ruminants. More studies are needed to confirm these results and to understand what are the most efficient ways to apply this information in production conditions.

In their review, Khiaosa-ard and Zebeli (2013) reported a reduction in methane production in beef cattle, an almost significant tendency for reduction in small ruminants, and no effects in lactating cows. However, in this review methane production was calculated based on the proportion of VFA, thus the results on methane were basically parallel to those on VFA discussed in the previous paragraph, whereas *in vivo* methane measurements were not considered.

4. Effects of essential oils on ammonia

Ammonia (NH_3) concentration is another important ruminal variable which has received great attention in the literature, because shortage of ammonia reduces animal performance but its excess favors high N excretion and pollution and can increase the incidence of various pathologies.

In the study conducted by Hristov et al. (2013) on primiparous lactating dairy cows, *Origanum vulgare* L. reduced (P=0.04) ammonia production compared with control (mean 6.3 mM in the control and 5.0 mM in the groups treated with 250, 500 and 750 g/cow/d). Similarly, in the study on castrated Angus steers conducted by Geraci et al. (2012), ammonia concentration decreased (P<0.03) from 20.05 in the monensin group to 10.78 mg/dL in the group fed plant extracts. In the study conducted by Hassan and Abdel-Raheem (2013) on male buffalo calves, ammonia concentration decreased (P=0.001) from 35.5 in the control group to 27.7 mg/dl in the group fed the mixture of caraway seed power and dried garlic power (CS+DG) 3 h after supplementation and from 27.1 mg/dl in the control group to 19.3 mg/dl in the CS+DG group 6 h after supplementation.

In Mehraban growing male lambs fed increasing doses of *Echium amoenum* extract (EAE) (from 0 to 3 ml of EAE/kg diet DM), Nooriyan Soroor et al. (2013) found a reduction of ammonia production compared with control on day 35 (mean 17.6 in the control and 10.5 mg/dl in the treated groups; P<0.001) and day 70 (mean 18.5 in the control and 12.6 mg/dl in the treated groups; P<0.001).

In the study of Lin et al. (2013) on Hu sheep supplemented with a blend of oil from leaves or a blend of active components, ammonia production was reduced (P=0.004) from 14.8 mg/dl in the control group (diet C = basal diet added with 25 g/day of mosodium fumarate) to 12 mg/dl in the group fed diet C plus 1 g/day of EO combination (a blend composed of oil from clove, oregano, cinnamon and lemon in equal ratio).

In accordance to these studies, Manh et al. (2012) also observed that ammonia production by non-lactating cows decreased from 14.8 mg/dL in the control group to 10.0 mg/dL in the group supplemented with 200 g/head/d of *E. camaldulensis* leaf meal powder. These results might be explained by a reduction of feed fermentability and proteolytic activity in the rumen of animals fed plant extracts, as previously observed in the *in vitro* studies of

Castillejos et al. (2006) and Fraser et al. (2007). However, these results are not in agreement with many other studies on cattle, sheep, and goats, in which differences on ammonia concentration were not observed (Tables 1 and 2). Rumen ammonia concentration can be affected by many different factors, so it is clear that more systematic investigations on the effects of EO on rumen ammonia production and concentrations are needed.

5. Effects of essential oils on microbial population

Rumen ecology has an important rolein the understanding of ruminant nutrition (Wallace, 1992). Several factors can modify the ruminal environment, including dietary fats (Hristov and Jouany, 2005). Indeed, in recent years some studies have been done to manipulate rumen microbiology and, consequently, rumen fermentation (Tables 1 and 2).

Cattle

Hristov et al. (2013) reported that increasing levels (i.e., 0, 250, 500 and 750 g/cow/d) of *Origanum vulgare* L. supplied to lactating dairy cows did not affect rumen archea, bacteria, and fungi, except for *Ruminococcus flavefaciens,* which slightly increased with the lowest dose of oregano and decreased with the two highest supplementation levels (quadratic effect, P=0.02).

Likewise, Benchaar et al. (2007b) reported that alfalfa or corn silage added with an EO blend (thymol, eugenol, vanillin, guaiacol, and limonene) at 750 mg/d in dairy lactating cows did not influence (P>0.20) rumen total viable bacteria (mean 2.80×10^9/ml), cellulolytic bacteria (mean 5.34×10^7/ml) and protozoa (mean 4.89×10^5/ml).

Successively, Benchaar et al. (2008b) found that feeding lactating dairy cows a total mixed ration without supplementation (control) or supplemented with cinnamaldehyde (1 g/d = 43 mg/kg of DMI) did not influence total protozoa (mean 5.89 log10/ml), *Dasytricha* spp. (mean 4.24 log10/ml), *Diplodinium* spp. (mean 2.97 log10/ml), *Entodinium* spp. (mean 5.62 log10/ml), and *Polyplastron* spp. (mean 3.37 log10/ml), but tended to increase (P=0.09) the number of *Isotricha* spp. from 4.23 log10/ml in the control group to 4.46 log10/ml in the group supplemented with cinnamaldehyde.

In another study with lactating dairy cows, Yang et al. (2007) did not find any effect of garlic (*Allium sativum,* 5 g/cow/d) or juniper berry (*Juniperus communis*, 2 g/cow/d) EO on total number of protozoa (mean 5.35 x 10^5), and of Isotricha and Entodina.

On the other hand, Manh et al. (2012) observed that *E. camaldulensis* leaf meal powder in the diet of non-lactating dairy cows reduced (P<0.05) protozoa population, total viable bacteria population, and proteolytic and cellulolytic bacteria but did not affect (P>0.05) fungal zoospore and amylolytic bacteria in the rumen.

Differently, in the experiment of Yang et al. (2010a) using beef heifers, the different doses of eugenol tested did not influence the total number of protozoa (mean 20.1 ×10^5/ml) and the proportions of Dasytrichia (mean 0.037) and Entodinium (mean 0.954), but tended (P=0.07) to cause a quadratic reduction of the proportion of Isotrichia as doses increased.

Sheep and goats

In the trial conducted by Lin et al. (2013) on sheep fed different blends (EOC, LEOAC and HEOAC, see above), the authors observed that protozoa population was reduced (P<0.001) from 3.50% of the total bacteria in the control group to 1.51% in the EOC group and to, on average, 0.5% in the LEOAC or HEOAC groups. The amount of rumen fungi did not change (P=0.6) among treatments, with a mean of about 0.30% of the total number of bacteria. The percentage of *F. succinogenes* bacteria (as % of total bacteria) decreased (P<0.001) from 0.46 in the control group to 0.33 in the EOC, 0.16 in LEOAC and 0.11 in HEOAC. The percentage of *B. fibrisolvens* was higher (P<0.001) in the control group or EOC (mean 1.06% of the total bacteria x10^{-2}) than in LEOAC or HEOAC (mean 0.19% of the total bacteria x10^{-2}). Methanogen bacteria were greater (P=0.002) in the control or EOC groups (mean 0.55 % of the total bacteria) than in the LEOAC or HEOAC groups (mean 0.44 % of the total bacteria), whereas *R. flavefaciens* bacteria did not differ (P=0.123) among treatments, with a mean of about 1.90% of the total bacteria x10^{-2}.

In the experiment of Nooriyan Soroor et al. (2013) on Mehraban growing male lambs fed increasing doses of *Echium amoenum* extract (EAE) (from 0 to 3 ml of EAE/kg diet DM), the pattern of protozoa population was similar on days 35 and 70. As doses increased, the total protozoa decreased (P=0.0001) linearly from 14.77 to 4.98 x 10^5/ml of rumen fluid (on day 35 and from 15.34 to 3.09 x 10^5/ml of rumen fluid on day 70). The population of

Entodininnae markedly decreased (P<0.0001) on 35 day from 13.65 to 4.44 x 105 /ml RF on day 35 and from 19.04 to 3.14 x 105 /ml RF on day 70 when doses increased. At both sampling times, the population of Isotrichidae did not change (mean 0.16 x 105 /ml RF and 0.18 x 105 /ml RF on days 35 and 70, respectively; P>0.373). The population of cellulolytic bacteria decreased (P=0.0008) linearly from 9.04 to 7.39 log/ml RF as doses increased.

In the study of Anassori et al. (2011) using Iranian Makoui male sheep, garlic oil reduced (P<0.05) protozoal population. In contrast, Newbold et al. (2004) found that protozoal population (mean 8.28 x 10^5/ml) of mature ewes was not affected by the blend of EO (thymol, guajacol and limonene) tested. This result was in agreement with those of Giannenas et al. (2011), who found that the EO (thymol, eugenol, vanillin, guaiacol, and limonene) tested on lactating dairy Chio ewes did not influence protozoal population (mean 4.85 × 10^5/ml) and total viable bacteria (mean 2.86 × 10^9/ml). However, the same authors found that the number of cellulolytic bacteria increased from 3.82 × 10^7/ml to 5.55 × 10^7/ml (P<0.05) and the ammonia-producing bacteria decreased from 5.32 × 10^7/ml to 4.22 × 10^7/ml (P<0.05) as doses of EO changed from 0 to 150 mg/kg of concentrate. In the study by Malecky et al. (2009) on Saanen and Alpine goats in early lactation, monoterpene blend supplementation did not affect protozoal population (mean 913/µL).

As in the case of rumen ammonia, microbial population can be affected by many different factors, so it is clear that it is difficult to isolate the effects of the many different types of EO from that of the other substrates used in the diet and from the many other factors that can affect microbial species growth and number. The very contradictory results observed both in cattle and in small ruminants reflect this complexity.

The review of Khiaosa-ard and Zebeli (2013) did not observe any specific effects of EO on microbial population, except for increased protozoa numbers when EO were used in low concentrations, and decreased numbers when used in doses higher than 0.20 g/kg of DM.

6. Effects of essential oils on *in vivo* digestibility

A very important variable in the nutrition and feeding of ruminant animals is the quantification of digestibility of feedstuffs (Firkins et al., 1998). This variable is influenced,

among other factors, by certain ingredients and additives of the diet. In fact, this is one of the reasons why essential oils have been studied in recent years.

In vivo digestibility results of experiments carried out on cattle and buffaloes are reported in Table 1, whereas those on small ruminants are presented in Table 2.

Dairy cattle

In the experiment by Manh et al. (2012), testing *E. camaldulensis* leaf meal powder in the diet of non-lactating cows, the three doses evaluated did not influence ruminal digestibility, which averaged 61.7% for DM, 66.0% for OM, 59.8% for CP, and 52.8% for NDF.

Similarly, Santos et al. (2010) found no effects of feeding an EO blend (eugenol, geranyl acetato and coriander oil) at two doses (0 and 1 g/d per head) to Holstein cows in early lactation on ruminal digestibility of OM, CP and aNDF (mean 67.0%, 63.4% and 46.8%, respectively).

Likewise, when testing three EO (cinnamaldehyde, eugenol and capsicum) at different doses in lactating Holstein cows, Tager and Krause (2011) did not find any effect of plant extract on digestibility, which averaged 52.5% for DM, 54.3% for OM, 30.7% for NDF, 30.7% for ADF, 50.2% for CP and 89.0% for starch.

In another study on lactating Holstein cows, Yang et al. (2007) observed that garlic or juniper berry EO increased ($P<0.02$) digestibility of DM (mean 49.4% for control and 56.1% for garlic and juniper berry groups), but did not find influence ($P>0.15$) digestibility of NDF, ADF and starch (mean 42.9, 39.7 and 69.2 % of intake for all treatments).

In the experiment performed by Benchaar et al. (2007b) on lactating dairy cows, the EO blend (thymol, eugenol, vanillin, guaiacol, and limonene) tested at 2 doses (0 and 750 mg/d) did not influence digestibility of DM, CP, ADF, NDF and starch, which averaged 66.4, 61.3, 52.2, 52.0 and 97.4%, respectively.

Similarly, in the other trial of Benchaar et al. (2008b) on lactating dairy cows, feeding a total mixed ration supplemented with cinnamaldehyde did not affect ($P>0.09$) digestibility of DM, OM, CP, NDF and ADF, which averaged, in unsupplemented and supplemented groups, 64.0, 66.1, 61.2, 55.0 and 48.0%, respectively.

These studies are in accordance with the more recent experiment lactating dairy cows by Hristov et al. (2013), who observed that increasing levels (i.e., 0, 250, 500 and 750 g/d per

cow) of *Origanum vulgare* L. did not affect (P>0.06) the digestibility of DM, OM, ADF, CP digestibility (mean 65.6, 66.9, 46.7 and 62.4%, respectively), but decreased (P = 0.04) NDF digestibility from 51.3% in the control group to 49.3% in all groups supplemented with oregano.

When Spanghero et al. (2009) fed lactating Holstein heifers EO from oregano, cinnamon, thyme and orange peel, which are rich in the active principles carvacrol, cinnamaldehyde and eugenol, thymol, and terpenes, respectively, the authors did not find significant differences (P>0.50) between supplemented and unsupplemented groups for digestibility of OM (mean 75.3%), CP (mean 75.2%), and NDF (mean 56.5%).

Beef cattle

In the trial conducted by Beauchemin and McGinn (2006), Angus beef heifers fed the EO and spice extract based on thymol, eugenol, vanillin, and limonene (1 g/d) had a decrease (P<0.001) in digestibility of DM (63.2% in control vs. 58.6% in treated group), gross energy GE (61.6% vs. 56.9%), for NDF (41.8% vs. 33.1%) and ADF (35.1% vs. 25.3%).

When Meyer et al. (2009) fed two mixes of EO, one composed of thymol, eugenol, vanillin, guaiacol, and limonene and the other composed of guaiacol, linalool, and α-pinene, to crossbred yearling steers (British and British × Continental), no differences occurred among treatments for digestibility of DM (mean 84.3%) and OM (mean 86.0%).

Similar results were reported by Yang et al. (2010a) on beef heifers, in which as doses of eugenol increased from 0 to 1600 mg/d per head, the digestibility of aNDF in the total tract tended (P=0.10) to decrease linearly (from 56.1% to 49.4%), whereas digestibilities of OM (mean 77.2%) and CP (mean 70.5%) were not affected. In the study conducted by Hassan and Abdel-Raheem (2013) on male buffalo calves fed different diets (control, CS, DG and CS+DG, see above), digestibility of OM (mean 62.3%) was not affected, whereas DM digestibility was lower (P<0.01) in the control group (58.3%) than in the DG and CS+DG groups (mean 60.9%), CP digestibility was lower (P>0.01) in the control group (69.6%) than in the CS or CS+DG groups (mean 72.7%), and EE digestibility was lower (P = 0.04) in the control and CS groups (mean 78.6%) than in the DG group (82.6%).

Sheep and goats

In the study on Mehraban growing male lambs, supplementation with *Echium amoenum* extract (EAE) influenced digestibility, with a mean digestibility of 87.2%, 69.6%, 81.0%, 82.7% and 63.4% for OM, CP, EE, aNDF and ADF, respectively (Nooriyan Soroor et al., 2013).

In the experiment conducted by Lin et al. (2013) on sheep (see above for details), treatments did not differ for DM, CP, NDF and ADF digestibilities, with a mean of about 67.7%, 70.8%, 61.7% and 50.6%, respectively. Differently, Anassori et al. (2011) reported a depression of OM, NDF and ADF digestibility by garlic oil but not by raw garlic supplementation in Iranian Makoui male sheep. Finally, Malecky et al. (2009) found that early lactating Saanen and Alpines goats fed the two doses of monoterpenes mixture did not differ from control goats for ruminal digestibility of DM (mean 41.2%), OM (mean 50.3%), aNDF (mean 54.2%) and ADF (mean 45.8%).

Overall, it appears that in the most common effect of EO addition is related to a decrease in NDF digestibility. The mechanisms of this effect have not been explored, even though it is possible to speculate that the addition of EO might have a negative effect on fiber digestion similar to that caused by the addition of fat, another water insoluble compound. It would be interesting to see if there is an interaction among these compounds, i.e. if the reduction of NDF digestibility occurs only when dietary fat concentration is high.

The review of Khiaosa-ard and Zebeli (2013) previously mentioned did not consider possible effects on dietary digestibility.

7. Effects of essential oils on dry matter intake and the milk productionand compositioninlactating ruminants

Different types of essential oil sources have been used in lactating ruminants (Tables 3 and 4). Several experiments used EO commercial complexes, whereas others used plant extracts or, only in one case, unprocessed leaves of plants containing EO.

Dairy cows

Table 3 reports the main results of 9 studies carried out on lactating cows. The studies differed for the EO source, for the EO dosage, and for the forage to concentrate ratio of the diets. They also differed for lactation stage and production level of the cows.

Few studies observed differences in dry matter intake (**DMI**) (Table 3). Santos et al. (2010) observed a non-significant decrease (−1.43 kg/d; P=0.13) and Tassoul and Shaver (2009) found a significant (−1.8 kg/d; P<0.05) decrease in DMI as a result of the inclusion of commercial EO complexes in the diets. In addition, Hristov et al. (2013) observed a linear decrease in DMI as the dosage of *Origanum vulgare* leaves increased in the diets. In contrast, Kung et al. (2008) observed an increase in DMI (+1.9 kg/d; P<0.05) as a result of the addition of a commercial EO complex. No obvious reasons could be found to explain these differences. In particular, for an EO commercial complex such as Crina (Crina S.A., Gland, Switzerland; see note 2 of Table 3 for its characteristics) DMI increased in one experiment, did not change in a second one and decreased in a third one (Table 3). It appears that the variation in DMI were not driven by the EO complex used.

Milk production was affected only in one experiment, where it tended to increase (+1.9 kg/d; P<0.16) when the EO commercial complex Crina was used (Kung et al., 2008). The authors suggested that earlier studies did not affect milk yield because they had been carried out in early lactation, whereas their study was carried out in mid-lactation. The importance of the stage of lactation was in part confirmed by Tassoul and Shaver (2009) in a long-term experiment using Crina as a source of EO. They found that until the 7th week of lactation milk yield was numerically lower in the EO diet, but from the 8th to the 15th week of lactation milk yield became progressively numerically higher in the EO group. Unfortunately, they interrupted the experiment when the difference was increasing at a high rate. All the other experiments which used EO complexes did not report milk yield differences, but they were all carried out in early lactation (Table 3). The only study that compared stages of lactation (51 DIM vs. 247 DIM) was that of Hristov et al. (2013), who used 3 dosages of *Origanum vulgare* leaves. They did not observe any milk yield or milk composition differences associated with the treatments, except for a linear decrease in DMI as the dosage of *Origanum vulgare* increased. It is clear that the studies that used EO complexes might not be fully comparable with this study, which used leaves of a plant rich in EO. It

should be pointed out that in the study of Hristov et al. (2013), as in those of Santos et al. (2010) and Tassoul and Shaver (2009), there was a marked increase in feed efficiency utilization (kg milk/kg of feed) by the cows.

Regarding milk composition, in general the experiments did not show any effect of EO on milk fat and protein concentration (Table 3). Only Santos et al. (2010) found a significant, even though small, increase in milk fat concentration, whereas Tassoul and Shaver (2009) reported a significant but numerically small decrease of milk protein concentration. In general, EO did not cause variations in BCS or body weight (Table 3). Only Santos et al. (2010) found a significant increase in BCS for the cows which used an EO complex.

Overall, it appears that in most cases EO did not induce noticeable effects on production performance of lactating cows. A possible positive effect on milk production after the peak of lactation needs to be confirmed with other experiments.

Dairy sheep

Few publications report studies on the utilization of EO on lactating ewes (Table 4).

Two studies were based on the supply of EO extracts to dairy ewes: Giannenas et al. (2011) tested two dosages of the EO complex Crina, whereas Chiofalo et al. (2012) tested two dosages of EO extracts of *Rosmarinus officinalis*. In both cases, treatments affected milk yield but did not affect DMI. In the study of Giannenas et al. (2011) milk yield was not significantly affected at the lowest dosage (0.075 g/d per head of EO complex) but markedly and progressively increased above this dosage (+20% and 35% for the dosages 0.15 and 0.217 g/d per head of EO complex, respectively; Table 4). Interestingly, in this study there was also a marked reduction of somatic cell count for all dosages considered, suggesting that this was one of the causes of the increase in milk yield. In the other study, Chiofalo et al. (2012) observed a 10% significant increase in milk yield at the highest dosage (1.2 g/d per head of EO extracts) (Table 4). In this study there were some effects on milk composition too and BCS increased in both treated groups.

The study of Manca et al. (2012b) supplied the vegetative components (leaves and small twigs) of three plants (*Melissa officinalis, Ocimum basilicum* and *Thymus vulgaris)* at 3 dosages (50, 125 and 200 g/d, DM basis) to lactating dairy ewes. The plants were mixed with a different proportion of corn and pea meal, to form isoproteic mixes, and supplied

individually (450 g/d of DM) to lactating dairy ewes. The results showed that the aromatic plants did not affect DMI, except for *Melissa officinalis*, which caused a 33% reduction of the intake of the mix when used at the highest dosage. This was probably caused by the very fibrous and ligneous structure of this plant. In addition, there were no effects of aromatic plants on milk yield and composition, except for a linear decrease of milk urea as the dosage of *Thymus vulgaris* increased and a decrease of milk lactose as the dosage of *Rosmarinus officinalis* increase (Table 4). However, the plants induced significant effects on milk fatty acid composition, as discussed later in this review.

Dairy goats

Four studies were found in which EO were supplied to lactating goats. One was based on the supply of a blend of monoterpenes at two dosages to Alpine and Saanen goats and reported no effects on DMI, milk yield and its composition (Malecky et al., 2009).

A second study (Heidarian Miri et al., 2013) supplied two dosages (at 1 and 2 g/L of rumen volume) of cumin seed extracts to lactating goats in early lactation. Milk yield was increased (+13%) by the lowest dosage (1 g/L of rumen volume). No effects were observed on milk fat and protein concentration, even though an increase in conjugated linoleic acid was observed for both dosages. A third study (Boutoial et al., 2013) provided two dosages of rosemary (*Rosmarinus officinalis* spp.) to lactating Murciano-Granadina goats from parturition to seven months of lactation. Milk fat, protein and somatic cells did not change ($P>0.05$), with means of about 5.51%, 3.37% and 2.74 log SCC mL^{-1}, respectively.

In the fourth study, three oil extracts (oil of cinnamon, garlic and ginger) rich in EO were compared with the control group (Kholif et al., 2012). Milk yield was significantly increased by all oils (+24%, +16% and +19% for cinnamon, garlic and ginger oils, respectively). In all treated groups, milk fat markedly decreased and milk protein increased even more strongly. The Authors attributed the decrease of milk fat concentration to both a dilution effect and a decrease of the rumen acetate to propionate ratio, and the increase in milk protein concentration to a possible increase in microbial synthesis group (Kholif et al., 2012).

Effects of essential oils on milk fatty acids

Benchaar et al. (2007b) found on dairy cattle that the profile of milk fatty acids (**FA**) of cows was not influenced by supplementation with 750 mg per day of a mixture of EO compounds.

Similarly, Hristov et al. (2013) did not observe variations in milk fatty acids when supplementing the diet of lactating cows with three dosages of *Origanum vulgare* leaves.

In lactating Damascus goats, Kholif et al. (2012) found that supplementation with garlic oil, cinnamon oil or ginger oilincreased unsaturated FA and conjugated linoleic acid (**CLA**), and that cinnamon oil also increased n3 linolenic acid.

Heidarian Miri et al. (2013) reported that cumin (*Cuminumcyminum*) seed extract fed at two doses (12.7 and 25.3 g/kg DMI) to lactating goats caused an increase in poly-unsaturated FA and CLA of the milk. In the study of Boutoial et al. (2013) on supplementation of rosemary (*Rosmarinus officinalis* spp.) to lactating Murciano-Granadina goats, polyunsaturated fatty acids (PUFA) increased ($P<0.05$) from 3.37 to 4.65 and then to 5.20 with increasing doses of 0, 10 and 20% of additive in the diet.

In the study of Manca et al. (2012a) on supplementation with vegetative parts of aromatic plants, the botanical species (*Melissa officinalis, Ocimum basilicum* and *Thymus vulgaris*) affected all milk FA groups (Table 7), except for the trans FA and the branched chain FA. In addition, as dose increased (50, 125 and 200 g/d, DM basis), branched chain fatty acids increased, suggesting a supportive effect of the plants on microbial activity. There was also an increase in n3 poly-unsaturated FA and in the sum of conjugated linoleic acid isomers (Table 7) suggesting a reduction of biohydrogenation process, since these FA are intermediates of this process.

Some commentson effects of essential oils on milk production and composition

The review of Khiaosa-ard and Zebeli (2013) did not find effects of EO on milk yield and compositionin dairy cows, except for an increase in milk protein concentration and yield, whereas production data on sheep and beef cattle were not sufficient for a meta-analysis.

The limited effects on milk yield and composition in dairy cows on one side and the marked effects in lactating ewes and goats reported above could be due to real differences between animal species or due to the different treatments applied.

In dairy cattle, a possible positive effect on milk production after the peak of lactation was suggested by the two experiments which evaluated EO complexes in mid lactation in long term studies (Kung et al., 2008; Tassoul and Shaver, 2009). In the case of lactating sheep and goats, in all experiments which used EO extracts or oils there was an increase in milk yield, at

least in one dosage. This effect was particularly strong (+35%) for the experiment with the highest duration (5 months, Giannenas et al., 2011). In most studies the doses used were comparably higher and the experiments were longer in sheep and goats than in the case of cows (Tables 3 and 4). Based on this information, it is possible to speculate that EO complexes are more effective after the beginning of lactation, i.e. periods of positive energy balance, in long term studies and at high dosages. Ruminants in early lactation are generally in negative energy balance and mobilize body fat and this might interact with the utilization of EO. Indeed, both in sheep (Pulina et al., 2006) and cows (Chilliard, 1993) the effects of the addition of fat in the diet were more evident in mid-late lactation than in early lactation. It would be interesting to study if the mechanisms involved in this phenomenon are similar between EO and supplemented fat, being both compounds water-insoluble.

The fact that small ruminants responded consistently better than cows could be also due to the higher rumen passage rate of liquids and solids which characterize ruminants of small body size compared to those of larger size (Van Soest, 1994; Cannas et al., 2003). The high rumen passage rate of sheep and goats might have reduced the interaction of EO at rumen level and increased their intestinal digestion. This would imply that the effects of EO are more related to an enhancement of metabolic pathways of milk synthesis than to modifications of the rumen environment, as often postulated. This hypothesis is supported by the studies reported above on milk FA. It appears that in the few studies available on dairy cows milk fatty acids were not affected, by the supply of EO, whereas all studies on lactating goats and ewes observed an increase of the unsaturation of FA and of CLA, suggesting that EO can reduce the biohydrogenation process, potentially improving the nutraceutical value of their milk. This animal species difference could be, also for FA, a result of the high feed rumen passage rate of small ruminants in comparison to large ones, which could limit the ability or the need of rumen bacteria to complete the biohydrogenation process.

8. Effects of essential oils on average daily gain of growing animals

Some studies tested the possibility of improving average daily gain (**ADG**) of growing animals by adding EO (Table 5). Some of them actually achieved an increase of ADG, even though the

results were somehow inconsistent. Geraci et al. (2012) observed a positive effect of a commercial EO complex on ADG of beef heifers. However, this effect was limited to the second half of the experiment. All the other studies on cattle which used EO commercial complexes did not report any effects on ADG. Inthe recent study of Nooriyan Soroor et al. (2013) on Mehraban male lambs, varying the doses of *Echium amoenum* extracts (from 0 to 3 ml/kg diet DM) did not influence ADG (mean about 183.3 g/day; P=0.7). Differently, Chaves et al. (2008b) observed an increase in ADG when cinnamaldehyde or juniper berry oils were added to the diet of lambs fed high starch diets. However, the same research group did not observe any effect in two other studies that used cinnamaldehyde on growing lambs fed high starch diets (Chaves et al., 2008b, 2011). As suggested by Patra (2011), it is possible that the results of the utilization of EO are diet dependent. However, not many studies on the utilization of EO to improve ADG are available and, therefore, it is not possible to define which feeding conditions may be more favorable for their utilization.

9. Effects of plant secondary metabolites on gastro-intestinal parasites

Since the anthelmintic resistance against drugs was first suspected to exist (Drudge et al., 1957), the gastrointestinal parasitism has been controlled by other strategies as well, such as vaccines, genetic selection, nutrition manipulation (Torres-Acosta and Hoste, 2008) and grazing management (Torres-Acosta et al., 2012b). In the last years, natural compounds have become an interesting alternative against parasites. In fact, plant secondary metabolites (**PSM**) have received a lot of attentionlately for their potential anti-parasitic effects. In the literature, some *in vivo* studies, described below, have found anti-parasitic properties of aromatic plants, plant extracts or essential oils in small ruminants, but their effects and mode of action are still controversial.

Adult sheep

Several *in vivo* studies evaluated the anthelmintic effects of plant extracts in adult sheep (Table 8). In some studies sheep were fed indoors and artificially infected (Eguale et al., 2007; Vatta et al., 2011), whereas in others sheep were naturally infected in indoors trials

(Tariq et al., 2009; Lone et al., 2012). Only one study was carried out in grazing conditions (Chagas et al., 2008; Table 8).

The anthelmintic activity of crude powder and crude methanolic extract of *Ferula costata* was tested and found the highest at the highest dosage (3 g/kg of BW) of utilization on day 14 after the treatment (Kakar et al., 2013). In another study on undefined sheep breed, Mesquita et al. (2013) evaluated the anthelmintic activity of *Eucalyptus staigeriana* essential oils and showed an efficacy of about 83.8%. In Kashmir Marino sheep, a crude aqueous extract and a methanolic extract of *Euphorbia helioscopia* L. had an anthelmintic dose-dependent effect, reducing faecal egg count (Lone et al., 2012). In male Menz sheep, a diet containing an extract of dried seeds of *Coriandrum sativum* showed anthelmintic effects, which were dose and concentration dependent (Eguale et al., 2007). Supplementation with an extract of dried seeds of *Carum copticum* had also anthelmintic effects (Lateef at al., 2006). The anthelmintic efficacy of essential oils (EO) of *Lippia sidoides*, expressed as reduction of faecal egg count from day 7 to day 14 after the treatment, varied from 38% to 30% with the lowest dose (0.23 g/kg) and from 45% to 54% with the highest dose (0.28 g/kg) (Camurça-Vasconcelos et al., 2008). On the contrary, extracts of *Cereus jamacuru* mature plants (Vatta et al., 2011) and *Azadirachta indica* A. Juss (Morada Nova breed; Chagas et al., 2008) at different doses did not have any anthelmintic effect in sheep (Table 8).

Lambs

The *in vivo* studies with lambs evaluated the anthelmintic effects of leaves, stems, oil and plant extracts and, to our knowledge, were all carried out in indoor conditions (Table 9). In some trials lambs were artificially infected (Githiori et al., 2003;Hördegen et al., 2003; Squires et al., 2010; Katiki et al., 2012) whereas in others the animals were naturally infected (Ademola et al., 2004, 2005, 2007; Whitney et al., 2013).

Three studies reported a faecal egg count reduction when the lambs were fed *Juniperus pinchotii* leaves and stems (Barbados Blackbelly and St. Croix breed; Whitney et al., 2013), *Spondiasmombin* extract (West African dwarf sheep breed; Ademola et al., 2005) or *Khaya senegalensis* extract (West African dwarf sheep breed; Ademola et al., 2004), whereas in Santa Ines breed Katiki et al. (2012) did not observe faecal egg count reduction. Two studies reported anthelmintic activity against *Haemonchus contortus* in lambs fed orange oil

(Squires et al., 2010) or supplied with *Nauclea latifolia* extract (West African dwarf sheep breed; Ademola et al., 2007). On the contrary, no anthelmintic effects were observed against *Trichostrongylus colubriformis* in lambs fed extracts of Ananas *comosus* and *Caesalpinia crista* (lambs of triple crossing of Swiss White Alpine, Swiss black-Brown Mountain and Charollais breeds; Hördegen et al., 2003) and against *Haemonchus contortus* in lambs fed the same extracts (Hördegen et al., 2003) of fed extract of *Albizia anthelmintica* (Dorper, Red Massai and their cross; Githiori et al., 2003).

Goats

In goats, only two *in vivo* studies had animals which were naturally infected (Burke et al., 2009; Botura et al., 2011, Table 10), Most studies were carried out in indoor conditions (Burke et al., 2009; Botura et al., 2011; Hernández-Villegas et al., 2012; Moreno et al., 2012), whereas two trials were performed on pasture (Burke et al., 2009; Macedo et al., 2010, Table 10). Several plant extracts, leaves, essential oil, juice or bulbs were tested.

Phytolacca icosandra extract (Criollo breed; Hernández-Villegas et al., 2012), *Agave sisalana* extract (Mixed breed; Botura et al., 2011), *Acacia salicina, Acacia nilotica, Eucalyptus corymbia, Casuarina cunninghamiana* and *Eucalyptus drepanophylla* fresh leaves (Moreno et al., 2012) and *Eucalyptus citriodora* or *Eucalyptus staigeriana* essential oils (Macedo et al., 2010, 2011) were effective against *Haemonchus contortus* (Table 10). The five Australian plants supplied by Moreno et al. (2012) also were effective, reducing total faecal egg output about *Trichostrongylus colubriformis*. On the contrary, *Agave sisalana* juice (Domingues et al., 2010), garlic juice, in a formula or freshly squeezed, and garlic bulbs (Spanish, Spanish x Boer Doe breed; Burke et al., 2009) did not have any anthelmintic effects in goats (Table 10).

Final remarks on anthelmintic effects of plants

In a recent review about the control of gastrointestinal nematodes in sheep and goats through nutritional manipulation, Torres-Acosta et al. (2012b) showed that several plants had anthelmintic effects. However, the main compounds considered as active by the authors were condensed tannins, saponins, alkaloids, flavonoids and polyethylene glycol, while essential oils were not considered as possible acting substances. However, in many of the studies cited the possible presence of EO was not investigated. Furthermore, in a paper

about the novel approaches for the control of nematodes, Torres-Acosta et al. (2012a) considered plants with possible anthelmintic effects identified on the bases of local traditional wisdom and knowledge. Most of these plants were identified and tested in Brazil, Mexico and Guadaloupe with *in vitro* trials. Hoste and Torres-Acosta (2011) affirmed the importance of finding out the origin of variability in the efficacy results of some plant materials, the biochemical characterization of the active compounds and their mode of action on worm proteins at molecular level and, finally, the optimal conditions of application under farm conditions.

In conclusion, the system "parasites-host-nutrition" is by itself very complex. The results and the interpretation of many experiments is also complicated by the fact that several papers with *in vivo* trials used low doses of plants and did not report which were the main secondary components present in the plants studied. Thus, it becomes difficult to have repeatable results and to identify the acting compounds. For these reasons, anthelmintic effects of plants should be studied with *in vivo* experiments by using plants or plant parts chemically well characterized and possibly in grazing conditions, so far rarely considered by the literature.

10. Conclusions

Based on *in vivo* studies on the effects of EO or of whole plants rich in EO on ruminants, it is possible to conclude that:

- in cattle the effects on rumen pH and VFA are very limited, whereas in sheep and goats several studies reported an increase of total VFA and a decrease of the acetate to propionate ratio;
- *in vivo* methane production was reduced in most of the studies reviewed, in cattle, sheep and goats;
- ammonia production was reduced in various experiments but not affected in many others. More research is needed to define the conditions in which ammonia reduction can occur;
- rumen microorganisms were often affected but not clear patterns could be observed;
- NDF digestibility was often reduced, but in many cases it was unchanged or increased. As for rumen microbial population, no clear patterns or mechanisms could be observed;
- milk yield was often positively affected after the first part of the lactation, in long term studies, at high dosages of EO, and in sheep and goats compared to dairy cows;
- milk composition was marginally affected, except for milk fatty acid composition, for which all studies on lactating goats and ewes, but none of those on cattle, observed an increase on the unsaturated FA and of CLA concentrations, with possible reductions of the rumen biohydrogenation processes;
- in some cases ADG was improved, but too few studies on growing animals are available to understand which conditions might favor this effect;
- anthelmintic effects were reported in various experiments carried out on small ruminants fed indoors, whereas few data are available on grazing ruminants.

In general, it appears that small ruminants are more responsive to the action of EO than cattle, possibly due to differences in the rumen feed and liquid passage rate, usually higher in small than in large ruminants.

Compared to *in vitro* studies, in which frequent measurements are possible, the variables of interest of *in vivo* studies are often measured few times, for the difficulties of taking measurements with alive animals. In addition, *in vivo* studies have to deal with the complex and difficult-to-control ruminal environment as well as with variations in plant species, plant

parts, oils and extracts tested. Despite these limitations, *in vivo* studies are fundamental for the transfer of the knowledge acquired to production conditions. Therefore, in the future it would be important to conduct *in vivo* studies with more frequent samplings, possibly taken at various intervals from the first supply of EO, and monitoring how and to what extent the rumen environment and the animals can adapt to these compounds, so that the mode of action of these compounds could be better understood.

Regarding the anthelmintic effects of EO, there is the need to carry out experiments by using plants or plant parts chemically well characterized and possibly in grazing conditions, so far rarely considered by the literature.

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Table 1. Main ***in vivo*** rumen values of experiments in which essential oils (EO) or aromatic plants were supplied to cattle.

EO and physical form	Daily dose, g/head	pH	Total VFA	A:P ratio	Methane	NH_3	Microbial population	Digestibility	Species	Reference
EO complex [1]	0.5, 10	NS	NS	NS	-	NS	-	NS	Lact. cow	Tager and Krause (2011)
EO complex [2]	1.0	-	-	-	-	-	-	NS	Lact. cow	Santos et al. (2010)
EO complex [3]	0.32, 0.64, 0.96	-	-	-	-	-	-	NS	Lact. cow	Spanghero et al. (2009)
EO complex [4]	0.75	NS	↑alfalfa [#], ↓corn sil. [#]	NS	-	NS	NS	NS	Lact. cow	Benchaar et al. (2007b)
Cinnamaldehyde	1.0	NS	NS	NS	-	NS	*Isotrichia*↑[#]	NS	Lact. cow	Benchaar et al. (2008b)
Garlic oil, Juniper berry EO	5.0, 2.0	NS	NS	NS	-	NS	NS	DM, OM ↑**	Lact. cow	Yang et al. (2007)
Origanum vulgare, leaves	500	NS	NS	NS	↓**	↑**	↑*Clostridium* &*Bacterioides* **; ↑*Butyrivibrio fibrisolvens* [#]	NS	Lact. cow	Tekippe et al. (2011)
Origanum vulgare, leaves	250, 500, 750	NS	NS	NS	↓[#]	↓*	*Ruminococcus flav.*↑*	NDF ↓*	Lact. cow	Hristov et al. (2013)
E.camaldulensis, leaf meal powder	100, 200	NS	↓*	↓*	↓*	↓*	↓ of protozoa & total, proteolytic and cellulolytic bacteria *	NS	Dry dairy cow	Manh et al. (2012)
EO complex [5]	0.39	NS	NS	NS	-	↓*	-	-	Castrated beef steers	Geraci et al. (2012)
Eugenol	0.4, 0.8, 1.6	NS	NS	↓[#]	-	NS	Isotrichia ↓[#]	NDF ↓[#]	Beef heifers	Yang et al. (2010a)
Cinnamaldehyde	0.4, 0.8, 1.6	NS	NS	NS	-	NS	NS	↓N, OM, NDF *	Beef heifers	Yang et al. (2010b)
EO complex[6]	22	↓**	NS	↓*	-	NS	-	-	Young bulls	Devant et al. (2007)
Carum carvi seed (CS), dried garlic powder (DG), CS+DG	CS & DG: 14.7 CS+DG: 14.8+14.8	NS	↑**	-	-	↓**	-	DM, CP, EE ↑**	Buffalo calves	Hassan and Abdel-Raheem (2013)

A:P = acetate to proprionate. NS = not significant (P>0.05) *P<0.05 **P<0.01 [#] considered a trend at 0.05 < P ≤ 0.10.

[1] XT 6965 (Pancosma S.A., Bellegarde-sur-Vlaserine, France): 170 mg cynnamaldehyde, 280 mg eugenol per gram of product.
[2] Agolin Ruminant (Agolin Ruminant, Biere, Switzerland): mixture of EO including eugenol, geranyl acetate and coriander oil as main components.
[3] RumaXol Feed (Soda Feed Ingredients, Monaco): mixture of EO including oregano, cinnamon, thyme and orange peel as main components.
[4] Crina ruminants (Crina S.A., Gland, Switzerland): mixture of EO including thymol, eugenol, vanillin, guaiacol and limonene as main components.
[5] XT 6965 (see note 1) plus XT 6933: 133 mg/head/d of capsicum oleoresin extract.
[6] Plant extract blend: cynarin, gingsen and fenugreek; Biostar®, Phytosynthese, France.

Table 2. Main ***in vivo*** rumen values of experiments in which essential oils (EO) or aromatic plants were supplied to sheep and goats.

EO and physical form	Daily dose, g/head	pH	Total VFA	A:P ratio	Methane	NH_3	Microbial population	Digestibility	Species	Reference
					Sheep					
EO complex[1]	EOC=0.5 EOAC=0.5 and 1	NS	↓**	↓*	-	↓**	↓ Protozoa, F. succin, B. fibris., Methanogen **	NS	Sheep	Lin et al. (2013)
Cinnamaldehyde	0.102, 0.210, 0.457	NS	NS	NS	-	NS	-	-	Ewe lambs	Chaves et al. (2011)
Raw and oil garlic	75, 0.5	NS	NS	↓**	-	↓**	Protozoa ↑**	↓ OM, CP, ADF, NDF or ↑ NDF ** (time interact.)	Male sheep	Anassori et al. (2011)
Raw and oil garlic	100, 0.75	NS	NS	↓**	-	↑ or ↓**	Protozoa ↓**	↓OM, ADF, NDF or ↑ NDF # (time interact.)	Male sheep	Anassori et al. (2011)
Raw and oil garlic	75, 100, 0.5, 0.75	NS	NS	NS	-	↓**	Protozoa ↓**	↓ OM, ADF, NDF #	Male sheep	Anassori et al. (2011)
Ropadiar from oregano extract	0.25	NS	↑**	NS	↓**	↓**	-	NS	Wethers	Wang et al. (2009)
Echium amoenum extract	0.3, 1.5, 3 ml/kg DM	NS	↑*	↓**	-	↓**	Protozoa, cellulolytic bacteria ↓**	NS	Lambs	Nooriyan Soroor et al. (2013)
Carvacrol or Cinnamaldehyde	0.259; 0.273	↓*	↑*	NS	-	NS	-	-	Lambs	Chaves et al. (2008a)
Cinnamaldehyde; garlic oil; juniper berry oil	0.24, 0.23, 0.24	NS	NS	NS	-	NS	-	-	Lambs	Chaves et al. (2008b)
EO complex[2]	0.11	NS	↑#	NS	NS	NS	NS	-	Mature sheep	Newbold et al. (2004)
EO complex[3]	0.075, 0.15, 0.217	NS	↑#	↓*	-	NS	Cellulolytic bacteria↑ Bacteria producing NH_3↓*	-	Lactating ewes	Giannenas et al. (2011)
Melissa officinalis[4]	50, 125, 200	NS	NS	NS	-	NS	-	-	Lactating ewes	Manca et al. (2012a)
Ocimum basilicum[4]	50, 125, 200	NS	NS	NS	-	NS	-	-	Lactating ewes	Manca et al. (2012a)
Thymus vulgaris[4]	50, 125, 200	NS	NS	NS	-	NS	-	-	Lactating ewes	Manca et al. (2012a)

Goats										
Cumin seed extracts	1.48, 2.96	-	-	-	↓**	-	-	-	Lactat. goats	Heidarian Miri et al. (2013)
Cinnamon, garlic, ginger	2 ml	NS	↑**	↓**	-	↓*	-	-	Lactat. goats	Kholif et al. (2012)
Monoterpene blend	0.116, 1.16	NS	NS	NS	-	NS	NS	NS	Lactat. goats	Malecky et al. (2009)

A:P = acetate to propionate. NS = not significant (P>0.05) *P<0.05 **P<0.01 [#] considered a trend at 0.05 < P ≤ 0.10.

[1] EOC: 1 g/day: mixture of EO including clove, oregano, cinnamon and lemon in an equal ratio; EOAC: 0.5 or 1 g/day: mixture of EO including eugenol, carvacrol, citral and cinnamaldehyde in an equal ratio.

[2] Crina ruminants (Crina S.A., Gland, Switzerland): mixture of EO including thymol, guajacol and limonene as main components.

[3] Crina ruminants (Crina S.A., Gland, Switzerland): mixture of EO including thymol, eugenol, vanillin, guaiacol and limonene as main components.

[4] vegetative components, supplied in isoproteic mixes with corn and pea meals separately from the basal ration.

Table 3. Main results of experiments in which essential oils (EO) or aromatic plants were supplied to lactating dairy cows.

EO and physical form	Daily dose, g/head	Exper. length	Initial DIM	Diet F:C	DMI variation (treated-control), kg/d	Milk yield Control, kg/d	Milk yield Treated kg/d	Fat, %	CP, %	BCS or BW (treated-control)	Reference
EO complex [1]	1.0	28 d	20 d	38:62	-1.43 NS	49.2	NS	+0.07 **	NS	BCS = -0.016/d **	Santos et al. (2010)
EO complex [2]	1.2	19 wk	-4 wk	70:30	-1.8 *	48.2	NS	NS	-0.15 *	BCS =NS	Tassoul and Shaver (2009)
EO complex [2]	0.75	28 d	61 d	50:50	NS	28.9	NS	NS	NS	-	Benchaar et al. (2007b)
EO complex [2]	1.2	9 wk	118 d	50:50	+1.9 *	39.8	41.7 (P<0.16)	NS	NS	BCS and BW = NS	Kung et al. (2008)
EO complex [3]	0.5, 10.0	21 d	43 d	42:58	NS	33.0	NS	NS	NS	-	Tager and Krause (2011)
EO complex [4]	0.32, 0.64, 0.96	21 d	40 d	56:44	NS	31.4	NS	NS	NS	-	Spanghero et al. (2009)
Cynnamaldehyde	1.0	28 d	87 d	40:60	NS	33.6	NS	NS	NS	NS	Benchaar et al. (2008b)
Garlic oil	5.0	21 d	113 d	40:60	NS	29.0	NS	NS	NS	NS	Yang et al. (2007)
Juniper berry EO	2.0	21 d	113 d	40:60	NS	29.0	NS	NS	NS	NS	Yang et al. (2007)
Origanum vulgare, leaves	500	42 d	80 d	60:40	NS	43.6	NS	+0.17 **	NS	BW = NS	Tekippe et al. (2011)
Origanum vulgare leaves	250, 500, 750	20 d	51 and 247 d	57:43	Linear ↓	43.4	NS	NS	NS	-	Hristov et al. (2013)

DIM, days in milk; DMI, dry matter intake. NS = not significant (P>0.05) * P<0.05 ** P<0.01.

[1] Agolin Ruminant (Agolin Ruminant, Biere, Switzerland): mixture of EO including eugenol, geranyl acetate, and coriander oil as main components.
[2] Crina ruminants (Crina S.A., Gland, Switzerland): mixture of EO including thymol, eugenol, vanillin, guaiacol, and limonene as main components.
[3] XT 6965 (Pancosma S.A., Bellegarde-sur-Vlaserine, France): 170 mg cynnamaldehyde, 280 mg eugenol per gram of product.
[4] RumaXol Feed (Soda Feed Ingredients, Monaco): mixture of EO including oregano, cinnamon, thyme, orange peel as main components.

Table 4. Main results of experiments in which essential oils (EO) or aromatic plants were supplied to lactating sheep and goats.

EO and physical form	Daily dose, g/head	Exper. length	Initial DIM	Diet F:C	DMI variation, kg/d	Milk yield Control, kg/d	Milk yield Treated, kg/d	Fat variation, %	CP variation, %	Others	Reference
Lactating ewes											
EO complex [1]	0.075	5 mo	-1 wk	54:46	NS	1.565	1.681 NS	NS	NS	Lower SCC	Giannenas et al. (2011)
	0.15	5 mo	-1 wk	54:46	NS		1.876 *	NS	NS	Lower SCC	
	0.217	5 mo	-1 wk	54:46	NS		2.119 *	NS	NS	Lower SCC	
EO extract of *Rosmarinus officinalis*[2]	0.6	150 d	0 wk	79:21 to 69:31	NS (rationed)	1.150	1162 NS	-0.52% **	NS	+0.023 BCS *	Chiofalo et al. (2012)
	1.2						1264 *	NS	-0.16 *	+0.035 BCS *	
Melissa officinalis[3]	50, 125, 200	56 d	4 mo	80:20	200 dosage: 33% refusal (P<0.05)	1.371	NS	NS	NS	-	Manca et al. (2012b)
Ocimum basilicum[3]					NS		NS	NS	NS	-	
Thymus vulgaris[3]					NS		NS	NS	NS	Lower milk urea	
Lactating goats											
Monoterpene blend	0.116 & 1.16	6 wk	11 wk	73:27	NS	3.159	NS	NS	NS	-	Malecky et al. (2009)
Cinnamon oil	2 ml	90 d	1 wk	60:40	+0.03		1389 *	-0.2% *	+0.37 *		
Garlic oil	2 ml	90 d	1 wk	60:40	NS	1.123	1308 *	-0.22% *	+0.42 *	-	Kholif et al. (2012)
Ginger oil	2 ml	90 d	1 wk	60:40	+0.03		1335 *	-0.25% *	+0.33 *		
Cumin seed extracts	1.48	32 d	-	50:50	NS	1.357	1.533 **	NS	NS	Increased CLA	Heidarian Miri et al. (2013)
Cumin seed extracts	2.96	32 d	-	50:50	NS		NS	NS	NS	Increased CLA	
Rosmarinus officinalis leaves	10 & 20%	7 mo	0 wk	-	-	-	-	NS	NS	Lower Lactose, High PUFA	Boutoial et al. (2013)

DIM, days in milk; DMI, dry matter intake. SCC, somatic cell count.
NS = not significant (P>0.05) * P<0.05 ** P<0.01.
[1] Crina ruminants (Crina S.A., Gland, Switzerland): mixture of EO including thymol, eugenol, vanillin, guaiacol, and limonene as main components.
[2] ROXP supplement (SEVECOM SpA, Italy): natural extract of *Rosmarinus officinalis* L. Main components: rosmarinic acid, carnasol, carnosic acid rosmanolo.
[3] vegetative components, supplied in isoproteic mixes with corn and pea meals separately from the basal ration.
PUFA: polyunsaturated fatty acid.

Table 5. Growth of experiments in which essential oils (EO) were supplied to cattle and sheep.

Essential Oil	Daily dose, g/head	Exper. Length, d	Daily gain, g/d	Species	Reference
			Cattle		
Carum carvi seed (CS), garlic powder (DG), CS+DG	CS and DG=2g CS+DG=4g/kg diet	180	NS	Buffalo calves	Hassan and Abdel-Raheem (2013)
EO complex[1]	0.39	84	d 1-44: NS d 45-84:+16.2% **	Castrated beef heifers	Geraci et al. (2012)
EO complex[2]	1	115	NS	Crossbred steers	Meyer et al. (2009)
EO complex[3]	22	84	NS	Young bulls	Devant et al. (2007)
EO complex[4]	1	84	NS	Beef heifers	Beauchemin and McGinn (2006)
			Sheep		
Echium amoenum extract	0.3, 1.5, 3 ml/kg DM	97	NS	Lambs	Nooriyan Soroor et al. (2013)
Cinnamaldehyde	0.102, 0.210, 0.457	126	NS	Lambs	Chaves et al. (2011)
Carvacrol or cinnamaldehyde	0.259; 0.273	77	NS	Lambs	Chaves et al. (2008a)
Cinnamaldehyde	0.24	91	+15.4% **	Lambs	Chaves et al. (2008b)
Garlic oil	0.23	91	NS	Lambs	Chaves et al. (2008b)
Juniper berry oil	0.24	91	+17.3% **	Lambs	Chaves et al. (2008b)

NS = not significant (P>0.05) ** P<0.01.

[1] XT 6965 (Pancosma S.A., Bellegarde-sur-Vlaserine, France): 170 mg cynnamaldehyde, 280 mg eugenol per gram of product, plus XT 6933: 133 mg/head/d of capsicum oleoresin extract.

[2] EOM, Crina ruminants (Crina S.A., Gland, Switzerland): mixture of EO including thymol, eugenol, vanillin, guaiacol and limonene as main components and EXP contained guaiacol, linalool, and α-pinene. Both mixtures are proprietary blends of essential oils on an organic carrier (DSM Nutritional Products Inc.).

[3] PE, cynarin, gingseng and fenugreek; Biostar®, Phytosynthese, France.

[4] Essential oil and spice extract (Crina Ruminants; Akzo Nobel Surface Chemistry S.A., Cedex, France).

Table 6. Effects of plant, period and dose on proportions of rumen fluid fatty acids (g/100 g of FAME) (Manca et al., 2012a).

	Fatty acids, g/100 g of FAME					
PLANT	**Σ SFA**	**Σ MUFA**	**Σ BCFA**	**Σ PUFA n3**	**Σ PUFA n6**	**Σ TFA**
Melissa officinalis	74.10	14.62	7.25 ^{a}	1.63 ^{b}	8.30	4.32
Ocimum basilicum	74.47	14.07	6.92 ^{ab}	2.02 ^{a}	7.97	4.03
Thymus vulgaris	75.38	13.59	6.49 ^{b}	1.68 ^{b}	8.05	4.19
P for Plant species	NS	NS	0.001	0.001	NS	NS
DOSE						
200 g/d	75.27	13.35	7.18 ^{a}	2.22 ^{a}	7.66	4.24
125 g/d	74.41	14.00	7.05 ^{ab}	1.91 ^{ab}	8.33	4.04
50 g/d	74.92	14.14	6.88 ^{ab}	1.61 ^{bc}	8.01	4.22
0 g/d	74.01	14.88	6.44 ^{b}	1.37 ^{c}	8.42	4.23
P for Dose	NS	NS	0.04	0.001	NS	NS

FAME = fatty acids methyl-esters; SFA = short chain fatty acids; MUFA = mono-unsaturated fatty acids; BCFA = odd branched chain fatty acids; TFA = trasn fatty acids; PUFA = poly-unsaturated fatty acids;
^{a,b} in the same column within factor = P<0.05.

Table 7. Effects of plant, period and dose on proportions of milk fatty acids (g/100 g of FAME) (Manca et al., 2012b).

	Fatty acids, g/100 g of FAME						
PLANT	**Σ SFA**	**Σ MUFA**	**Σ BCFA**	**Σ PUFA n3**	**Σ PUFA n6**	**Σ TFA**	**Σ CLA**
Melissa officinalis	77.45^{a}	18.15 ^{b}	2.71	0.89 ^{b}	2.06 ^{b}	1.26	0.86 ^{b}
Ocimum basilicum	76.42^{ab}	18.94 ^{ab}	2.68	0.98 ^{b}	2.11 ^{b}	1.11	0.84^{b}
Thymus vulgaris	75.79^{b}	19.17 ^{a}	2.72	1.04 ^{a}	2.42 ^{a}	1.18	0.93^{a}
P for Plant species	0.001	0.03	NS	0.001	0.001	NS	0.02
DOSE							
200 g/d	76.67	18.36	2.89 ^{a}	1.10 ^{a}	2.24	1.30	0.99 ^{a}
125 g/d	76.13	19.09	2.78 ^{ab}	1.01 ^{ab}	2.19	1.28	0.94^{ab}
50 g/d	76.45	18.87	2.60 ^{b}	0.93 ^{bc}	2.24	1.03	0.89 ^{bc}
0 g/d	76.97	18.68	2.54 ^{b}	0.85 ^{c}	2.13	1.12	0.82 ^{c}
P for Dose	NS	NS	0.001	0.001	NS	NS	0.001

FAME = fatty acids methyl-esters; SFA = short chain fatty acids; MUFA = mono-unsaturated fatty acids; BCFA = odd branched chain fatty acids; TFA = trasn fatty acids; PUFA = poly-unsaturated fatty acids; CLA = conjugated lioleic acid.
^{a,b} in the same column within factor = P<0.05.

Table 8. Main ***in vivo*** anti-parasitic effects of plants rich in secondary metabolites supplied to adult sheep.

Plant	Dose tested	Infection	Feeding	Nematode specie	Parasitic effect	Reference
Ferula costata extract[1]	1, 2, 3 g/kg BW	Natural	-	Mixed	Time and dose dependent	Kakar et al. (2013)
Eucalyptus staigeriana essential oil	0.365 g/kg	Natural	Indoors	Mixed	Effective	Mesquita et al. (2013)
Euphorbia helioscopia extract[2]	1.0 g/kg BW	Natural	Indoors	Mixed	Dose dependent	Lone et al. (2012)
Cereus jamacuru plant[3]	32.3, 64.6 per head	Artific.	Indoors	*Haemonchus contortus*, *Trichostrongylus colubriformis*	Not effective	Vatta et al. (2011)
Artemisia absinthium extract[4]	1, 2 g/kg BW	Natural	Indoors	Mixed	↓*H. contortus* worm motility and FEC	Tariq et al. (2009)
Azadirachta indica A. Juss[5]	1.6 g/head/d	-	Pasture	Mixed	Not effective	Chagas et al. (2008)
Azadirachta indica A. Juss leaves	12.5, 25.0, 37.5 g/head/d	-	Pasture	Mixed	Not effective	Chagas et al. (2008)
Lippia sidoides essential oil	0.23, 0.28 g/kg (5 days)	Natural	-	Mixed	Effective	Camurça-Vasconcelos et al. (2008)
Coriandrum sativum seed extract[6]	0.45, 0.9 g/kg	Artific.	Indoors	*Haemonchus contortus*	Dose and concentration dependent	Eguale et al. (2007)
Carum copticum seed[7]	1, 2, 3 g/kg BW	Natural	-	Mixed	Effective	Lateef et al. (2006)

[1] Extract of aerial parts with mature flowers of *Ferula costata*; [2]Extract of mature plant (*Euphorbia helioscopia*) at peak of flowering; [3]Fresh plant *Cereus jamacuru* without thorns; [4]Extract of dried plant (*Artemisia absinthium*) parts; [5]Homeopathic product Fator Vermes (according to the recommendations of its marker, Laboratório Arenales Fauna e Flora) or dried leaves; [6]Extract of dried seed of *Coriandrum sativum*; [7]Extract of dried seed of *Carum copticum*; FEC = faecal egg count.

Table 9. Main ***in vivo*** anti-parasitic effects of plants rich in secondary metabolites supplied to lambs.

Plant	Dose tested	Infection	Feeding	Nematode specie	Parasitic effect	Reference
Juniperus pinchotii leaves and stems[1]	30%	Natural	Indoors	*Haemonchus contortus*	↓FEC	Whitney et al. (2013)
Cymbopogon schoenantus essential oil	0.18, 0.36 g/kg BW (3 days)	Artificial	Indoors	*Haemonchus contortus*	Not effective	Katiki et al. (2012)
Orange oil[2]	0.6 g/kg (once or 3 days)	Artificial	Indoors	*Haemonchus contortus*	Effective	Squires et al. (2010)
Nauclea latifolia extract[4]	0.13, 0.25, 0.5 g/kg BW (2 days)	Natural	Indoors	Mixed	↓*Haemonchus contortus*, *Trichostrongylus* spp., *Strongyloides* spp. e *Trichuris* spp.	Ademola et al. (2007)
Spondias mombin extract[5]	0.13, 0.25, 0.5 g/kg BW (2 days)	Natural	Indoors	Mixed	↓FEC	Ademola et al. (2005)
Khaya senegalensis extract[6]	0.13, 0.25, 0.5 g/kg BW (2 days)	Natural	Indoors	Mixed	↓FEC	Ademola et al. (2004)
Albizia anthelminica extract[7]	*	Artificial	Indoors	*Haemonchus contortus*	Not effective	Githiori et al. (2003)
A.comosus[8], *C. crista*[9]	1, 0.028 g/kg BW	Artificial	Indoors	*Haemonchus contortus*, *Trichostrongylus colubriformis*	Not effective	Hördegen et al. (2003)

[1] Dried leaves and stems of *Juniperus pinchotii* plant; [2] 40% orange terpene oil, 20% orange valencia oil, 4% polysorbate 80, 1.5% hydrogen peroxide and 34.5% water; [3] Crude powder or Crude methanol extract of *Ziziphus nummularia* and *Acacia nilotica*; [4] Extract of leaves of *Nauclea latifolia*; [5] Extract of leaves of *Spondias mombin*; [6] Extract of bark of *Khaya senegalensis*; [7] Extract of dried bark of *Albizia anthelminica*; [8] Extract of dried leaves of *Ananas comosus*, [9] Extract of dried seed of *Caesalpiniacrista*; *Traditional dose, half of traditional dose and double of traditional dose; FEC = faecal egg count.

Table 10. Main ***in vivo*** anti-parasitic effects of plants rich in secondary metabolites supplied to goats.

Plant	Dose tested	Infection	Feeding	Nematode specie	Parasitic effect	Reference
Phytolacca icosandra extract[1]	0.25 g/kg BW (2 days)	Artificial	Indoors	*Haemonchus contortus*	Effective	Hernández-Villegas et al. (2012)
A. salicina, A. nilotica, E. corymbia, C. cunninghamiana and *E. drepanophylla*[2]	*Ad libitum*	Artificial	Indoors	*Haemonchus contortus*, *Trichostrongylus colubriformis*	Effective	Moreno et al. (2012)
Agave sisalana extract[3]	1.7 g/kg (8 days)	Natural	Indoors	Mixed	Moderately effective against eggs and free-living stages	Botura et al. (2011)
Eucalyptus citriodora essential oil[4]	0.5 g/kg (3 days)	Artificial	-	*Haemonchus contortus*	Effective	Macedo et al. (2011)
Eucalyptus staigeriana essential oil[5]	0.5 g/kg	Artificial	Pasture	*Haemonchus contortus*	Effective	Macedo et al. (2010)
Agave sisalana juice[6]	0.92 g/kg (4 or 8 days)	-	-	Mixed	Not effective	Domingues et al. (2010)
Garlic juice[7]	50 ml	Natural	Indoors	Mixed	Not effective	Burke et al. (2009)
Garlic juice or garlic bulbs[8]	40 ml or 3 bulbs	Natural	Pasture	Mixed	Not effective	Burke et al. (2009)

[1] Extract of dried leaves of *Phytolacca icosandra*; [2] Fresh leaves of *Acacia salicina, Acacia nilotica, Eucalyptus corymbia, Casuarina cunninghamiana* and *Eucalyptus drepanophylla*; [3] Extract of *Agave sisalana* waste; [4] Essential oil of *Eucalyptus citriodora* was purchased from Dierberger Óleos Essenciais Ltda (Barra Bonita, State of São Paulo, Brazil); [5] Essential oil of *Eucalyptus staigeriana* was purchased from Dierberger Óleos Essenciais Ltda (Barra Bonita, State of São Paulo, Brazil, lot 061153); [6] Juice of leaves of *Agave sisalana*; [7] Garlic juice (1:1 dilution of 99.3% formula Garlic Barrier, Garlic Research Labs, Inc., Glendale, CA); [8] Garlic juice (freshly squeezed) or garlic bulbs (approximate equivalent of that used to extract juice).

Objectives of the dissertation

This dissertation was carried out within the objectives and the research planned for the Techeese Project. The goals of the dissertation were focused in several aspects regarding the effects of the utilization of aromatic plants on diet utilization, milk production, parasitic load and health status of lactating Sarda sheep.

To respond these aspects we did 3 different Chapters with specific goals:

Chapter 2: -Evaluate the effects of *Carum* sp., *Coriandrum* sp. and *Satureja* sp. at three different doses on milk yield and quality, feed intake, blood and ruminal parameters and digestibility of lactating Sarda dairy ewes.

Chapter 3: - Examine possible anthelmintic effects of supplements containing either *Satureja* sp. alone or blends of *Satureja* sp. with *Carum* sp. and *Coriandrum* sp. in non-lactating and pregnant Sarda ewes naturally infested by gastro-intestinal parasites.

Chapter 4: - Test possible interactions and synergistic effects of blends of the same plants on milk production and composition, rumen function and health status of lactating Sarda ewes.

Effects of the utilization of increasing doses of aromatic plants on milk production, feed utilization and health status of lactating dairy ewes

CHAPTER 2

Oscar Boaventura Neto

Effects of the utilization of increasing doses of aromatic plants on milk production, feed utilization and health status of lactating dairy ewes

1. Introduction

After the ban of the utilization of antibiotics on ruminant nutrition by the European Community (Regulation 1831/2003/EC), there has been a vast interest in the identification and utilization of natural products (Benchaar et al., 2008). In this sense, Wallace (2004) postulated that plant extracts are interesting natural antimicrobial sources, because of the biological properties of their secondary compounds. Among the studied compounds, essential oils (**EO**) have been extensively studied *in vitro* (Cardozo et al., 2005, 2006; Castillejos et al., 2005; Busquet et al., 2006; Benchaar et al., 2007a; Castillejos et al., 2008; Patra et al., 2010; Bhatta et al., 2012). Differently, fewer long term studies have been conducted *in vivo*, as described in Chapter 1 of this dissertation. In a previous research of the Dipartimento di Agraria, University of Sassari Cannas, personal communication, after a series of *in vitro* and *in vivo* tests, including a short term lactation trial, it was found that caraway (*Carum* sp.), winter savoury (*Satureja* sp.), and coriander (*Coriandrum* sp.) did not limit milk production and did not cause negative effects on the health of dairy ewes. However, the long term supply of plants with antimicrobial EO might induce negative effects on microbial fermentations and animal's health that might not be observed in short term tests.

The aim of this long-term study was to examine the effects of three aromatic plants (*Carum* sp., *Coriandrum* sp. and *Satureja* sp.) at three different doses on milk yield and quality, feed intake, blood and ruminal parameters and digestibility of lactating Sarda dairy ewes.

2. Materials and Methods

This research consisted of a long term feeding trial and a digestibility trial carried out at its end. In both trials, the aromatic plants *Carum* sp., *Coriandrum* sp., and *Satureja* sp. were studied at three doses. For simplification, aromatic plants will be called **Carum**, **Coriandrum** and **Satureja** and the three doses will be called **Low dose**, **Medium dose** and **High dose** in the rest of this chapter.

The trials were conducted in accordance with the principles and specific guidelines on animal care and welfare required by Italian law (Gazzetta Ufficiale, DL no. 116, January 27, 1992).

Experiment 1. Long term feeding trial

Location and duration

This study was conducted on Sarda dairy ewes, which were selected in the experimental farm of AGRIS, located in Bonassai, Olmedo (Northwestern Sardinia, latitude: 41° N, longitude: 8° E, average annual rainfall = 547 mm) at approximately 20 km from Sassari, Italy. The trial was carried out between the beginning of February and the end of April 2012 and lasted 84 days, with 21 days of preliminary period (until the 26th of February) and 63 days of experimental period (from the 27th of February on), divided into three periods of 21 days.

Preliminary period

The main steps of the experimental design are reported in Table 1.

All ewes at the beginning of the preliminary period were treated orally with a therapeutic dose (4ml per 20 kg live weight of albendazole (Alben; Virbac Animal Health, Australia)) to remove any possible nematode infection, and then after thirty days the feces were collected to confirm the treatment efficacy.

During the preliminary period, 96 lactating Sarda ewes were group-fed a diet made by chopped dehydrated alfalfa (1.0 kg/d per head), beet pulps (0.50 kg/d per head), and a mix of ground corn and pea grains (0.50 kg/d per head). Water was always available. At the end of the preliminary period, all the ewes were subjected to measurements of milk yield and quality, body weight (**BW**) and body condition score (**BCS**) based on Russel (1969).

In addition, individual blood samples were taken early in the morning before feeding for analysis.

Based on milk, body weight andbody condition score measurements, 88 ewes were selected to be used in the experimental period. These ewes were divided into 4 groups (22 ewes each), homogenous for milk yield (mean±S.D, 1.99±0.37kg/d) and composition (fat, 5.00±0.59 %, and protein, 5.05±0.36 %, content) and for body weight (45.11±4.62kg) and BCS (2.34±0.19, in a range from 0 to 5). One ewe was removed from the control group due to neurological problems.

On average, ewes were 2.7-year-old (±0.78 S.D.) in the control group and 3-year-old (±1.16 S.D.) in the other three groups (Carum, Coriandrum and Satureja) (Table 3). The average lengthof lactation was 150, 141, 146 and 143 days for the control, Carum, Coriandrum and Satureja groups, respectively (Table 3).

Experimental period and experimental design

In the first experimental day, each of the 4 groups was randomly assigned to an aromatic plant to be used throughout the experiment or to the control. As said before, the three plants used were *Carum* sp., *Coriandrum* sp. and *Satureja* sp., tested at three increasing doses (low, medium and high), in chronological order, corresponding to the first, second and third experimental periods (each of 21 days), respectively (Table 1).

All the ewes were confined in a large indoor pen and group-fed a diet made by chopped dehydrated alfalfa (1.0 kg/d per head), beet pulps (0.50 kg/d per head) and alfalfa hay (0.50 kg/d per head). Water was always available. The mix was divided into two doses of 250 g as fed, individually supplied each day during the two daily machine milkings (8.00 AM and 3.00 PM). Based on the chemical composition of the various ingredients (Table 2), the proportion between corn grains and pea grains was changed for the various groups and dosages in a way that the concentrate mixes supplied at milking were isoproteic.

The mixes were prepared daily in the laboratory of the Dipartimento di Agraria, University of Sassari, Italy, and then stored in plastic bags until use. At the beginning of each milking, the

mixes were put in individual buckets placed in the milking parlour. The ewes then entered the milking parlor and were placed in one of the three main areas assigned for each group. Within each area, each ewe received individually the appropriate mix. After 20 min from the beginning of the meal, i.e. when the milking had been completed, the buckets were taken out of the parlor to be weighed and to allow sampling of the orts for subsequent analysis.

Measurements and samplings

One time in the first week of the preliminary and experimental periods and 2 days in a row in the last week of each period, the individual milk yield of all ewes was measured at each of the two daily milkings. Individual milk samples were taken at each milking. The fresh milk samples were stored in a refrigerator (4° C) for, at most, 48 h and then analyzed for standard milk composition (fat, protein, lactose, total microbial count, somatic cell count, casein and urea) in the laboratories of the Associazione Regionale Allevatori of Sardinia (Oristano, Sardinia, Italy). Fat-corrected milk (**FCM**, 6.5% fat) and fat-protein-corrected milk (FPCM, 6.5% fat and 5.8% protein) were calculated based on Pulina and Nudda (2004) as follows:

$FCM^{(6.5)} = M (0.37 + 0.097 F)$;

$FPCM^{(6.5; 5.8)} = M (0.25 + 0.085 F + 0.035 P)$

where: M = milk yield (kg); F and P = fat and protein concentration (%), respectively.

In the last days of the preliminary period, blood samples were drawn from each animal early in the morning before milking, from the jugular vein, by using a needle gauge of 18, a holder and two different tubes depending on the analysis required: complete blood count (**CBC**) and biochemical profile. For the analysis of CBC, 3 ml vacutainer tubes with purple cap, which contained EDTA to prevent the coagulation of the blood sample, were used. For the analysis of biochemical profile, 5 ml vacutainer tubes with red cap, without anticoagulant substances, were used. Once collected, the samples for CBC were shaken gently for about 10 times to mix the blood with EDTA. After that, the tubes were kept upright in a cooler (4-8°C). Within 4 hours from collection, all blood samples were delivered to the laboratory of the Istituto Zooprofilattico Sperimentale della Sardegna (Sassari, Sardinia, Italy) for analysis. Just after taking the blood samples, the body weight and BCS of the animals were also determined.

In the last experimental day of each period, rumen liquid samples were taken from half of the ewes (44 in total, 11 per group), 2 hours after the morning milking and meal, using a stomach tube. The first portion (about 30-50 ml) of rumen liquor collected was discarded to avoid or reduce saliva contamination. Soon after sampling, the pH was measured with a pH meterand the sample was then divided into two subsamples, which immediately frozen (-80 °C) until analyses. In the last day of each period, just after taking the rumen liquid samples, the body weight and BCS of the animals were also determined. In the last experimental day of each period (first, second and third), blood samples were drawn with the same procedure described above for the preliminary period from the same ewes that the rumen liquid samples were collected (44 in total, 11 for each group).

Experiment 2. *In vivo* digestibility trial

Animal selection, adaptation and experimental period

Just after the conclusion of the long term trial, part of the ewes were selected for an *in vivo* digestibility trial. The trial was carried out between the beginning and the middle of May 2012 and lasted 14 days in total. The four experimental groups of the last period (High dose of aromatic plant) of the long term trial were kept on the experimental diet for few days after the conclusion of the trial. Five ewes per group were selected to form 4 experimental groups homogeneous for milk production (mean±S.D, 1.37±0.18kg/d), milk fat (6.03±0.56%) and protein (5.16±0.21%) content, BW (46.13±4.51kg) and BCS (2.65±0.18, range from 0 to 5). The ewes were placed in individual metabolic cages for a period of 9 days of adaptation to the experimental conditions. The ewes were milked twice a day, at 8.00 AM and 3.00 PM, by a portable milking machine. The experimental period lasted 5 days, at the beginning of which the ewes were 156.3±19.2 days in milk (**DIM**, Table 20).

Measurements and feeding schedule

At the beginning andend of the experimental period, all the ewes were subjected to measurements of body weight. The animals were fed the same experimental concentrate

mixes used in the last period of the long term trial, in which the highest dosage (Table 1) of aromatic plants was used. Each morning, at the beginning of milking, the experimental concentrate mixes were put in individual buckets placed in the metabolic cages. Each ewe received individually the appropriate mix (250 g as fed of a mix), after 20 min from the beginning of the meal, i.e. when the milking had been completed, the buckets were taken out of the parlor to be weighed and to allow sampling of the orts. Thereafter, 0.5 kg/head of chopped dehydrated alfalfa were supplied, and then at the second milking time the other half of the appropriate mix (250 g as fed of a mix) was made available for 20 min. Soon after, 0.5 kg of chopped dehydrated alfalfa, 0.5 kg of beet pulps and 0.5 kg of alfalfa hay were provided to each ewe and made available until the next morning. Water was always available through an automatic drinker. During the 5 days of experimental period, group milk yield was recorded, whereas individual intake and total feces excreted were measured daily. Feed samples were collected twice for subsequent chemical analysis. The orts and feces of each ewe were weighed and mixed each day, and an aliquot (about 10%) was sampled and immediately stored at -20°C. Daily individual samples of orts and feces were pooled to have individual samples for the subsequent chemical analysis.

The coefficient of digestibility (%) was calculated as follows:

$$\text{Coefficient of digestibility (\%)} = \frac{\text{nutrient intake} - \text{nutrient in feces}}{\text{nutrient intake}} \times 100$$

Chemical analysis

Feed and feces analyses

Samples of each of the aromatic plants received from the partners of the project were taken each week during the trial. For each plant the samples were pooled, thoroughly mixed and subsampled. The final samples were then stored at room temperature (max 18 °C) until chemical analyses. Half of each sample was then ground using a 1 mm screen for subsequent chemical analysis. Feed and feces samples were analyzed for neutral detergent fiber (**NDF**), acid detergent fiber (**ADF**),and acid detergent lignin (**ADL**) (Van Soest et al., 1991), using an Ankom 220 fiber analyzer (Ankom™ technology, Fairport, NY, USA), for dry matter (**DM**) and for ether

extract (AOAC, 1990a). The extract ether content was determined by the Soxtec System HT 1043 Extraction Unit (Tecator, Foss, Amersfoort, The Netherlands). Ash and crude protein (**CP**) content were determined following the AOAC (1990b). For CP determination, mineralization was carried out with a Tecator™ Digestion Unit 8 (FOSS Slangerupgade 69, DK-3400 Hilleroed, Denmark) and a Kjeltec™ 2200 Auto Distillation unit (FOSS Analytical 69, Slangerupgade DK-3400 Hilleroed Denmark).

For all the samples that had more than 4% of ether extract, a pre-treatment with ethane was carried out before NDF analyses, to solubilize the lipids of the samples and avoid interferences, as suggested by Van Soest et al. (1991).

Milk analyses

Milk fat, protein, casein and lactose were measured with an infrared method (Milkoscan 4000, Foss Electric, Hillerød, Denmark) and milk somatic cell count was determined with a flow-citometry method (Fossomatic 5000, Foss Eletric, Hillerød, Denmark). The Milkoscan and the Fossomatic equipments were previously calibrated for sheep milk based on the calibration method of the Italian Association of Breeders (AIA, Rome, Italy). The reference methods for the calibration were the following: for milk fat, the Rose-Gottlieb method (FIL/IDF; 1D:1996); for milk protein, the Kjeldahl method (N x 6.38) (FIL/IDF-20B:1993); for lactose, the differential pH-metry; and for somatic cell count, the fluorometric method with flux cell (FIL/IDF 148 1995, method C). Milk urea was analyzed with an automatic system (Chem spec 150 based on infrared reading, Bentley Instruments, Chaska, Minnesota, USA), previously calibrated with an enzymatic-colorimetric method.

Fatty acid analyses of feeds and milk

Feed analyses. The fatty acid profile of the feed ingredients of the mixtures was performed according to Kramer et al. (1997). About 1 mg of sample was weighed into a tube whose neck was wrapped using Teflon tape. Then 1 ml of hexane and 2 ml of sodium methoxide in methanol (0.5 M) were added. Afterwards, the samples were slightly shaken in a vortex and incubated at 50°C in a water bath for 10 minutes. After 5 minutes of cooling, 3 ml of methanolic HCl were added and samples wereagitated by vortexing, and incubated at 50°C

for 10 minutes. After 7 minutes of cooling, 3 ml of hexane and 7.5 ml of 6% K_2CO_3 were added and samples were then centrifuged at 1200 rpm for 10 minutes to separate the phases. The hexane phase was used for the gas-chromatographic analyses.

Milk analyses. The analysis of milk fatty acids was performed only on the samples taken in the first (lowest aromatic plant dosage) and the last (highest aromatic plant dosage) period. Milk fat extraction was performed according to the Rose-Gottlieb method (AOAC, 1990) modified by Nudda et al. (2005). Briefly, ammonia 25% (0.4 mL), ethyl alcohol 95% (1 mL), and hexane (5 mL) were added to 1 g of sample. Samples were centrifuged at 3000 rpm and the upper layer was collected. The extraction was repeated a second time using ethyl alcohol 95% (1 mL) and hexane (5 mL); samples were centrifuged at 3000 rpm and the upper layer was collected. A third extraction was repeated using 5 mL of hexane; samples were centrifuged at 3000 rpm and the upper layer was collected. The fatty acid methyl esters (FAME) were prepared with a base-catalyzed trans-esterification according to the FIL-IDF standard procedure (1999). Briefly, approximately 25 mg of lipid extract was mixed with 0.1 mL of 2 *N* methanolic KOH and 1 mL of hexane containing the internal standard (0.5 mg/mL of C19:0), vortexed for 2 min, and then centrifuged at 3000 rpm for 1 min. After addition of 0.08 g of sodium hydrogensulfate monohydrate, the samples were centrifuged at 3000 rpm for 3 min and the supernatant was used for gas chromatography.

For feed and milk,the FAME were separated on 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) as an internal standard. The injector and flame ionization detector temperatures were 255°C. The programmed temperature was 75°C for 1 min, increased to 165°C at a rate of 8°C/min, maintained at 165°C for 35 min, increased to 210°C at a rate of 5.5°C/min, and then increased to 240°C at a rate of 15°C/min. The split ratio was 1:40 and helium was the carrier gas with a pressure of 37 psi. Individual FAME of milk and ingredients of diet mixture were identified by comparison with the relative retention time of FAME peaks from samples, with the standard mixture 37 Component FAME Mix (Supelco, Bellefonte, PA, USA). The standards PUFA-2, non-conjugated 18:2 isomer mixture, individual *cis*-5,8,11,14,17 C20:5, *cis*-4,7,10,13,16,19 C22:6 (Supelco,Bellefonte, PA, USA), *cis*-6,9,12 C18:3, and *cis*-9,12,15 C18:3 (Matreya Inc., Pleasant Gap, PA, USA) were used to identify polyunsaturated fatty

acids. High purity individual CLA c9,t11 and t10,c12 (Matreya Inc.Pleasant Gap, PA, USA) were used to identify the CLA isomers of interest. Additional standard CLA c9c11, t9t11, 11-13 (77% c,t; 2% c,c; 6% t,t) (Matreya Inc.,Pleasant Gap, PA, USA), CLA mix standard (Sigma Chemical Co.), and published isomeric profile (Kramer et al., 2004) were used to help identify the CLA isomers in ovine milk. Individual t9 C18:1, t11 C18:1, t12C18:1, t13 C18:1 (SupelcoBellefonte, PA, USA) and published isomeric profile (Griinari et al., 1998) were used to identify *trans* C18:1 isomers of interest. The content of each FAME was expressed as weight percentage of total FAME present.

Calculations of fatty acids quality indexes. The following milk fat nutritional indices were considered as predictors of the atherogenic and thrombogenic potential of the diet: n-6/n-3, atherogenic index (**AI**), thrombogenic index (**TI**) and the hypocholesterolemic to hypercholesterolemic ratio (**h/H**). The AI and TI were calculated according to Ulbricht and Southgate (1991) as follows: AI = [12:0 + (4 x 14:0) + 16:0]/[(∑PUFA) + (∑MUFA)], and TI = [14:0 + 16:0]/[(0.5 x ∑MUFA) + (0.5 x n-6) + (3 x n-3) + (n-3/n-6)]. The h/H was calculated according to Fernández et al. (2007) as follows: h/H = [(sum of 18:1 cis9, 18:1 cis11, 18:2n-6, 18:3n-6, 18:3n-3, 20:3n-6, 20:4n-6, 20:5n-3, 22:4n-6, 22:5n-3 and 22:6n-3)/(sum of 14:0 and 16:0)].

Blood analyses

Red blood cells (RBC), hemoglobin (HGB), hematocrit (Hct), mean corpuscular volume of red blood cells (MCV), mean corpuscular hemoglobin concentration (MCHC), total platelets (PLT), white blood cells (WBCB), neutrophils cells (NEUTS), lymphocytes cells (LYMPHS), monocytes cells (MONOS), eosinophils cells (EOS), and basophils cells (BASOS) were measured by using a LaserCyte Analyzer (IDEXX Laboratories, Milan, Italy). The biochemical parameters, albumin (ALB), alkaline phosphatase (ALP), total bilirubine (BT), creatinine (CRE), gamma glutamil transpeptidase (GGT), glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and total protein (PROT), were determined by a clinical analyzer spectrophotometric method (Dimension RXL Chemistry Analyzer, Dade Behring, Munich, Germany).

Rumen analyses

Rumen liquid was sampled with a stomach tube, after discarding the first 50 ml of liquid obtained from the sampling. The pH of rumen content wasmeasured, immediately after the sampling, with a pH meter (Orion Research Inc., model 250A, Boston, MA, USA) equipped with thermometer and a penetrating glass-electrode (Hamilton Company USA, model 238405, Reno, NV, USA).

Statistical analysis

Experiment 1. Long term trial

Body weight, body condition score, milk production and quality data

Body weight, body condition score, milk yield and composition data were analyzed by a completely randomized ANOVA design considering separately each period (Tables 8 and 9). An alternative statistical analysis, described below, was also carried out to take into account the effects of period (Tables 8 and 9 and Figures 1 and 2).

First step. All data ofthe control group (21 ewes) were analyzed using the PROC GLM procedure of SAS (2002; SAS Institute Inc., Cary, NC, USA). The model used was the following:

$Y_{ij} = \mu + P_i + e_{ij}$,

where Y is the dependent variable, μ is the overall mean, P is the fixed effect of period (i=4, i.e. preliminary period, experimental periods 1, 2 and 3) and e_{ij} is the residual error assumed to be normally distributed, with mean = 0 and constant variance.

Second step. For each plant within each period a "new Y" was calculated by subtracting the mean of the control group in the corresponding period, as described above, from the actual value as follows:

New Y = actual value – mean of control group in each period

where *actual value* is the observed value for each plant species and each animal within each period.

Third step. Thereafter, the "new Y" variable was used. Only data ofthe three groups of aromatic plants (Carum, Coriandrum and Satureja, 66 ewes in total, 22 per group) were used here. Data were analyzed with the PROC MIXED procedure of SAS (2002; SAS Institute Inc., Cary, NC), using the following model:

$Y_{ijkl} = \mu + D_i + P_j + (D \times P)_{ij} + A_k(i) + e_{ijkl}$,

where Y is the dependent variable, μ is the overall mean, D_i is the fixed effect of diet (i=4), P_j is the fixed effect of period=dose(j=4), $(D \times P)_{ij}$ is the interaction between diet and period=dose, $A_k(i)$ is the random effect of animal (k=66) nested within diet i, and e_{ijkl} is the residual error assumed to be normally distributed, with mean = 0 and constant variance. Means were separated by pairwise *t*-test (PDIFF option of PROC MIXED).

Intake and rumen data

Intake and rumen data were analyzed by a completely randomized ANOVA design with the PROC MIXED procedure of SAS (2002; SAS Institute Inc., Cary, NC, USA),by usingthe following model:

$Y_{ijk} = \mu + D_i + P_j + (D \times P)_{ij} + e_{ijk}$,

where Y is the dependent variable, μ is the overall mean, D is the fixed effect of diet (i=4), D is the fixed effect of period=dose(j=3), D × Pis the fixed effect of the interaction between diet and period=dose, and e_{ijk} is the residual error, assumed to be normally distributed, with mean = 0 and constant variance. Means were separated by pairwise *t*-test (PDIFF option of PROC MIXED).

Milk fatty acids and blood parameters

The data on milk fatty acids (measured only for the periods 1, lowest dosage, and 3, highest dosage) and blood parameters were analyzed following a completely randomized ANOVA design. All data were analyzed using the PROC MIXED procedure of SAS (2002; SAS Institute Inc., Cary, NC, USA). The model used was as follows:

$Y_{ij} = \mu + D_i + e_{ij}$,

where Y_{ij} is the dependent variable, μ is the overall mean, D_i is the fixed effect of diet (i=4) and e_{ij} is the residual error, assumed to be normally distributed, with mean = 0 and constant variance. Means were separated by pairwise *t*-test (PDIFF option of PROC MIXED).

Experiment 2. Digestibility trial

Nutrient intake and digestibility data were analyzed by a completely randomized ANOVA design. All digestibility data were analyzed using the PROC MIXED procedure of SAS (2002; SAS Institute Inc., Cary, N, USA). The model used was as follows:

$Y_{ij} = \mu + D_i + e_{ij}$,

where Y_{ij} is the dependent variable, μ is the overall mean, D_i is the fixed effect of diet (i=4) and e_{ij} is the residual error, assumed to be normally distributed, with mean = 0 and constant variance. Means were separated by pairwise *t*-test (PDIFF option of PROC MIXED).

For both experiments the statistical differences were declared significant at $P<0.05$. Differences between treatments at $0.05<P<0.10$ were considered as a trend towards significance.

3. Results and Discussion

Composition of the feeds

The chemical composition varied among the aromatic plants used in this study (Table 2). In particular, CP ranged from 11.6 to 26.3% of DM, in Satureja and Carum, respectively, NDF ranged from 34.1 to 75.7% of DM, in Satureja and Coriandrum, respectively, and EE ranged from 2.8 to 8.7% of DM, in Satureja and Carum, respectively (Table 2). The chemical analysis of the other ingredients (Table 2) of the diets was in line with literature values (Martillotti et al., 1996).

Fatty acid profile

The fatty acid profiles of the plants used are shown in Table 10.

Total saturated fatty acids (as percentage of total fatty acids) were much higher in Satureja (28.3% of total FA) than in Carum and Coriandrum (9.3% and 7.8% of total FA, respectively), mostly because of the very high concentration of palmitic acid (C16:0) in Satureja.

The monounsaturated fatty acids ranged from 13.5% in Satureja to 54.7% in Carum and 70.6% in Coriandrum, with oleic acid (C18:1 cis-9) being the most represented. Among the analyzed plants, the percentage of C18:1 cis-9 was considerably higher in Carum and in Coriandrum, probably because of the plants used. It is important to note, however, that such high value of oleic acid in Coriandrum 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 ranged from 21.6% in Coriandrum to 58.2% in Satureja, being represented mainly by linoleic acid (C18:2 n-6) and by acid α-linolenic (C18:3 n-3). Generally, C18:2 n-6 was higher in Carum and Coriandrum, whereas C18:3 n-3 was higher in Satureja.

Experiment 1. Long term trial

Feed intake

The intake of the basal diet supplied in the pens was almost complete for all feeds supplied (Table 4). Regarding the individual intake of the experimental mixes during the milking time, there was not interaction (P>0.05) between plant effect and time (dosage) effect. There was a significant difference due to the plant effect, with the Control group having significantly (P<0.05) higher intake (501.3 g as fed/d per head) than the Coriandrum (485.2 g as fed/d per head) and the Satureja (485.1 g as fed/d per head) groups, with the Carum group having intermediate values (492.2 g as fed/d per head; Table 5). The effect was significant but numerically very small. It was probably due to a small negative effect in terms of palatability of these plants. However, there were no negative effects of the increasing degree of inclusion of aromatic plants (dosage effect) on the intake of the mixes throughout the experiment (Table 5).

Body weight, body condition score, and milk yield and composition

Statistical analysis carried out separately for each period

Body weight and body condition score markedly increased during the trial in all groups, denoting that the energy balance of the animals was always positive (Table 6). Based on the statistical analysis carried out separately for each period, no differences associated to the plant used were observed for body weight and body condition score.

Milk yield decreased throughout the experiment, with a corresponding increase in the concentration of milk fat, protein, casein and urea and a decrease of lactose (Table 7).

Based on the statistical analysis carried out separately for each period, no differences associated with the plant used were observed for milk yield and composition, with the exception of milk urea, which was lower for Coriandrum (2nd period) and Carum (3rd period) than for Control (Table 7).

Statistical analysis based on differences from the Control group

Body weight, body condition score, milk yield and composition data were also statistically analyzed, as described in the Material and methods paragraph, by subtracting the individual values, for each plant within each period, from the mean of the control group in the corresponding period.

For BW and BCS the plant × dose interaction was significant ($P<0.05$). Thus, the results are reported as figures (Figure 1 and 2, for BW and BCS, respectively). Carum ($P<0.05$) and, to a lower extent, Satureja ($P<0.05$) had higher BW variations than the Control from the dose 0 to the Medium dose, whereas Coriandrum behaved similarly to the Control groups. At the High dose, Satureja values ($P<0.05$) declined abruptly and Coriandrum slightly increased ($P<0.05$), reaching the values of Carum($P<0.05$). BCS variations followed the same general patterns observed for BW, with Coriandrum always lower ($P<0.05$) than the Control. All treated groups showed an increase ($P<0.05$), relative to the Control, of their BCS within the first period and a decrease ($P<0.05$) in the following periods, especially for Satureja, whose BCS markedly decreased ($P<0.05$) at the highest dosage.

There were no significant ($P>0.05$) plant × dose interactions regarding milk yield and composition, thus the data are presented in separated tables for the effects of type of plant supplied (Table 8) for and of dosage (Table 9).

The type of supplied plant did not influence ($P>0.3$) any of the milk parameters studied (Table 8). Regarding milk yield, it can be noted that, even if not significant, the value of Carum (0.054, kg/d), calculated as a difference from the Control, was slightly higher than that of Coriandrum (0.024, kg/d) and Satureja (0.017, kg/d).

The same pattern was observed for fat and protein corrected milk yield (**FPCM**) and fat corrected milk yield (**FCM**), as shown in Table 8.

The dose of aromatic plants used, instead, had a significant effect (P<0.01) for some variables, such as milk yield, FPCM, FCM, lactose and urea content, whereas no differences were observed for milk fat, milk protein, and milk casein concentrations and somatic cell count (**SCC**)(Table 9). Milk yield, FPCM and FCM had the same pattern, showing that their difference from the Control at the Low dose and at the Medium dose was higher (for milk yield: 0.080 and 0.050 kg/d, respectively; P=0.007) than that at day zero and dose 0 (for milk yield: no aromatic plants, -0.008 kg/d). In contrast, the High dose did not differ from day zero.

These findings on milk yield suggest that the effect of the aromatic plants was fairly limited and could be evidenced only when the values of the treated groups were aggregated and compared to the same groups at day zero. The difference in milk yield decreased going from the lowest dose to the highest, for which the effects of plants were not significant anymore. This might depend on a dose-related effect or on the stage of lactation. As the experiment progressed, milk yield declined and the energy balance increased, with increased BW and BCS (Table 6). Thus, it is possible that the animals became less responsive to dietary changes, because they were already over fed. However, it is also possible that there was a direct effect of the dosage used, with negative effects when high doses of aromatic plants were used. This is suggested by the fact that going from dose 0 to the lowest dosage, treated groups increased their BCS in relation to the Control, whereas at higher dosages they decreased it (Figure 2).

Three studies tested the effects of the supply of EO to lactating ewes.

Two were based on the supply of EO extracts to dairy ewes: Giannenas et al. (2011) tested two dosages of the EO complex Crina, whereas Chiofalo et al. (2012) tested two dosages of EO extracts of *Rosmarinus officinalis*. In both cases, treatments affected milk yield but did not affect DMI. In the study of Giannenas et al. (2011) milk yield was not significantly affected at the lowest dosage (0.075 g/d per head of EO complex) but markedly and progressively increased above this dosage (+20% and +35% for the dosages 0.15 and 0.217 g/d per head of EO complex, respectively; Table 3). Interestingly, in this study there was also a marked reduction of somatic cell count for all dosages considered, suggesting that this was one of the causes of the increase in milk yield. In the other study, Chiofalo et al. (2012)

observed a significant increase of 10% in milk yield at the highest dosage (1.2 g/d per head of EO extracts) (Table 3). In this study there were also some effects on milk composition and BCS increased in both treated groups. Unfortunately, these studies used a EO complex or an extract derived from plant species different from those used in our study. In addition, it is impossible to compare doses of EO complexes or extracts with those of whole plant parts, as used in our study.

Lactose was higher for all three dosages of aromatic plants compared to the dosage zero (Table 9). It is hard to explain this fact, because it was impossible to separate the effect of the dosage from that of the stage of lactation. It is possible that the aromatic plants favoured the integrity of the mammary gland, thus reducing the flow of lactose to the blood, contrary to what normally occurs when this integrity is reduced by inflammations. However, no effects on SCC, which usually increase as lactose decreases, were observed. In agreement with our results, Chiofalo et al. (2012) found an increase of lactose when Valle del Belice ewes were supplied an extract of *Rosmarinus officinalis* L., possibly, as suggested by the authors, due to effects of phenolic compounds present in the aromatic plant used. In contrast, Boutoial et al. (2013) reported for dairy goats a decrease of lactose as the dose of *Rosmarinus officinalis* increased. Other studies with dairy ewes fed a EO complex (Giannenas et al., 2011) and with lactating dairy cows fed *Origanum vulgare* L. leaves (0, 250, 500 and 750 g/d) (Hristov et al., 2013) did not report changes in lactose concentration. The reason for this disagreement is unclear.

Milk urea at the Medium dose had a higher difference from the Control than that at the zero dose (experimental groups – Control: -5.889 mg/dl and -2.097 mg/dl for Medium dose and zero dose, respectively; P= 0.004). Probably, the secondary compounds of the aromatic plants had some effects on the ruminal protein degradation and metabolism, reducing the rate of degradation of protein in the rumen, due to a lower microbial activity, or favoring ammonia utilization, thus causing a reduction of urea content in milk. In agreement withour milk urea data, Hristov et al. (2013) found a decrease (P=0.04) in milk urea when lactating cows received *Origanum vulgare* L. leaves.

Fatty acids

The fatty acid (**FA**) composition of the feeds used in the ration consumed by the ewes is presented in Table 10. Saturated fatty acids (**SFA**) ranged from 7.8% in Coriandrum to 28.3% in Satureja. Among SFA, the palmitic acid (16:0) was higher in Satureja than in Carum, Coriandrum, corn and pea. Monounsaturated fatty acids (**MUFA**) ranged from 13.5% in Satureja to 70.6% in Coriandrum, where the most abundant was the oleic acid (18:1 cis-9), which was more abundant in Coriandrum and Carum than in Satureja. The values of polyunsaturated fatty acids (**PUFA**) ranged from 21.6% in Coriandrum to 58.2% in Satureja and the main FAs were linoleic acid (18:2 n-6), which was more abundant in Coriandrum and Carum, and α-linolenic acid (18:3 n-3), which was higher Satureja. All these differences among the aromatics plants used could be related to the species or to the plant parts used, or both.

The effects of the aromatic plants on milk fatty acid composition for the Low dose and the High dose tested are presented separately in Tables 11 and 12, respectively. During the trial, the concentrate mixtures (supplied at a dosage of 500 g/d per head) were almost completely eaten by the ewes of all groups (Table 5).

Low dose

Considering the various classes of milk FA (Table 11), the Low dose of aromatic plants caused a shift in only one class of milk FA, the long-chain FA (**LCFA**), whose proportions increased (P=0.004) in Carum (+25%) and in Satureja (+17%) compared to the Control group. Carum had also higher LCFA than Coriandrum.

Regarding the individual milk FA (Table 11), the supply of aromatic plants caused a reduction (P<0.05) of the saturated FA 10:0, 12:0, 14:0 (neosynthesis), and an increase of the preformed 18:0 (Table 11). Nudda et al. (2013), in a study on goats, also showed a decreased in the same FA and suggested a benefit in milk FA profile in terms of human health, because, according to Tholstrup et al. (2003), these FA play an important positive role in the formation of blood cholesterol.

The atherogenic index (**AI**) is generally used as a measure of dietary fat quality. According to Addis et al. (2005), fat with a high AI value is harmful to human health. In this sense, our results showed a reduction (P<0.05) of about -18% of this index in Carum and Satureja

groups compared with the Control group (Table 11). This pattern is similar to that observed by Addis et al. (2005) with sheep supplied with *Chrysanthemum coronarium* and by Nudda et al. (2013) with goats fed extruded linseed.

High dose

The effects of aromatic plants on FA where much stronger at the High dose than at the Low dose, with changes being observed for almost all individual and classes of milk FA (Table 12). Short-chain fatty acids, total PUFA, PUFA n-3 and total conjugated linoleic acid (**CLA**) did not differ (P>0.061) among treatments, whereas variations were observed for all other FA classes.

MCFA decreased (P=0.001) by 12%, and SFA decreased (P=0.001) by 7% in both Carum and Coriandrum groups compared to Control and Satureja groups. In contrast, LCFA increased (P<0.0001) by 46%, MUFA increased (P<0.0001) by 42%, and TFA increased (P=0.001) by 5% in Carum and Coriandrum groups compared to Control and Satureja groups.

The concentration of odd- and branched-chain fatty acids (**OBCFA**), was higher (P=0.003) in Satureja than in all other groups, whereas Coriandrum had the lowest value, even if not statistically different from that of Carum. Diedrich and Henschel (1990) declared that OBCFA occur at trace levels in most plants, whereas Kaneda (1991) affirms that OBCFA are mainly present in bacterial membrane lipids. In addition, Keeney et al. (1962) proposed that OBCFA in ruminant milk comes mainly from rumen bacteria. In this sense, according to Vlaeminck et al. (2006), our results indicate a shift in ruminal metabolism with the addition of Satureja (stimulatory effect) and Coriandrum (inhibitory effect) to sheep diets.

At the higest dosage, the AI and trombogenic index were markedly lower (-33% and -25% respectively; P<0.001) in Carum and Coriandrum groups than in the Control and Satureja groups. At the same time, the hypocholesterolemic/hypercholesterolemic index increased (P=0.001) by 50% in Carum and Coriandrum groups compared to Control and Satureja. These parameters indicate that sheep milk enriched with Carum and Coriandrum might be used as human health promoter. In general, the Carum and Coriandrum groups had the same pattern, probably because similar plant parts were used and due toa similar fatty acid composition of their oil (Table 10).

Hemogram and blood serum biochemistry

Blood data regarding only the preliminary and the third period are presented, due to technical problems. The ewes assigned to the 4 experimental groups did not differ for the values of their hemogram parameters (Table 13), total and differential white blood cells (Table 14) and biochemical blood parameters (Table 15) in the preliminary period. All the 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. Only red blood cells, hemoglobin and hematocrit were slightly lower than the reference values, indicating a possible, even though limited, iron deficiency. However, this might be a characteristic of the Sarda breed.

During the third experimental period, the values of all hemogram parameters were within the reference ranges and were not different among treatments (P>0.25 for all treatments, except for P=0.039 for MCHC) (Table 16). Total and differential white blood cells were not significantly affected by the plants either, even if the monocytes values tended (P=0.114) to differ among treatments, having lower values in Coriandrum and Satureja (0.23 x 10^3 and 0.17 x 10^3 cells/µL, respectively) than in Carum and control (0.28 x 10^3 and 0.26 x 10^3 cells/µL, respectively) (Table 17). Similarly, the blood biochemical parameters (Table 18) were all within the normal reference values and did not differ significantly among treatments (P>0.25), except for urea which tended (P=0.130) to be affected by treatments, ranging from 52.00 mg/dl in Carum to 58.55 mg/dl in Coriandrum. In summary, during the third experimental period, in the groups treated with aromatic plants almost all values of hematogram parameters, total and differential white cells and blood serum biochemistry were not significantly (P>0.25) different from the control, suggesting that the aromatic plants tested did not exert any negative effects on the health of the ewes.

Ruminal pH

Rumen pH was influenced by type of aromatic plant (P=0.004) and dose level (P=0.001), but not by the interaction plant × dose (P>0.25) (Table 19).

The pH of rumen fluid was significantly higher for Coriandrum than for the Control (6.95 vs. 6.80; Table 19). This was probably due to differences in the proportion of ground corn and pea grains in the two groups or to a direct effect of that aromatic plant species on rumen

fermentation, considering that the ruminal pH for Carum and Satureja was intermediate (6.86 and 6.87, respectively; Table 19) and not statistically different from the other two treatments. The values were for all cases above the minimum values (pH >6.2) for ruminants fed diets with sufficiently high fiber content. The Low dose of aromatic plant used had the lowest ruminal pH value (6.80) compared to the other two doses tested (6.93 and 6.90 for the Medium and High doses, respectively). Such differences can be explained by the higher content of corn grains and pea grains used in the first experimental period.

Experiment 2. In vivo digestibility trial

To our knowledge, in literature there are no other studies on *in vivo* digestibility measurements of diets containing aromatic plants fed to lactating dairy ewes.

Chemical composition of the diet

The diets used were the same described for the long term feeding trial (Experiment 1), except that only the Control and the High dose diets of the three aromatic plants, Carum, Coriandrum and Satureja, were used. The quantity of feed provided was the same for all groups and, even though the protein content differed among the aromatic plants, the mixtures were balanced to be isoproteic (18% of DM).

Intake and digestibility of the diets containing aromatic plants

The animals quickly adapted to the metabolic cages, with all ewes showing a normal feeding behavior and dietary intake.

Body weight, body condition score and milk production did not differ among groups during the digestibility trial (Table 20).

No differences (P>0.104) among treatments were observed for DM, OM and CP intake (Table 21). The main essential oils of Carum (carvone, according to Laribi et al., 2013), Coriandrum (linalool, according to Eikani et al., 2007) and Satureja (carvacrol and thymol, according to Damjanović-Vratinica et al., 2011) did not influence DM intake (DMI) in our trial. In contrast, Hristov et al. (2013) studied the effect of *Origanum vulgare* leaves on lactating cows and suggested that the carvacrol may negatively influence the DMI of the animals.

In contrast to DM, OM and CP intake, NDF, ADF, ADL, NFC and EE intake were significantly affected by the aromatic plants added to the diets of the lactating ewes. In particular, NDF intake was increased (by about 27%, P=0.013) by Coriandrum compared with the Carum and Control group. ADF and ADL intake had a similar pattern: both increased in Coriandrum and Satureja (by about 35% and 66%, respectively, P=0.001) compared to the Control and Carum group. The EE intake increased (by about 26%, P=0.013) in Carum and Coriandrum compared to the Control group. These differences were mostly a result of the direct effect of the composition of the aromatic plants supplied on the composition of the resulting concentrate mixes.

In general, the addition of the High dose of aromatic plants on the diets of Sarda ewes influenced the digestibility of all parameters (Table 22).

The digestibility of DM increased (by 5.6%, P=0.051) inthe Satureja group compared with the Coriandrum group, with Control and Carum having intermediate values. Satureja had a significantly higher (P=0.012) OM digestibility (from 3 to 4 percentage points) than the other groups, which did not differ among them. In contrast, Anassori et al. (2011) showed a decrease in OM digestibility in Iranian Makoui rams fed a diet supplemented with *Allium sativum*, whereas Hristov et al. (2013) did not observe any variation in OM digestibility in lactating cows fed *Origanum vulgare* leaves.

The digestibility of NDF was markedly higher (by 22%, P=0.001) in the three treated groups, which did not differ among them, than in the Control group. Similar results were obtained for ADF digestibility. These important results are confirmed by the results of Anassori et al. (2011), who also foundan increasein NDF digestibility when the diet of ram Makoui was supplemented with fresh garlic at two different dosages (75 and 100 g/kg of DM). However, those authors affirmed that the variations could be due to the experimental protocols or to variations in the forage to concentrate ratio of the diet. According to Cannas et al. (2013), it seems that the most common effect of EO addition is connected witha decrease in NDF digestibility, although there are studies that go in the opposite direction. The mechanisms at the basis of these contrasting results have not yet been clarified. It is possible to hypothesize that in some cases EO could have negative effects on the digestion of fiber similar to those caused by the addition of fat, because both EO and fat are insoluble in water and rich in unsaturated bonds and may interfere with microbial activity.

In other cases, EO might favour NDF digestibility, with a mechanism that needs to be further studied, especially considering the results of our digestibility trial.

Interestingly, the rumen pH of the treated groups was numerically (Carum and Satureja) or significantly (Coriandrum) higher than that of Control in the long term experiment (Table 19), and the digestibility of NFC was significantly lower (by 3.7%, P=0.001) in Carum and Coriandrum than in the other diets. Thus, it appears that the markedly higher digestibility of NDF and ADF in the treated groups than in the Control group could be due to a stimulation of fibrolytic bacteria associated with conditions not favorable, at least for Carum and Coriandrum diets, for amylolytic activity. It should be noted that the concentration of NDF in the ration actually ingested (Table 21) was much lower than planned, due to the selection of feed made by the animals. This could have favored conditions of low rumen pH. In addition, based on the results on the digestibility of the fiber, it can be inferred that there was an increase in the anaerobic fungi, such as the *Neocallimastix* sp. (Krause et., 2003), or an increase of rumen protozoa, which may have an important role in the digestibility of the fiber (Devillard et al., 2003). It is not plausible that the different fiber digestibility observed between the treatments depends on the level of feeding of the fiber, because in reality the treated rations had a higher NDF intake than the Control, and thus presumably higher fiber rumen passage rate, and higher ADL to NDF ratio, which should cause a reduction of NDF digestibility and not an increase, as it actually occurred (Figure 3).

In general, EE digestibility was very low in all groups (Table 22), with Satureja having 44.1%a lower digestibility (P=0.014) than that of the other groups. This plant species had a much lower EE concentration than the other aromatic plants (Table 2), even though the whole Satureja diet differed little in terms of EE concentration compared to the others (Table 21). Interestingly, Satureja had higher (by 4.3%, P=0.020) CP digestibility than the other three treatments.

Overall, the Satureja group had the highest numerical or statistical digestibility values for all nutrients considered, except for EE, for which it had the lowest values. These results suggest that this plant ensured the best ruminal conditions among the four groups studied.

4. Conclusions

The research carried out to compare diets which included 3 different doses of the aromatic plants *Carum* sp., *Coriandrum* sp., and *Satureja* sp. showed that:

- in this study the aromatic plants used did not influence the feed intake of the basal diet in the pens. The aromatic plants only slightly reduced the feed intake of the concentrate mixes in which they were included. This effect was observed numerically, but not statistically, at the highest level of inclusion of the plants in the mixes;
- milk yield did not differ among aromatic plant species, even ifthe Carum group showed numerically higher milk yields than the other groups;
- milk yield was affected by the effect of dose. In particular, the difference between treated groups and the Control group was significant and the highest at the Low dose of aromatic plant integration, intermediate for the Medium dose and not significant for the High dose of aromatic plant;
- milk lactose was higher for all three dosages of aromatic plants than for dosage zero;
- milk urea was in general higher in the control dose than in the treated groups, suggesting a certain effect of aromatic plants in reducing the degradation rate of proteins;
- no negative effects of the aromatic plants on the health status of the ewes were observed even at the highest level of inclusion in the diets;
-the low dose of aromatic plant supply moderately modified milk fatty acids, increasing the long chain fatty acids and reducing the atherogenic index of about -18% in Carum and Satureja groups compared with the Control group;
- the high dose of aromatic plant supply markedly modified milk fatty acids, with a decrease inmedium chain, odd chain and trans fatty acids, and an increase in long chain increasing the long chain, monounsaturated and polyunsaturated fatty acids in Carum and Coriandrum compared to the Control and Satureja groups. In addition, Carum and Coriandrum reduced the atherogenic and the trombogenic indexes and increased the hypocholesterolemic/hypercholesterolemic index compared to the Control and Satureja groups;
- there were no adverse effects of the utilization of aromatic plants on DM digestibility, whereas OM digestibility was the highest for the Satureja group;

- NDF digestibility was markedly higher (by 22%) in the three treated groups, despite the high level of lignification of the aromatic plants, compared to the Control group;
- the Satureja group had the highest numerical or statistical digestibility values for all nutrients considered, with the exception of EE, for which it had the lowest values. These results suggest that, among the four groups studied, Satureja ensured the best ruminal conditions.

These results suggest that the aromatic plants tested can be safely used in the diet of lactating sheep and that they can have positive effects on milk yield, milk fatty acid profile and fiber digestibility.

5. Acknowledgments

I thank gratefully Dr. Giovanni Molle and Dr. Mauro Decandia, researchers of the Department of Animal Production of Agris, the technicians Mr. Stefano Picconi and Mr. Emiliano Deligios, and the laboratory staff of the same Department for their contribution to the experiments described in this chapter.

I am also grateful to the members of the Sezione di Scienze Zootecniche of the Dipartimento di Agraria of the University of Sassari, in particular to Dr. Ana Helena Dias Francesconi for reviewing and editing the chapter and to Mr. Gesumino Spanu, Mr. Antonio Fenu, Mr. Antonio Mazza and Mr. Roberto Rubattu for their technical assistance.

I also would like to acknowledge Dr. Paola Nicolussi, Dr. Piero Bonelli and the staff of the Istituto Zooprofilattico Sperimentale della Sardegna (Sassari, Sardinia) for the contribution in the blood analyses.

I am also grateful to Dr. Maria Grazia Manca for the help and the suggestions in the conduction of the milk fatty analyses and, last but not least, I would like to thank Dr. Normand St-Pierre for invaluable suggestions regarding the statistical analyses.

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Table 1. Scheme of the sequence of treatments applied during each period.

Treatment	Ewes	Preliminary (21 d)	Period 1 (21 d)	Period 2 (21 d)	Period 3 (21 d)
Control	21	0	0	0	0
Carum sp.	22	0	Low dose	Medium dose	High dose
Coriandrum sp.	22	0	Low dose	Medium dose	High dose
Satureja sp.	22	0	Low dose	Medium dose	High dose

Table 2. Chemical compositionof the feeds used in the experiment.

Feed	Chemical composition				
	DM % as fed	Ash % DM	CP % DM	EE % DM	NDF % DM
Dehydrated alfalfa	85.16	10.04	23.70	2.1	31.99
Alfalfa hay	-	-	-	-	-
Beet pulps	86.13	4.96	11.70	-	50.69
Corn grains	88.63	1.30	9.28	3.70	15.86
Pea grains	87.01	3.02	24.25	0.69	31.28
Carum sp.	87.49	8.14	26.25	8.67	53.88
Coriandrum sp.	89.15	6.35	16.19	8.16	75.72
Satureja sp.	87.43	9.11	11.57	2.75	34.14

Table 3. Age and days in milk (DIM) of the ewes as a mean of the experimental period.

Variable	Treatment			
	Control	Carum	Coriandrum	Satureja
Age (year), min	2	2	2	2
Age (year), mean	2.7	3.0	3.0	3.1
Age (year), max	5	5	5	5
DIM, min	127	121	117	125
DIM, mean	150	141	146	143
DIM, max	187	157	187	185

Table 4. Intake (kg/d as fed per sheep) of the basal diet of the ewes while kept in the group pens.

Feed	Treatment			
	Control	Carum	Coriandrum	Satureja
Beet pulp	0.50	0.50	0.50	0.50
Alfalfa hay	0.48	0.48	0.47	0.48
Dehydrated alfalfa	1.03	1.03	1.03	1.03

Table 5. Mean Intake (g/d as fed per sheep) of the concentrate mixes fed during the milking time.

Variable	Treatment				SED[1]	P value
	Control	Carum	Coriandrum	Satureja		
Daily intake, g/d	501.3 [a]	492.2 [ab]	485.2 [b]	485.1 [b]	5.234	0.006
	Dose (g DM)					
	0	Low	Medium	High		
Daily intake, g/d	-	493.1	493.0	486.7	4.533	ns

[1] SED = standard error of the difference, ns = not significant, P>0.25, [a, b] in the same row = P<0.05.

Table 6. Effect of aromatic plant species supplied to Sarda dairy ewes on their body weight and body condition score.

Item		Treatment					
	Period*	Control	Carum	Coriandrum	Satureja	SEM[1]	P value
Live weight, kg	0	42.3	43.8	42.3	43.2	21.74	ns
	1	45.8	47.9	45.3	46.3	21.39	ns
	2	46.3	47.5	46.2	47.0	22.80	ns
	3	48.0	48.4	48.4	47.2	25.63	ns
Body condition score	0	2.4	2.4	2.3	2.3	0.04	ns
	1	2.5	2.5	2.4	2.5	0.03	ns
	2	2.6	2.6	2.5	2.6	0.04	0.075
	3	2.8	2.7	2.7	2.7	0.05	ns

[1] standard error of the means; ns = not significant, P>0.1. *0 = no aromatic plant; 1 = Low dose of aromatic plant; 2 = Medium dose of aromatic plant; 3 = High dose of aromatic plant.

Table 7. Effect of aromatic plant species supplied to Sarda dairy ewes on their milk yield and composition.

Item	Period*	Treatment				SEM[1]	P value
		Control	Carum	Coriandrum	Satureja		
Milk yield, kg/day	0	1.83	1.80	1.82	1.84	0.08	ns
	1	1.54	1.65	1.62	1.63	0.12	ns
	2	1.56	1.53	1.53	1.54	0.12	ns
	3	1.31	1.32	1.29	1.22	0.11	ns
Fat content, %	0	5.0	4.8	5.1	5.0	0.414	ns
	1	5.5	5.5	5.6	5.5	0.297	ns
	2	5.8	5.8	5.8	5.9	0.426	ns
	3	6.2	6.0	6.3	6.1	0.387	ns
Protein content, %	0	5.1	5.1	5.0	5.0	0.133	ns
	1	5.1	5.1	5.0	5.0	0.140	ns
	2	5.3	5.2	5.2	5.3	0.189	ns
	3	5.4	5.1	5.1	5.3	0.160	0.074
Lactose content, %	0	4.9	4.9	4.9	4.9	0.033	ns
	1	4.8	4.9	4.8	4.9	0.040	ns
	2	4.7	4.8	4.8	4.8	0.043	ns
	3	4.7	4.7	4.8	4.7	0.049	ns
Casein content, %	0	3.9	3.9	3.9	3.9	0.090	ns
	1	4.0	4.0	3.9	3.9	0.098	ns
	2	4.1	4.0	4.0	4.1	0.112	ns
	3	4.2	4.0	4.0	4.1	0.110	0.072
Urea, mg/dl	0	48.6	44.6	46.7	48.3	76.91	ns
	1	54.1	49.4	53.3	53.4	68.33	ns
	2	54.1 [a]	48.9 [ab]	47.6 [b]	48.6 [ab]	54.41	0.025
	3	57.2 [a]	48.8 [b]	54.3 [ab]	52.3 [ab]	56.15	0.004

[1] standard error of the means; ns = not significant, P>0.1. *0 = no aromatic plant; 1 = Low dose of aromatic plant; 2 = Medium dose of aromatic plant; 3 = High dose of aromatic plant.

Table 8. Effect of aromatic plant species supplied to Sarda dairy ewes on their milk yield and composition. The values were calculated, for each animal, as the difference between the actual values of the animal in each experimental period and the mean of the Control group for that period.

Variable	Aromatic Plant				
	Carum	Coriandrum	Satureja	SED[1]	P value
Milk					
Yield, kg/d	0.054	0.024	0.017	0.081	ns
FPCM[2], kg/d	0.042	0.020	0.012	0.073	ns
FCM[3], kg/d	0.044	0.027	0.014	0.075	ns
Fat, %	-0.057	0.025	-0.031	0.121	ns
Protein, %	-0.054	-0.129	-0.048	0.083	ns
Lactose, %	0.048	0.040	0.043	0.042	ns
Casein, %	-0.048	-0.108	-0.043	0.068	ns
Urea, mg/dl	-5.466	-3.096	-3.498	1.692	ns
SCC[4], log_{10} cell/ml*	0.280	0.179	0.152	0.131	ns

[1] Standard error of the difference; [2 (6.5; 5.8)]Fat-Protein-corrected milk and [3 (6.5)]Fat-corrected milk according by Pulina and Nudda (2004); [4] Somatic cell count *In thousands; The statistical analysis was carried out on log_{10} transformed values, ns = not significant, P>0.332.

Table 9. Effect of four dosages of aromatic plants supplied to Sarda dairy ewes on their milk yield and composition. The values were calculated, for each animal, as the difference between the actual values of the animal in each experimental period and the mean of the Control group for that period.

Variable	Dosage (g/head/d)					
	0	Low	Medium	High	SED[1]	P value
Milk						
Yield, kg/d	-0.008 [c]	0.080 [a]	0.050 [ab]	0.005 [bc]	0.028	0.007
FPCM[2], kg/d	-0.002 [c]	0.069 [a]	0.047 [ab]	0.009 [bc]	0.027	0.003
FCM[3], kg/d	-0.029 [c]	0.075 [a]	0.050 [ab]	0.016 [bc]	0.027	0.001
Fat, %	-0.075	-0.018	0.021	-0.012	0.092	ns
Protein, %	-0.044	-0.066	-0.056	-0.141	0.051	ns
Lactose, %	-0.015 [b]	0.068 [a]	0.070 [a]	0.052 [a]	0.026	0.004
Casein, %	-0.051	-0.061	-0.046	-0.109	0.042	ns
Urea, mg/dl	-2.097 [a]	-3.328 [ab]	-5.889 [c]	-4.768 [bc]	1.100	0.004
SCC, log_{10} cell/ml*	0.253	0.153	0.213	0.195	0.083	ns

[1] Standard error of the difference; [2 (6.5; 5.8)]Fat-Protein-corrected milk and [3 (6.5)]Fat-corrected milk according by Pulina and Nudda (2004); *In thousands; the statistical analysis was carried out on log_{10} transformed values, [a,b,c] in the same row = P<0.05, ns = not significant, P>0.215.

Table 10. Fatty acid composition of the feeds consumed by the ewes.

Fatty acids, g/100 g of FAME	Ingredients				
	Corn	Pea	*Carum* sp.	*Coriandrum* sp.	*Satureja* sp.
C14:0	0.05	0.11	0.10	0.09	0.26
C16:0	13.69	12.32	5.71	4.82	20.05
C18:0	2.40	4.15	1.80	0.86	2.66
C18:1 cis-9	35.06	24.83	41.32	69.48	8.20
C18:2 n-6	45.82	49.90	35.07	20.47	13.76
C18:3 n-3	1.26	7.13	0.66	0.37	43.13
SFA[1]	17.51	17.67	9.32	7.82	28.28
MUFA[2]	35.38	25.20	54.66	70.61	13.54
PUFA[3]	47.11	57.13	36.02	21.58	58.18

FAME = fatty acid methyl esthers, [1] Saturated fatty acid, [2] Monounsaturated fatty acid, [3] Polyunsaturated fatty acid.

Table 11. Fatty acid (FA) composition of milk from Sarda ewes fed a low dose of aromatic plants.

FA, g/100 g of FAME[2]	Treatment[1]					
	Control	Carum	Coriandrum	Satureja	SED[3]	P value
SFA						
4:0	3.52	3.45	3.62	3.34	0.242	ns
6:0	2.67	2.54	2.66	2.38	0.186	ns
8:0	2.64	2.38	2.49	2.20	0.196	ns
10:0	10.64 [a]	8.98 [b]	9.50 [ab]	8.58 [b]	0.699	0.049
12:0	6.84 [a]	5.36 [b]	5.73 [b]	5.22 [b]	0.433	0.007
14:0	14.97 [a]	13.20 [b]	13.65 [b]	13.52 [b]	0.604	0.046
16:0	31.78	32.40	33.31	34.33	1.114	ns
18:0	3.89 [c]	6.00 [a]	5.00 [ab]	4.60 [bc]	0.516	0.007
OBCFA						
iso 13:0	0.05	0.04	0.04	0.04	0.007	ns
anteiso 13:0	0.01	0.01	0.01	0.02	0.006	ns
iso 14:0	0.16	0.18	0.14	0.18	0.024	ns
iso 15:0	0.24	0.29	0.24	0.27	0.027	ns
anteiso 15:0	0.63 [a]	0.57 [bc]	0.55 [c]	0.59 [abc]	0.026	0.044
iso 16:0	0.33	0.35	0.30	0.34	0.033	ns
iso 17:0	0.39	0.42	0.35	0.39	0.036	ns
anteiso 17:0	0.48	0.51	0.46	0.48	0.035	ns
MUFA						
cis-9 14:1	0.28	0.20	0.22	0.25	0.052	ns
cis-9 16:1	0.96	0.81	0.87	1.00	0.154	ns
cis-9 17:1	0.26	0.22	0.24	0.26	0.024	ns
cis-9 18:1	9.44	11.68	10.63	10.88	0.886	ns
PUFA						
18:2 n-6	2.01	2.21	1.81	2.07	0.223	ns
18:3 n-3	0.84	0.84	0.75	0.90	0.111	ns
cis-9, trans-11 CLA	0.67	0.58	0.57	0.73	0.101	ns
20:4 n-6	0.11	0.11	0.09	0.11	0.016	ns
20:5 n-3 (EPA)	0.06	0.07	0.06	0.07	0.007	ns
22:5 n-3 (DPA)	0.08 [ab]	0.10 [a]	0.07 [b]	0.10 [a]	0.012	0.049
22:6 n-3 (DHA)	0.02	0.03	0.03	0.02	0.008	ns
TFA						
trans-4 18:1	0.01 [b]	0.01 [b]	0.02 [b]	0.08 [a]	0.020	0.005
trans-6-8 18:1	0.06	0.06	0.11	0.15	0.038	ns
trans-9 18:1	0.08	0.06	0.08	0.15	0.032	0.075
trans-10 18:1	0.18	0.23	0.19	0.26	0.055	ns
trans-11 18:1	1.04	1.32	1.42	1.26	0.255	ns

Table 11. Continued

Summary						
∑ SCFA	19.88	17.68	18.64	16.83	1.188	ns
∑ MCFA	59.92	57.13	58.80	59.62	1.284	ns
∑ LCFA	20.21 [c]	25.20 [a]	22.57 [bc]	23.55 [ab]	1.155	0.004
∑ OBCFA	4.43	4.60	4.31	4.58	0.169	ns
∑ SFA	81.54	79.02	80.45	79.01	1.073	0.086
∑ MUFA	13.96	16.15	15.39	15.98	0.990	ns
∑ PUFA	4.50	4.83	4.16	5.02	0.423	ns
PUFA n-3	1.02	1.07	0.95	1.15	0.121	ns
PUFA n-6	2.26	2.52	2.05	2.40	0.236	ns
∑ CLA	0.92	0.90	0.87	1.08	0.109	ns
∑ TFA	1.76	2.06	2.20	2.36	0.385	ns
Ratio or index						
AI	5.38 [a]	4.34 [b]	4.82 [ab]	4.51 [b]	0.345	0.040
TI	3.50	3.06	3.43	3.27	0.191	ns
h/H	0.27	0.33	0.29	0.30	0.022	0.074
n-6/n-3	2.23	2.39	2.17	2.10	0.147	ns

[1] Control, basal diet without aromatic plant,Carum, basal diet plus *Carum* sp., Coriandrum, basal diet plus *Coriandrum* sp. and Satureja, basal diet plus *Satureja* sp., FAME = Fatty acid methyl esters, SFA = satury fatty acids, OBCFA = odd and branched chain fatty acids, MUFA = monounsaturated fatty acids, SCFA = short chain fatty acids, MCFA = medium chain fatty acids, LCFA = long chain fatty acids, PUFA = polyunsaturated fatty acids, CLA = conjugated linoleic acid, EPA = eicosapentaenoic acid, DPA = docosapentaenoic acid, DHA = docosahexaenoic acid, TFA = trans fatty acids, AI = atherogenic index, TI = thrombogenic index, h/H = hypocholesterolemic/hypercholesterolemic ratio, n-6/n-3, [2] SFA (sum of 4:0, 6:0, 8:0, 10:0, 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 22:0, 24:0 and odd-branched fatty acids), OBCFA (sum of 11:0, iso13:0, anteiso13:0, 13:0, iso14:0, iso15:0, anteiso15:0, 15:0, iso16:0, iso17:0, anteiso17:0, 17:0), MUFA (sum of 10:1, 14:1c9, 15:1c10, ∑16:1, ∑17:1, ∑18:1, ∑22:1, 24:1c15), SCFA (from 4:0 to 10:1), MCFA (from 11:0 to 17:0), LCFA (from 18:0 to DHA), PUFA (sum of ∑18:2, 18:3, 18:4, ∑CLA, ∑20:2, 20:3, 20:4, 22:2, 22:4, EPA, DPA, DHA), ∑CLA (sum of isomers of CLA), ∑TFA (sum of 16:1t6-7, 16:1t8, 16:t9, 16:1t10, 18:1t4, 18:1t6-8, 18:1t9, 18:1t10, 18:1t11, 18:2t9, 18:2t12, 18:2t8c13, 18:2c9t2), [3] SED, standard error of the difference, [a,b,c] in the same row = P<0.05, ns = not significant, P>0.074.

Table 12. Fatty acid (FA) composition of milk from Sarda ewes fed a high dose of aromatic plants.

FA, g/100 g of FAME[2]	Treatment[1]					
	Control	Carum	Coriandrum	Satureja	SED[3]	P value
SFA						
4:0	3.04 [b]	3.30 [ab]	3.36 [ab]	3.59 [a]	0.182	0.057
6:0	2.32	2.27	2.23	2.44	0.135	ns
8:0	2.27	1.99	1.94	2.18	0.162	ns
10:0	9.03 [a]	7.12 [bc]	6.94 [c]	8.27 [ab]	0.628	0.012
12:0	6.06 [a]	4.26 [b]	4.11 [b]	5.29 [a]	0.391	0.001
14:0	15.47 [a]	12.90 [b]	12.85 [b]	14.58 [a]	0.749	0.006
16:0	34.33[ab]	32.41 [bc]	31.90 [c]	35.74 [a]	1.097	0.010
18:0	4.05 [b]	6.92 [a]	7.99 [a]	4.21 [b]	0.645	<.0001
OBCFA						
iso 13:0	0.06 [a]	0.03 [b]	0.03 [b]	0.05 [ab]	0.001	0.013
anteiso 13:0	0.02	0.01	0.01	0.02	0.003	ns
iso 14:0	0.16 [ab]	0.12 [b]	0.12 [b]	0.20 [a]	0.030	0.045
iso 15:0	0.27	0.25	0.23	0.28	0.024	ns
anteiso 15:0	0.53 [ab]	0.49 [bc]	0.44 [c]	0.60 [a]	0.038	0.006
iso 16:0	0.31 [abc]	0.29 [bc]	0.26 [c]	0.37 [a]	0.033	0.022
iso 17:0	0.38	0.36	0.35	0.37	0.023	ns
anteiso 17:0	0.49 [a]	0.44 [ab]	0.40 [b]	0.48 [a]	0.027	0.020
MUFA						
cis-9 14:1	0.37	0.26	0.27	0.33	0.062	ns
cis-9 16:1	1.23	1.05	0.96	1.19	0.199	ns
cis-9 17:1	0.26 [ab]	0.22 [bc]	0.20 [c]	0.28 [a]	0.021	0.008
cis-9 18:1	9.72 [b]	13.72 [a]	14.68 [a]	10.04 [b]	0.923	<.0001
PUFA						
18:2 n-6	1.96 [abc]	2.08 [a]	1.65 [c]	1.70 [bc]	0.150	0.029
18:3 n-3	0.86	0.76	0.68	0.86	0.093	ns
cis-9, trans-11 CLA	0.60	0.66	0.50	0.51	0.075	ns
20:4 n-6	0.12	0.13	0.11	0.11	0.009	ns
20:5 n-3 (EPA)	0.07 [b]	0.06 [b]	0.06 [b]	0.09 [a]	0.008	0.015
22:5 n-3 (DPA)	0.08	0.08	0.08	0.09	0.009	ns
22:6 n-3 (DHA)	0.02	0.02	0.03	0.02	0.005	ns
TFA						
trans-4 18:1	0.01	0.06	0.05	0.01	0.025	0.086
trans-6-8 18:1	0.06 [b]	0.26 [a]	0.29 [a]	0.06 [b]	0.065	0.002
trans-9 18:1	0.07 [b]	0.20 [a]	0.14 [ab]	0.07 [b]	0.039	0.010
trans-10 18:1	0.16 [b]	0.34 [a]	0.02 [b]	0.12 [b]	0.054	0.005
trans-11 18:1	0.87 [c]	1.62 [b]	2.19 [a]	0.79 [c]	0.213	<.0001

Table 12. Continued

Summary						
∑ SCFA	17.06	14.97	14.79	16.87	0.996	0.065
∑ MCFA	62.24 [a]	55.43 [b]	54.31 [b]	62.43 [a]	1.709	0.001
∑ LCFA	20.70 [b]	29.60 [a]	30.85 [a]	20.70 [b]	1.872	<.0001
∑ OBCFA	4.18 [b]	3.91 [bc]	3.62 [c]	4.68 [a]	0.234	0.003
∑ SFA	80.95 [a]	75.27 [b]	75.11[b]	81.23 [a]	1.382	0.001
∑ MUFA	14.46 [b]	19.93 [a]	20.99 [a]	14.43 [b]	1.272	<.0001
∑ PUFA	4.59	4.80	3.91	4.34	0.314	0.061
PUFA n-3	1.08	0.96	0.88	1.12	0.105	ns
PUFA n-6	2.26 [abc]	2.40 [a]	1.92 [c]	2.00 [bc]	0.162	0.032
∑ CLA	0.88	1.01	0.81	0.79	0.093	ns
∑ TFA	1.58 [b]	2.99 [a]	3.32 [a]	1.49 [b]	0.367	0.001
Ratio or index						
AI	5.40 [a]	3.61 [b]	3.58 [b]	5.31 [a]	0.368	<.0001
TI	3.64 [a]	2.76 [b]	2.78 [b]	3.77 [a]	0.218	0.001
h/H	0.26 [b]	0.38 [a]	0.39 [a]	0.26 [b]	0.030	0.001
n-6/n-3	2.13 [bc]	2.54 [a]	2.21 [ab]	1.79 [c]	0.184	0.008

[1] Control, basal diet without aromatic plant, Carum, basal diet plus *Carum* sp., Coriandrum, basal diet plus *Coriandrum* sp. and Satureja, basal diet plus *Satureja* sp., FAME = Fatty acid methyl esters, SFA = satury fatty acids, OBCFA = odd and branched chain fatty acids, MUFA = monounsaturated fatty acids, SCFA = short chain fatty acids, MCFA = medium chain fatty acids, LCFA = long chain fatty acids, PUFA = polyunsaturated fatty acids, CLA = conjugated linoleic acid, EPA = eicosapentaenoic acid, DPA = docosapentaenoic acid, DHA = docosahexaenoic acid, TFA = trans fatty acids, AI = atherogenic index, TI = thrombogenic index, h/H = hypocholesterolemic/hypercholesterolemic ratio, n-6/n-3, [2] SFA (sum of 4:0, 6:0, 8:0, 10:0, 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 22:0, 24:0 and odd-branched fatty acids), OBCFA (sum of 11:0, iso13:0, anteiso13:0, 13:0, iso14:0, iso15:0, anteiso15:0, 15:0, iso16:0, iso17:0, anteiso17:0, 17:0), MUFA (sum of 10:1, 14:1c9, 15:1c10, ∑16:1, ∑17:1, ∑18:1, ∑22:1, 24:1c15), SCFA (from 4:0 to 10:1), MCFA (from 11:0 to 17:0), LCFA (from 18:0 to DHA), PUFA (sum of ∑18:2, 18:3, 18:4, ∑CLA, ∑20:2, 20:3, 20:4, 22:2, 22:4, EPA, DPA, DHA), ∑CLA (sum of isomers of CLA), ∑TFA (sum of 16:1t6-7, 16:1t8, 16:t9, 16:1t10, 18:1t4, 18:1t6-8, 18:1t9, 18:1t10, 18:1t11, 18:2t9, 18:2t12, 18:2t8c13, 18:2c9t2), [3] SED, standard error of the difference, [a, b,c] in the same row = P<0.05, ns = not significant, P>0.061.

Table 13. Hemogram in the preliminary period.

Variable	Normal range	Mean	Standard error
RBC, x 10^6 cells/µl	8.85-16.0	6.97	1.13
HGB, g/dl	8.9-15.4	8.52	0.83
HCT, %	25.8-44.0	22.64	3.56
MCV, fl	21.6-34.9	32.55	2.05
MCH, pg	8.3-12.3	12.39	1.22
MCHC, g/dl	32.7-37.3	38.09	3.38
PLT, x 10^3 cells/µl	247-765	853.80	218.26

RBC = Red Blood Cells, HGB = Hemoglobin, HCT = Hematocrit, MCV = Mean Corpuscular Volumeof Red Blood Cells, MCHC = Mean Corpuscular Hemoglobin Concentration, PLT= Total Platelets.

Table 14. Total and differential white blood cells count in the preliminary period.

Variable	Normal range	Mean	Standard error
WBCB, x 10^3 cells/µL	4.0-13.0	7.90	1.90
NEUTS, x 10^3 cells/µL	1.4-6.0	2.76	0.97
LYMPHS, x 10^3 cells/µL	2.0-9.5	4.00	1.06
MONOS, x 10^3 cells/µL	0-0.9	0.29	0.20
EOS, x 10^3 cells/µL	0-1.3	0.62	0.44
BASOS, x 10^3 cells/µL	0-0.2	0.06	0.03

WBCB = White Blood Cells, NEUTS = Neutrophil Cells, LYMPHS = Lymphocytes Cells, MONOS = Monocytes Cells, EOS = Eosinophils Cells, Basos = Basophils Cells.

Table 15. Blood serum biochemistry in the preliminary period.

Variable	Normal range	Mean	Standard error
ALB, g/dl	2.0-3.5	2.76	0.14
ALP, U/L	45-250	200.69	80.87
BT, mg/dl	0.15-0.65	0.22	0.08
CRE, mg/dl	0.3-0.9	0.44	0.14
GGT, U/L	60-120	97.81	19.31
GOT, U/L	70-200	114.15	51.66
GPT, U/L	15-45	26.18	4.65
PROT, g/dl	6.0-8.5	7.34	0.53
UREA, mg/dl	25-60	48.24	8.55

ALB = Albumin, ALP = Alkaline Phosphatase, BT = Total Bilirubine, CRE = Creatinine, GGT = Gammaglutamiltranspeptidase, GOT = Glutamic Oxaloacetic Transaminase, GPT=GlutamicPyruvicTransaminase, PROT = Total Protein.

Table 16. Hemogram as affected by the treatments during the third experimental period (High dose of aromatic plant, except for control which had no aromatic plant).

Variable	Normal range	Treatment				SED[1]	P value
		Control	Carum	Coriandrum	Satureja		
RBC, x 10^6 cells/µl	8.85-16.0	9.10	8.87	9.04	9.28	0.364	ns
HGB, g/dl	8.9-15.4	9.82	9.38	9.42	9.85	0.300	ns
HCT, %	25.8-44.0	27.29	27.02	27.33	28.56	1.080	ns
MCV, fl	21.6-34.9	30.19	30.42	30.29	30.91	0.915	ns
MCH, pg	8.3-12.3	10.87	10.58	10.45	10.64	0.283	ns
MCHC, g/dl	32.7-37.3	36.07 [a]	34.85 [b]	34.51 [b]	34.47 [b]	0.606	0.039
PLT, x 10^3 cells/µl	247-765	555.82	548.18	543.36	543.55	47.781	ns

[1] Standard error of the difference, RBC = Red Blood Cells, HGB = Hemoglobin, HCT = Hematocrit, MCV = Mean Corpuscular Volumeof Red Blood Cells, MCHC = Mean Corpuscular Hemoglobin Concentration, PLT= Total Platelets, ns = not significant, P>0.25.

Table 17. Total and differential white blood cells count during the third experimental period (High dose of aromatic plant, except for control which had no aromatic plant).

Variable	Normal range	Treatment				SED[1]	P value
		Control	Carum	Coriandrum	Satureja		
WBCB, x 10^3 cells/µl	4.0-13.0	6.71	7.24	6.83	7.52	0.717	ns
NEUTS, x 10^3 cells/µl	1.4-6.0	1.97	2.12	2.22	2.90	0.474	ns
LYMPHS, x 10^3 cells/µl	2.0-9.5	3.98	4.34	3.95	4.11	0.462	ns
MONOS, x 10^3 cells/µl	0-0.9	0.26	0.28	0.23	0.17	0.048	0.114
EOS, x 10^3 cells/µl	0-1.3	0.43	0.42	0.35	0.25	0.107	ns
BASOS, x 10^3 cells/µl	0-0.2	0.05	0.04	0.04	0.04	0.008	ns

[1] Standard error of the difference, WBCB = White Blood Cells, NEUTS = Neutrophil Cells, LYMPHS = Lymphocytes Cells, MONOS = Monocytes Cells, EOS = Eosinophils Cells, BASOS = Basophils Cells, ns = not significant, P>0.25.

Table 18. Blood serum chemistry during the third experimental period (High dose of aromatic plant, except for control which had no aromatic plant).

Variable	Normal range	Treatment				SED[1]	P value
		Control	Carum	Coriandrum	Satureja		
ALB, g/dl	2.0-3.5	2.97	2.94	3.00	3.04	0.059	ns
ALP, U/l	45-250	155.27	161.27	110.36	151.64	31.104	ns
BT, mg/dl	0.15-0.65	0.27	0.26	0.22	0.27	0.031	ns
CRE, mg/dl	0.3-0.9	0.46	0.49	0.45	0.46	0.029	ns
GGT, U/l	60-120	82.82	86.64	96.27	84.91	7.793	ns
GOT, U/l	70-200	195.82	182.91	179.18	187.45	42.291	ns
GPT, U/l	15-45	28.55	28.91	28.46	30.73	2.100	ns
PROT, g/dl	6.0-8.5	7.10	7.03	7.22	7.24	0.130	ns
UREA, mg/dl	25-60	56.55	52.00	58.55	53.91	2.883	0.13

[1] Standard error of the difference, ALB = Albumin, ALP = Alkaline Phosphatase, BT = Total Bilirubine, CRE = Creatinine, GGT = Gamma Glutamil Transpeptidase, GOT = Glutamic Oxaloacetic Transaminase, GPT=GlutamicPyruvicTransaminase, PROT = Total Protein, ns = not significant, P>0.25.

Table 19. Means pH values of the rumen fluid of the ewes fed aromatic plants and of the control ewes.

	Treatment				Dose of plant (g DM)			P value		
	Control	Carum	Coriandrum	Satureja	Low	Medium	High	Treat	Dose	T x D
Rumen pH	6.80 [b]	6.86 [ab]	6.95 [a]	6.87 [ab]	6.80 [b]	6.93 [a]	6.90 [a]	0.004	0.001	ns

ns = not significant, P>0.25, [a, b] in the same row = P<0.05.

Table 20. Average of body weight (BW), body condition score (BCS), age, days in milking (DIM), production and composition of milk by ewes at the beginning of the digestibility trial (High dose of aromatic plant, except for control which had no aromatic plant).

Variable	Treatment				SEM[1]	P value
	Control	Carum	Coriandrum	Satureja		
BW, kg	45.5	47.0	46.2	45.8	1.009	ns
BCS	2.68	2.68	2.65	2.60	0.040	ns
Age, year	2.6	2.4	2.0	3.0	0.185	ns
DIM*, d	156.4	149.2	161.0	158.6	4.276	ns
DIM**, d	160.4	153.2	165.0	162.6	4.276	ns
Milk						
Production, kg/d	1.4	1.3	1.4	1.4	0.039	ns
Fat, %	6.3	5.8	6.1	5.9	0.125	ns
Protein, %	5.2	5.1	5.1	5.2	0.047	ns

[1] SEM = standard error of the means, ns = not significant, P>0.290, *Refers to the first day of the digestibility trial, **Refers to the last day of the digestibility trial.

Table 21. Nutrient intake of dairy sheep fed aromatic plants.

Variable	Treatment[1]				SED[2]	P value
	Control	Carum	Coriandrum	Satureja		
Intake,[3] g/d						
DM	1823	1815	1972	2040	100.89	ns
OM	1427	1408	1566	1626	104.26	ns
NDF	453 ^{c}	498 ^{bc}	604 ^{a}	588 ^{ab}	45.79	0.013
ADF	254 ^{b}	283 ^{b}	367 ^{a}	356 ^{a}	27.75	0.002
ADL	34 ^{b}	43 ^{b}	68 ^{a}	60 ^{a}	6.38	0.001
NFC	643 ^{ab}	567 ^{c}	592 ^{bc}	665 ^{a}	34.08	0.039
EE	37 ^{c}	45 ^{ab}	48 ^{a}	39 ^{bc}	3.35	0.013
CP	294	298	322	334	23.48	ns
Intake, % DM						
OM	78.3	77.6	79.4	79.7	-	-
NDF	24.8	27.4	30.6	28.8	-	-
ADF	13.9	15.6	18.6	17.4	-	-
ADL	1.9	2.4	3.4	2.9	-	-
NFC	35.3	31.2	30.0	32.6	-	-
EE	2.0	2.5	2.4	1.9	-	-
CP	16.1	16.4	16.3	16.4	-	-

[1] (n=5 for each treatment), Carum = basal diet + high dose of *Carum* sp., Coriandrum= basal diet + high dose of *Coriandrum* sp., Satureja= basal diet + high dose of *Satureja* sp., Control = basal diet (without aromatic plant), [2] SED = standard error of the difference, [3] Included: dehydrated alfalfa, alfalfa hay, beet pulps and a mix, average of 5 days, DM = dry matter, OM = organic matter, NDF = neutral detergent fiber, ADF = acid detergent fiber, ADL = acid detergent lignin, NFC = non fiber carbohydrate (was calculated: total carbohydrate (g) – NDF (g)), EE = extract ether, CP = crude protein, ns = not significant, P>0.104.

Table 22. Effects of aromatic plants supplementation on digestibility of nutrients in lactating sheep.

Digestibility*, %	Treatment[1]				SED[2]	P value
	Control	Carum	Coriandrum	Satureja		
DM	73.0 [ab]	72.8 [ab]	71.3 [b]	75.3 [a]	1.291	0.051
OM	71.3 [b]	71.1 [b]	69.9 [b]	74.2 [a]	1.146	0.012
NDF	44.0 [b]	52.3 [a]	52.6 [a]	56.1 [a]	1.973	0.001
ADF	24.2 [c]	36.8 [b]	40.0 [ab]	44.6 [a]	2.623	<.0001
NFC	91.0 [a]	87.9 [b]	87.7 [b]	91.1 [a]	0.917	0.001
EE	52.2 [a]	54.5 [a]	53.6 [a]	44.1 [b]	3.043	0.014
CP	72.5 [b]	73.0 [b]	72.1 [b]	75.6 [a]	1.066	0.020

[1] (n=5 for each treatment), Carum = basal diet + high dose of *Carum* sp., Coriandrum = basal diet + high dose of *Coriandrum* sp., Satureja = basal diet + high dose of *Satureja* sp., Control = basal diet (without aromatic plant), [2] SED = standard error of the difference, *DM = dry matter, OM = organic matter, NDF = neutral detergent fiber, ADF = acid detergent fiber, NFC = non fiber carbohydrate (calculated as total carbohydrate (%) – NDF (%)), EE = extract ether, CP = crude protein.

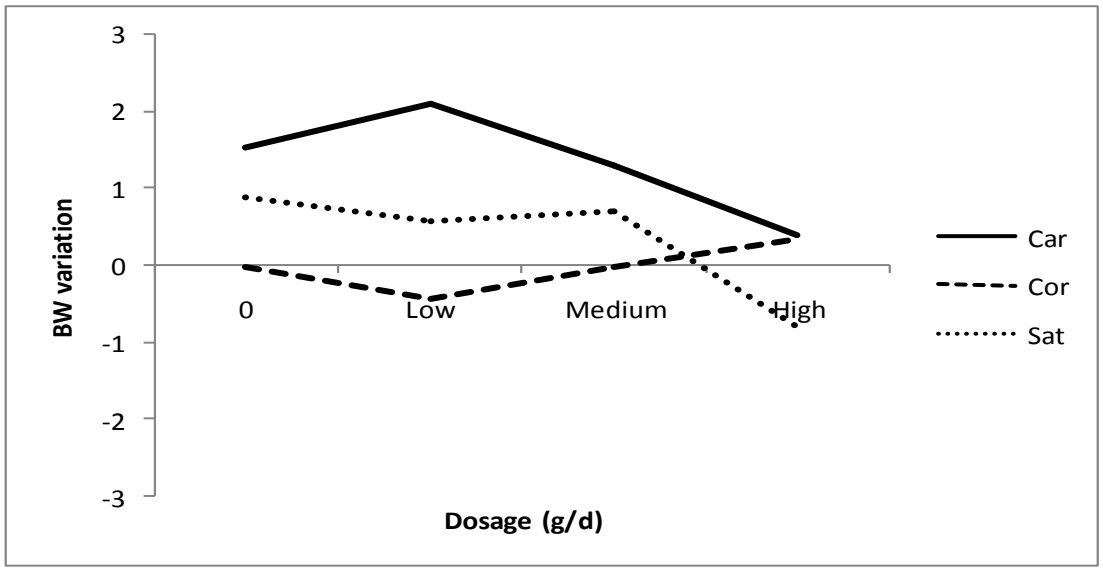

Figure 1. Body weight (BW) variations of the treated groups (Car=*Carum* sp.; Cor=*Coriandrum* sp.; Sat=*Satureja* sp.), reported as difference (P<.0001) from the Control group.

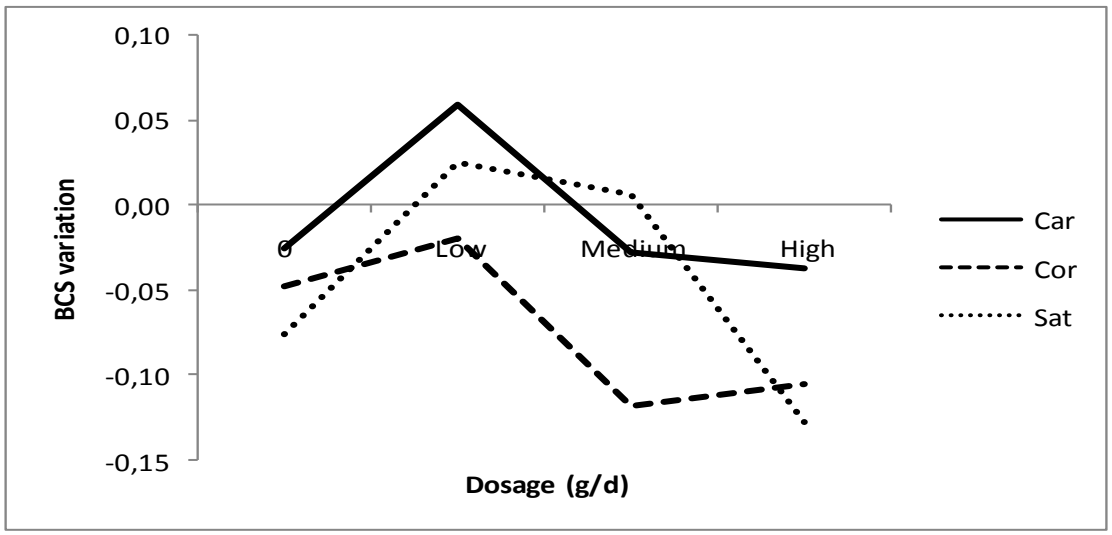

Figure 2. Body condition score (BCS) variations of the treated groups (Car=*Carum* sp.; Cor=*Coriandrum* sp.; Sat=*Satureja* sp.), reported as difference from the Control group.

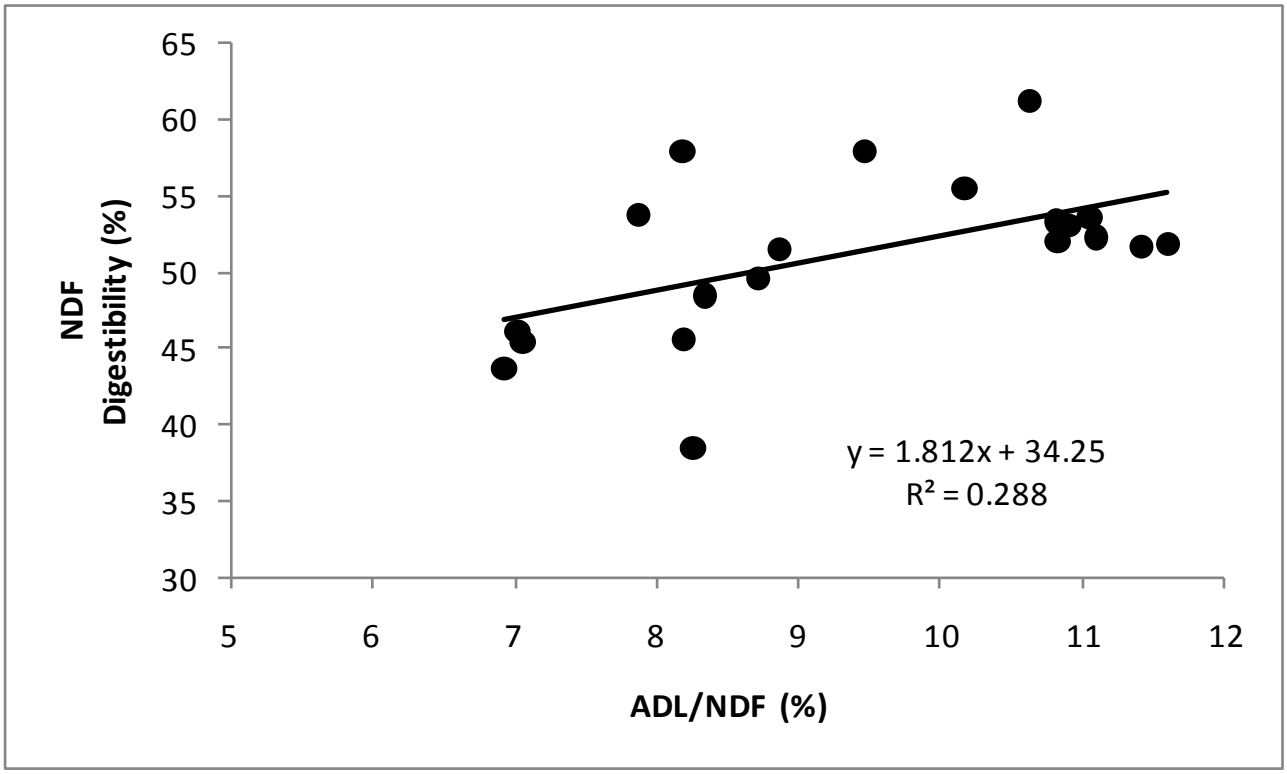

Figure 3. Relationship between the ratio of acid detergent lignin to NDF and *in vivo* NDF digestibility.

Anthelmintic effects of diets containing blends of aromatic plants fed to non-lactating pregnant Sarda ewes naturally infested by gastro-intestinal parasites

CHAPTER 3

Oscar BoaventuraNeto

Anthelmintic effects of diets containing blends of aromatic plants fed to non-lactating pregnant Sarda ewes naturally infested by gastro-intestinal parasites

1. Introduction

The Mediterranean regionproduces 66% of the world's sheep milk (Pandya and Ghodke, 2007). In the Mediterranean countries, dairy sheep are raised mainly on pasture (Molle et al., 2008), an environment where internal parasites become particularly dangerous and problematic (Jackson et al., 2012). Since the anthelmintic (**AH**) resistance was first suspected (Drudge et al., 1957), new strategies have been conceived to control the gastrointestinal parasitism, such as vaccines, genetic selection, nutrition manipulation (Torres-Acosta and Hoste, 2008) and grazing management (Torres-Acosta et al., 2012). With this aim, the essential oils from aromatic plants can play an interesting role because they can putatively affect egg shedding and worm growth, as demonstrated by *in vitro* (Camurça-Vasconcelos et al., 2007; Katiki, et al., 2011; Cala et al., 2012; Carvalho et al., 2012; Elandalousi et al., 2013) and *in vivo* (Githiori et al., 2006; Eguale et al., 2007; Chagas et al., 2008; Tariq et al., 2009; Vatta et al., 2011; Lone et al., 2012) experiments. For example, in a study on an undefined sheep breed (3 to 6 month-oldmales and females), Mesquita et al. (2013) evaluated the anthelmintic activity of *Eucalyptus staigeriana* encapsulated essential oils and found an efficacy of about 83.8%. In male Menz sheep, a diet containing an extract of dried seeds of *Coriandrum sativum*had anthelmintic effects, which were dose and concentration dependent (Eguale et al., 2007). Supplementation with an extract of dried seeds of *Carum copticum* also had anthelmintic effects (Lateef at al., 2006).

According to some authors, such as Cardozo et al. (2005), Castillejos et al. (2005, 2006, 2007) and Spanghero et al. (2008), one important factor that can influence the effectiveness of essential oils is whether they are provided alone or in mixtures. To our knowledge, there are no studies that tested possible anthelmintic effects of blends of aromatic plants in non lactating and pregnant ewes.

Thus, taking also into account the results described in Chapter 2, an experiment was planned and carried out to evaluate possible AH effects of supplements containing either *Satureja* sp.

alone or blends of *Satureja* sp. with *Carum* sp. and *Coriandrum* sp. in non-lactating and pregnant Sarda ewes naturally infested by gastro-intestinal parasites.

2. Materials and Methods

Location and duration

The trial was conducted at the experimental farm "Ovile Sardo" of the Department of Research in Animal Production (DiRPA) of the Agricultural Research Agency of Sardinia (Agris), situated in Monastir (Southern, Sardinia, Italy, latitude: 39°N, 9°E, average annual rainfall = 482 mm). It was conducted in compliance with the principles and specific guidelines on animal care and welfare as required by Italian law (Gazzetta Ufficiale, DL no. 116, January 27, 1992).

The trial was carried out from 13 July to 31 October 2012 and lasted 110 days in total. The study was divided into a preliminary (from 13 July to 5 August 2012) and an experimental period, which in turn was subdivided into an adaptation (from 6 to 12 August 2012) and a measurement (from 13 August to 31 October 2012) sub-period.

Preliminary period

During the preliminary period, 120 non-lactating Sarda ewes, recently submitted to artificial insemination, were group-fed a diet consisting of pasture stubbles and a commercial concentrate (300 g/d per head). All ewes were naturally infested with gastro-intestinal *Strongylidae* parasites (range: 1000–3000 eggs per gram of feces (**EPG**), as assessed by the McMaster counting technique (see below for details). This range of infestation was considered adequate to test AH treatments, in agreement with the studies of Ademola et al. (2004, 2005, 2007), who used sheep with a feces egg count (**FEC**) exceeding 750 eggs EPG. Furthermore, in small ruminants usually FEC of 50–800 EPG is considered a light infection, 800–1.200 EPG a moderate infection and >1.200 EPG a heavy infection (Abebe et al., 2010). For our trial, 80 ewes were selected and divided into 4 groups (5 three-year-old animals and 15 two-year-old animals in each group), homogeneous for infestation level of gastro-intestinal *Strongylidae* parasites, body weight (**BW**) and body condition score (**BCS**; Table 1).

Experimental period and experimental design

In the first adaptation day of the experimental period (6 August 2012), each of the 4 homogeneous groups (20 ewes/group) described above was randomly assigned a different diet, to test the effects of *Carum* sp., *Coriandrum* sp. and *Satureja* sp. on the infestation by gastro-intestinal *Strongylidae* parasites in dairy ewes.

All animals fed on pasture and were submitted to one of the following experimental treatments: 1) Control, supplemented with a concentrate made of ground corn(70%) and pea grains (30%), at 400 g/d per head, without the addition of aromatic plants; 2) Satureja (Sat), supplemented with the same amount and type of concentrate of the Control group added with *Satureja* sp.; 3) Satureja+Coriandrum (Sat+Cor), fed the same amount and type of concentrate of Control group, added with *Satureja* sp. and *Coriandrum* sp.; and 3) Satureja+Coriandrum+Carum (Sat+Cor+Car), fed the same amount and type of concentrate of Control group added with *Satureja* sp., *Coriandrum* sp. and *Carum* sp.

The ingredients of the experimental diets were accurately mixed before each supplementation meal. Feeds were supplied at the morning meal (6:30 am) in the yoke of a milking parlour to singly captured ewes, in order to avoid differences in intake among individuals of the same group. During the rest of the day, all ewes (four groups together) were kept on the same pasture for approximately 3-4 h/day, starting at c.a. 7:30 am. After being brought back to the stall, ewes were group-fed chopped grass hay (Italian ryegrass and oats) at varying amounts (average 0.60 kg/d per head; ranging from 0.50-0.75 kg/d), depending on the availability of grass. During the experimental phase, ewes grazed on pasture, rotating at 2-week intervals in 2 areas of 1 ha each, with irrigated multi-species grass seeded in spring, predominantly millet (*Panicumglaucum* cv. Daily Double). However, from 25 to 31 October 2012, the ewes grazed a different area of 1 ha of recently established Italian ryegrass (*Lolium multiflorum* cv. Teanna) pasture, because of the low availability of the summer grass pasture.

Measurements and samplings

Every time the experimental supplements (the mix of ground corn and peas with or without the aromatic plants) were given to the animals, the amount supplied to each ewe of the

experimental groups (Control, Sat, Sat+Cor and Sat+Cor+Car) and, subsequently, the orts were weighed. The hay supplied to each group of ewes after grazing was also weighed.

Every month, starting from 19 July 2012, body weight (**BW**) was measured using an electronic scale and body condition score (**BCS**) was estimated using the scale from 0 to 5 of Russel et al. (1969). On two occasions during the experiment, samples of concentrate, pasture and hay were collected for chemical analysis.

To determine the level of parasite infestation of the ewes, every 15 days fecal samples of a minimum weight of 5 g fresh matter were collected directly from the rectum. When the rectum was found empty, sampling was repeated at least 4 times within the subsequent 48 huntil the minimum total weight of the sample was reached. After sampling, feces samples were kept refrigerated at 4°C for a maximum of 2 days until being sent for analysis, inside a cooler bag, to the Laboratory of Parasitology of the Department of Veterinary Medicine of the University of Sassari, Italy.

Chemical analysis

Feed analyses. Samples of the feeds were oven dried at 70°C until constant weight, ground in a hammer mill to pass through a 1-mm-diameter screen and then analyzed for DM, byoven drying at 105°C for 24 h, for neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL), using the methods of Van Soest et al. (1991), for ether extract, with the Soxhlet method (AOAC, 1990a), for crude protein (CP), using the Kjeldahl method (AOAC, 1990b), and for ash, using a muffle at 550 °C.

Fecal analyses. To determine the FEC, expressed as number of eggs per gram of feces, samples of feces were submitted to microscopic examination using the qualitative and quantitative McMaster technique (Reynaud, 1970), with a floating solution of NaCl (p.s. 1200) and a cut-off of 15 EPG. To determine the genus of the parasites of the *Trichostrongylidae* nematodes present, pool of feces were sampled from 5 animals per group at day 0 of the trial and submitted to culture. Each pool of 50 g of feces was homogenized and placed in a Petri dish, where it was slightly humidified and incubated for 7 days at 22-27°C and 80% of relative humidity. Successively, feces were processed using the Baermann instrument, to isolate the larvae, which were then classified according to the

morphometric keys indicated by the guidelines of the British Ministry of Agriculture, Fisheries and Food (MAFF, 1986). The number of specimens of each genus was then expressed as percentage. For each pool, at least 100 larvae were classified.

Fecal egg count reduction. The fecal egg count reduction (**FERC**) was calculated using arithmetic means according to Coles et al. (1992):

$$\text{FECR (\%)} = 1 - \left[\frac{T_2}{C_2} \right] \times 100$$

where C_2 is the mean post-treatment FEC for the Control group and T_2 is the mean post-treatment FEC for the treated groups.

For comparison, the FECR was also calculated according to Dash et al. (1988), by using the arithmetic means as follows:

$$\text{FECR (\%)} = 1 - \left[\frac{T_2}{T_1} \times \frac{C_1}{C_2} \right] \times 100$$

where C_1 and C_2 are the pre- and post-treatment means of FEC for the Control group and T_1 and T_2 are the pre- and post-treatment means of FEC for the treated groups. Pre-treatment FEC is the FEC for the day of treatment in the week preceding the post-treatment FEC.

Statistical analysis

All data were analyzed using the PROC MIXED procedure of SAS (2002; SAS Institute Inc., Cary, NC). The difference in body weight, body condition score and FEC of the experimental treatments over time were analyzed using the repeated measures in a completely randomized ANOVA design. The model used was the following:

$Y_{ij} = \mu + P_i + s_{j:i} + T_k + PT_{ik} + e_{ijk}$,

where Y_{ij} is the dependent variable, μ is the overall mean, P_i is the fixed effect of plant treatment (i= 4 levels; 0, 1, 2 and 3), $s_{j:l}$ is the random effect of sheep within treatment, T_k is the fixed effect of the time, PT_{ik} is the fixed effect of the plant treatment × time interaction and e_{ij} is the residual error, assumed to be normally distributed, with mean = 0 and constant variance. Means were separated by pairwise *t*-test (PDIFF option of PROC MIXED). Because data on fecal egg count were not normally distributed, they were submitted to $\log_{10}$

transformation before statistical analysis. Differently, data regarding the percent distribution of nematodes determined by coproculture and the protozoa present in the feces were submitted to a Chi-square analysis, to assess differences between their expected and observed frequency distribution.

The quoted statistics are based on transformed data. Statistical differences were considered significantat $P \leq 0.05$. Differences between treatments at $0.05 < P < 0.10$ were considered as a trend towards significance.

3. Results and Discussion

Feed chemical composition and intake

The sheep ration was composed of the experimental mixes (Table 2) and a basal diet. The three aromatic plants used in the mixtures differed notably for all parameters considered (Table 2). In particular, the crude protein concentration was 11.57% in *Satureja*,16.19% in *Coriandrum* and 26.25% in *Carum*. The NDF concentration was 34.14% in *Satureja,* 53.88% in *Carum* and 75.72% in *Coriandrum.* The EE concentration was 2.75% in *Satureja*, 8.16% in *Coriandrum* and 8.67% in *Carum* (Table 2).

The experimental mixes were composed of a fixed amount of coarsely-ground corn and pea grains added with aromatic plants. Therefore, the amount of mix supplied daily increased as the number of aromatic plants included in the treatment increased. In addition, even if the supplements were planned to be isoproteic and isolipidic, they showed some variability for these parameters, with the Sat+Cor+Car group having a slightly higher concentration of CP, NDF and EE than that of the other experimental supplements.

The animals adapted quickly to the experimental mixes containing the aromatic plants, ingesting all the amount of mixes supplied after only 3 or 4 days from the beginning of their inclusion in the diet (Table 4). For this reason, the amounts of mixes ingested from the second experimental week onwards were equal to the planned ones. The supply of aromatic plant mixed with palatable concentrates helps to avoid or reduce neophobia phenomena, which often occurs when ruminants are offered an unknown feed (Cannas et al., 2009).

The basal diet was composed of grass hay and grass pasture (Table 3). During the experiment, grass hay from two different sources with similar chemical composition were used. The composition of the pastured millet grass varied over time due to changes in the plant phenological stage (Table 3). No data is available on the Italian ryegrass herbage, although the quality was certainly high (high CP and high energy content, see data by Molle et al., 2008).

Body weight and body condition score

There were no significant differences (P>0.8) among groups for BW and BCS at the beginning of the experimental period (day 0). During the experimental period, an interaction between treatment × time for live BW (P=0.0004) and for BCS (P=0.03) was found. BW increased considerably with the advancement of pregnancy, and this trend was paralleled by an increase of BCS (Figure 1 and 2). The latter was never in excess considering the sheep physiological stage, reaching levels close to 3 in all groups. The absolute values and temporal variations of live weight and BCS did not differ significantly among groups (P>0.108), even if the ewes from the Control group had numerically lower gains of BW and BCS over time than those of the ewes supplemented with aromatic plants. The trend towards a better performance of the sheep consuming aromatic plants could be explained by the ability of plant secondary metabolites to improve, at low levels of dietary inclusion, animal intake and performance, as shown by Khiaosa-ard and Zebeli (2013).

Gastrointestinal parasites

Nematodes

At day -25, all ewes were moderately to highly infested by strongyles, with no difference among groups (P>0.8) and an average FEC of 1927±539 EPG.

Despite the level of infestation, the ewes were in good health and had a sufficiently high BCS (Figure 2). Figure 3 shows the trend of strongyles egg count during the trial and the curve is in agreement with what found by Di Loria et al. (2009) who showed a peak of FEC values in July (in our case day -25, summer peak) and then a drop which can be regarded as a typical trend for sheep raised under Mediterranean climate conditions. In reality, the distribution of parasites in the grazed herbage and in the animal body is directly influenced by the climatic conditions (Roeber et al., 2013). The analysis shows that there were significant differences among sampling days, and a highly significant treatment × time interaction (P<.0001; Figure 3). In general, the FEC of Control group was higher than those of treated groups. On the days 15 and 30, the group supplemented with the three plants (Sat+Cor+Car) had lower (P<0.001) values of FEC than the Control group, but after that, this difference disappeared (Figure 3).

The group intake of the experimental supplements (concentrate plus aromatic plants) was numerically higher in the treated groups than in the Control group (Table 4) but this could hardly explain only part of the positive effect of the dietary essential oils. In contrast, the AH effect was probably discontinued due tothe climatic condition. In fact, on day 45 post-treatment application, the trial entered the autumn season, and in this period the newly established grasses possibly favoured re-infestation. Moreover, we cannot discard a possible confounding effect of mob grazing (all sheep grazing the same paddock) which could have smoothed the putative advantage of the aromatic plant-based supplementation after day 60 (Figure 3).

The fecal egg count reduction (FECR) calculated according to Coles et al. (1992) showed the highest percentage within Sat+Cor+Car group at day 15 post-treatment (11%) and within Sat+Cor group at day 30 post-treatment (24%)(Table 5). When the FECR was calculated according to Dash et al. (1988), the maximum percentage was 19% at day 75 post-treatment in the Sat group, 22% at day 15 post-treatment in the Sat+Cor group and 34% at day 75 post-treatment in the Sat+Cor+Car group. The highest absolute value of FECR found among treatment groups in this study (34% in Sat+Cor+Car) is far below the level (90–98%) indicated by Wood et al. (1995) as a minimum for assuming a synthetic anthelmintic as effective. This comparison, however, should be considered with caution, because Wood et al. (1995) refer mainly to the evaluation of the efficacy of specific chemical compounds concentrated in drugs, not to whole plants or plant parts.

The two FECR calculation approaches gave overall similar results, but the formula developed by Dash et al. (1988) seems able to reduce the variation within treatment groups because the pre-experimental data is taken into account in the equation for both Control and treated groups.

Results from FEC and FECR show an AH effect of treated groups including *Coriandrum* at day 15. In fact, Figure 3 displays a clear difference on day 15 post-treatment, when the groups supplied with Sat+Cor and Sat+Cor+Car had lower ($P<0.0001$) values compared with the Control group and these results are in agreement with the FECR results, because on day 15 day both FECR estimates showed positive values for the above groups. Another interesting result shown in Figure 3 is that, after day 60 the Control group increased its values, whereas the treated groups tended to be constant until day 75, when their FECR were all positive by

using Dash et al. (1988) equation. In indoors conditions Tariq et al. (2009) observed a fecal egg count reduction when the sheep were fed a crude aqueous extract of *Artemisia absinthium* at the two dosages tested (1 and 2 g/kg of BW). In other indoor studies, when other plants and their extracts were supplied to lambs, some authors also showed a fecal egg count reduction (Ademola et al., 2004, 2005; Whitney et al., 2013).

The relative presence of different genera of gastrointestinal nematodes, as identified on day -25 from the beginning of the treatment, is reported in Table 6. *Haemonchus contortus* was the dominant genus (more than 50%), followed by *Teladorsagia* (c.a. 24%) and *Trichostrongylus* (c.a. 15%). The remaining genera (*Chabertia*, *Oesophagostomum*, *Cooperia* and *Bunostomum*) constituted about 10% of the total genera. The relative presence of the different genera of gastrointestinal nematodes in the various treatments was identified in the last day of the trial (day 90) with the coproculture (Table 6). This determination showed that the most important genera were *Haemonchus contortus*, *Teladorsagia* spp. and *Trichostrongylus* spp., which were already present in high percentages on day -25. In contrast, the genera *Chabertia* spp. and *Oesophagostomum* spp. were observed at day -25 but only in the groups Control (2%) and Sat (9.5%) on day 90. The genera *Cooperia* spp. and *Bunostomum* spp. were present on day -25, even though with low percentages (2.0% and 1.0%, respectively), but were not observed at all in any group on day 90. Regarding *Cooperia* spp. and *Bunostomum* spp., their disappearance in the Autumn (last experimental day) was probably due to seasonal factors, because at the end of the experiment they were not present even in the Control group.

Considering more specifically the genus *Haemonchus contortus*, its percentual distribution between the last experimental day (day 90) and day -25 did not show differences for the groups Control and Sat+Car+Cor ($P>0.05$), whereas it showed a significant reduction ($P<0.0001$) for the groups Sat (from 51.5% to 17.1%) and Sat+Cor (from 51.5% to 24.7%). Considering the genus *Teladorsagia* spp., its percentual distribution between day 90 and day -25 increased in all groups, probably due to seasonal factors and not due to the treatments applied, considering that in the last experimental day there were not differences among experimental groups for this genus ($\chi 2$ with 3 degrees of freedom = 5.06; $P=0.168$).

For the genus *Trichostrongylus* spp. there were not differences between day 90 and day -25 for the Control group, whereas differences were observed for Sat (higher percentage; P=0.0369) and Sat+Cor+Car (lower percentage; P=0.038).

Based on these results, it is possible to conclude that the only differences on gastrointestinal nematodes distribution caused by the treatments tested were on the distribution of the genus *Haemonchus contortus,* which decreased over the experiment in the animals fed Sat or Sat+Cor. The small differences observed for the genus *Trichostrongylus* spp. were not consistent, because in respect to day -25 its concentration increased for the Sat group (26.7% *vs.* 14.8%) and decreased for the Sat+Cor+Car group (5.9% *vs.* 14.8%).

With regard to other parasites, the frequency of positive (infested) samples was the highest for *Eimeria* spp. (Table 7), followed by *Monieizia* spp. (Table 8), and by *Nematodirus* spp. (Table 9). In particular, the frequency of positive cases of *Eimeria* spp. differed significantly among groups on day 60 and day 75, in a decreasing order for the groups Sat+Cor+Car, Sat+Cor, Sat and Control. However, it is important to notice that the first two groups (Sat+Cor+Car and Sat+Cor) already had a numerically higher number of positive cases of *Eimeria* spp. at the preliminary period (Table7). Regarding *Monieizia* spp. infestation, the number of infested animals was already low during the preliminary period, decreased during the experiment and was not detected at all in the last sampling date (Table 8). Even if no significant differences occurred among groups at each sampling date, the total amount of positive cases of *Monieizia* spp. was significantly higher in the Sat+Cor group than in the other groups. However, it should be noticed that the initial level of infested animals was numerically higher in the Sat+Cor group (Table 7). The number of animals infested with *Nematodirus* spp. was already low at the preliminary period, decreased noticeably during the trial, becoming absent on day 75 (Table 9), without significant differences among groups.

To summarise the parasite measurements results, the types and amounts of plant tested using our protocol influenced the level of parasite infestation, in terms of EPG, in Sarda ewes under the initial parasitic conditions described above. Data obtained in this trial confirmed the anthelmintic properties of these plants, in terms of reduction of gastro-intestinal nematodes in small ruminants by ingestion of essential oils, reported in some studies (Pessoa et al., 2002; Macedo, 2008; Camurça-Vasconcelos et al., 2008; Silveira et al., 2012; Mesquita et al., 2013; Ribeiro et al., 2013).

It remains to clarify if the results obtained in the Sarda breed could be confirmed in other sheep breeds. Another important aspect to be explored is the dose-response relationship of these plants when included at higher dietary doses.

4. Conclusions

Based on the results of this experiment, it is possible to conclude that:

- Sarda ewes adapted in few days to the experimental mixes of the aromatic plants, despite the animals were unfamiliar to their marked smell and taste;
- the presence of aromatic plants in the ration of pregnant ewes did not cause any negative effect on the animals, which increased their body weight and BCS similarly to or better than the Control animals;
- the addition of aromatic plants such as *Satureja* sp., *Coriandrum* sp. and *Carum* sp. to the diet showed quantitative anthelmintic effects (fecal egg count reduction) in the treated Sarda sheep;
- some effects were observed in terms of genera distribution. In particular, the genus *Haemonchus contortus* decreased over the experiment in the animals fed Sat or Sat+Cor. The small differences observed for the genus *Trichostrongylus* spp. were not consistent, considering that its concentration increased over the experiment for the Sat group and decreased for the Sat+Cor+Car group.

Future studies would be important to confirm the AH properties of these aromatic plants in conditions different from the ones described in this trial, in terms of sheep breed, composition of the parasite population and pasture on offer (herbage quality and biomass availability).

5. Acknowledgments

I would like to thank Dr. Giovanni Molle, researchers of AGRIS Sardegna, for the suggestions in planning the experiment and for revising the manuscript, and for the other people of the same institution that collaborated in this experiment: Mr. Salvatore Deiana, Mr. Severino Tolu, Mr. Andrea Carta. I am also grateful to the members of the Sezione di Scienze Zootecniche of the Dipartimento di Agraria of the University of Sassari: Dr. Ana Helena Dias Francesconi for editing the English of the chapter, and, Mr. Gesumino Spanu, Mr. Antonio Fenu and Mr. Antonio Mazza for their technical assistance.

I also desire to thank also Prof. Antonio Scala, Professor of Parasitology, for the fundamental involvement in the experiment, and the collaborators of the Laboratory of Parasitology of the Department of Veterinary Medicine at the University of Sassari, in particular Dr. Giuliana Sanna and Dr. Francesco Dore, for the analyses of fecal samples.

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Table 1. Average of strongyles (egg per gram of feces, EPG), body weight and body condition score of Sarda sheep fed different experimental supplements.

Item	Treatment[1]			
	Control	Sat	Sat+Cor	Sat+Cor+Car
Strongyles, EPG	1882±544	2010±557	1956±570	1859±511
Body weight, kg	42.4±6.3	41.9±6.6	43.4±4.9	42.6±5.5
Body condition score	2.5±0.3	2.6±0.2	2.6±0.3	2.5±0.3

[1]Control = no aromatic plant, Sat = 25 g DM of *Satureja*, Sat+Cor = 25 g DM of *Satureja* + 25 g DM of *Coriandrum*, Sat+Cor+Car = 25 g DM of *Satureja* + 25 g DM of *Coriandrum* + 25 g DM of *Carum*.

Table 2. Chemical composition of the ingredients of the experimental supplements.

Feed	Composition (%, DM basis)					NEL [1] Mcal/kg DM
	DM, % a.f.	Ash	CP	EE	NDF	
Ground corn	88.63	1.30	9.28	3.70	15.86	1.98
Peas	87.01	3.02	24.25	0.69	31.28	2.01
Carum sp.	87.49	8.14	26.25	8.67	53.88	1.19
Coriandrum sp.	89.15	6.35	16.19	8.16	75.72	0.82
Satureja sp.	87.43	9.11	11.57	2.75	34.14	0.82

DM = dry matter(as fed), CP =crude protein, EE = ether extract, NDF = neutral detergent fiber, NEL =net energy for lactation, [1]estimated with the Small Ruminant Nutrition System.

Table 3. Chemical composition of the hay and pasture used in the trial.

Feed	Period or growth phase	DM % a.f.	Ash % DM	CP % DM	EE % DM	NDF % DM	ADF % DM	ADL % DM
Graminaceous hay[1]	15 August - 15 September	89.34	7.61	8.39	2.10	60.86	34.16	3.73
Graminaceous hay[1]	15 September-31 October	83.67	8.86	5.95	1.72	66.31	42.23	5.39
Millet grass[2]	Vegetative phase	14.10	14.26	16.10	4.04	53.33	27.02	3.12
Millet grass[2]	Reproductive phase	27.16	16.49	7.62	2.47	62.41	35.42	4.23

DM = dry matter, CP = crude protein, EE = ether extract, NDF = neutral detergent fiber, ADF = acid detergent fiber, ADL = acid detergent lignin, [1]Hay mix of Italian ryegrass and oats, [2]Results from samples of simulated grass grazing where the dominant species was millet (*Panicum glaucum*).

Table 4. Average group and individual concentrate intake (as fed) of Sarda sheep fed different experimental supplements.

Feed	Treatment[1]			
	Control	Sat	Sat+Cor	Sat+Cor+Car
Concentrate, kg/group per day	7.94	8.35	8.86	9.38
Concentrate, kg/sheep per day	0.40	0.42	0.44	0.47

[1]Control = no aromatic plant, Sat = Satureja, Sat+Cor = Satureja + Coriandrum, Sat+Cor+Car = Satureja + Coriandrum + Carum.

Table 5. Arithmetic mean fecal egg counts (FEC) in eggs per gram of feces (EPG) for ewes fed aromatic plants on days 0, 15, 30, 45, 60, 75 and 90 or not treated (Control) and the percentage fecal egg count reduction (FERC) by day post initial treatment (day).

Day[a]	Treatment									
	Control FEC (EPG)	Sat FEC (EPG)			Sat+Cor FEC (EPG)			Sat+Cor+Car FEC (EPG)		
			FECR (%)			FECR (%)			FECR (%)	
			C[b]	D[c]		C[b]	D[c]		C[b]	D[c]
0	1714	1770	-	-	1781	-	-	1736	-	-
15	1188	1321	-11	-8	968	19	22	1055	11	12
30	857	858	0	10	653	24	6	1157	-35	-52
45	414	545	-32	-31	445	-7	-41	647	-56	-16
60	235	531	-126	-72	296	-26	-17	718	-206	-96
75	404	742	-83	19	473	-17	7	817	-102	34
90	416	701	-68	8	468	-12	4	662	-59	21

Control = no aromatic plant, Sat = Satureja, Sat+Cor = Satureja + Coriandrum, Sat+Cor+Car = Satureja+ Coriandrum + Carum.

[a] Day after initial treatment

[b] Coles et al. (1992): FECR (%) = (1 - (T_2/C_2)) x 100, where C_2 is the mean post-treatment FEC for the Control group and T_2 is the mean post-treatment FEC for the treated groups.

[c] Dash et al. (1988): FECR (%) = (1 - (T_2/T_1 x C_1/C_2)) x 100, where C_1 and C_2 are the pre- and post-treatment means of FEC for the Control group and T_1 and T_2 are the pre- and post-treatment means of FEC for the treated groups.Pre-treatment FEC is the FEC for the day of treatment in the week preceding the post-treatment FEC.

Table 6. Percentage distribution of the species of parasites determined by coproculture in each experimental group at the beginning (day -25) and at the end (day 90) of the experiment.

Species	Day -25	Day 90			
		Control	Sat	Sat+Cor	Sat+Cor+Car
Haemoncus contortus	51.5	42.6	17.1[A]	24.7[A]	57.4
Teladorsagia spp.	23.8	41.6[A]	46.7[A]	51.5[A]	36.7[a]
Trichostrongylus spp.	14.8	13.8[a]	26.7	23.8	5.9[a]
Chabertia spp. and *Oesophagostomum* spp.	6.9	2.0	9.5	0	0
Cooperia spp.	2.0	0	0	0	0
Bunostomum spp.	1.0	0	0	0	0
TOTAL	100	100	100	100	100

Control = no aromatic plant, Sat = Satureja, Sat+Cor = Satureja + Coriandrum, Sat+Cor+Car = Satureja + Coriandrum + Carum.

[A] = significantly different from the preliminary period at the $\chi 2$ analysis, $P<0.001$, [a] = significantly different from the preliminary period at the $\chi 2$ analysis, $P<0.05$.

Table 7. Positive cases of *Eimeria* spp. at various sampling dates as affected by dietary treatments.

Day	Treatment[1]				Total	P value
	Control	Sat	Sat+Cor	Sat+Cor+Car		
-25	5	5	11	10	31	0.091
0	6	4	4	5	19	ns
15	4	4	3	2	13	ns
30	7	4	6	6	23	ns
45	6	9	11	8	34	ns
60	1^{c}	4^{bc}	9^{ab}	11^{a}	25	0.002
75	4^{b}	4^{b}	5^{ab}	11^{a}	24	0.044
90	6	7	2	5	20	ns
Total cases	34	36	40	48	189	ns
Cases/Ewe	0.21	0.22	0.27	0.31	1.00	ns

[1]Control = no aromatic plant, Sat = Satureja, Sat+Cor = Satureja + Coriandrum, Sat+Cor+Car = Satureja + Coriandrum + Carum.

ns = not significant, P>0.10, $^{a, b, c}$ = P<0.05.

Table 8. Positive cases of *Monieizia* spp. at various sampling dates as affected by dietary treatments.

Day	Treatment[1]				Total	P value
	Control	Sat	Sat+Cor	Sat+Cor+Car		
-25	1	2	4	2	9	ns
0	2	1	2	2	7	ns
15	1	2	3	2	8	ns
30		1	3	1	5	ns
45		1	3	1	5	ns
60			3		3	ns
75			2		2	ns
90						ns
Total cases	3^{b}	5^{b}	16^{a}	6^{b}	30	0.003
Cases/Ewe	0.10	0.18	0.51	0.21	1.00	

[1]Control = no aromatic plant, Sat = Satureja, Sat+Cor = Satureja + Coriandrum, Sat+Cor+Car = Satureja + Coriandrum + Carum.

ns = not significant, P>0.10, $^{a, b}$ = P<0.05.

Table 9. Positive cases of *Nematodirus* spp. at various sampling dates as affected by dietary treatments.

Day	Treatment[1]				Total	P value
	Control	Sat	Sat+Cor	Sat+Cor+Car		
-25		2	2	4	8	ns
0	3	3	3	2	11	ns
15	1	4	4	1	10	ns
30		2	2	3	7	ns
45			2	1	3	ns
60		1			1	ns
75						ns
90						ns
Total cases	4	10	11	7	4	ns
Cases/Ewe	0.10	0.30	0.33	0.28	1.00	

[1]Control = no aromatic plant, Sat = Satureja, Sat+Cor = Satureja + Coriandrum, Sat+Cor+Car = Satureja + Coriandrum + Carum.

ns = not significant, P>0.05.

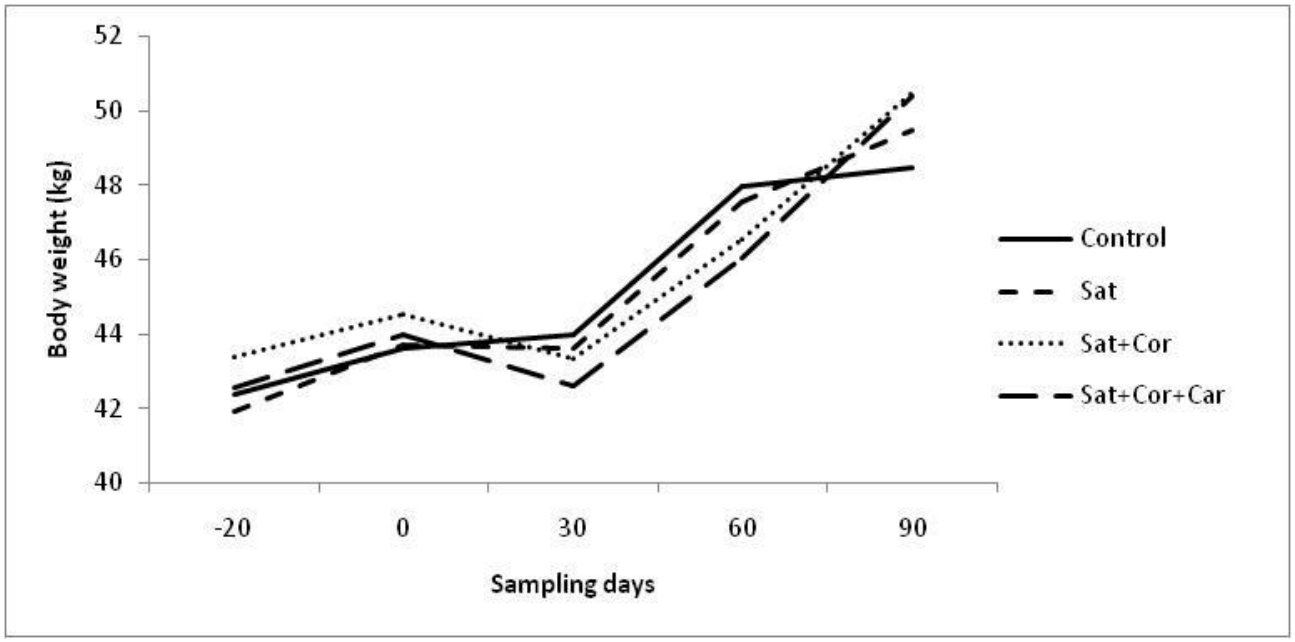

Figure 1. Trend of sheep body weight during the trial as affected by dietary treatments. Control = no aromatic plant, Sat = Satureja, Sat+Cor = Satureja + Coriandrum, Sat+Cor+Car = Satureja + Coriandrum + Carum.The treatment× time interaction was significant at P=0.0004.

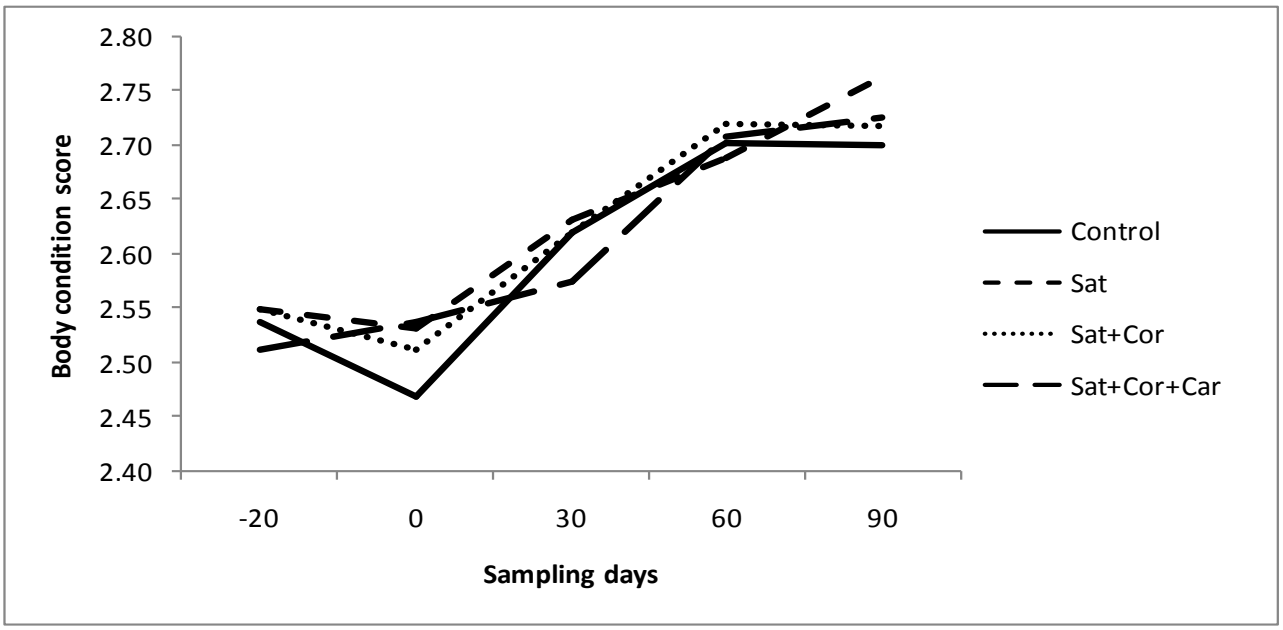

Figure 2. Trend of sheep body condition score during the trial as affected by dietary treatments. Control = no aromatic plant, Satureja, Sat+Cor = Satureja + Coriandrum, Sat+Cor+Car = Satureja + Coriandrum + Carum. The treatment × time interaction was significant at P=0.031.

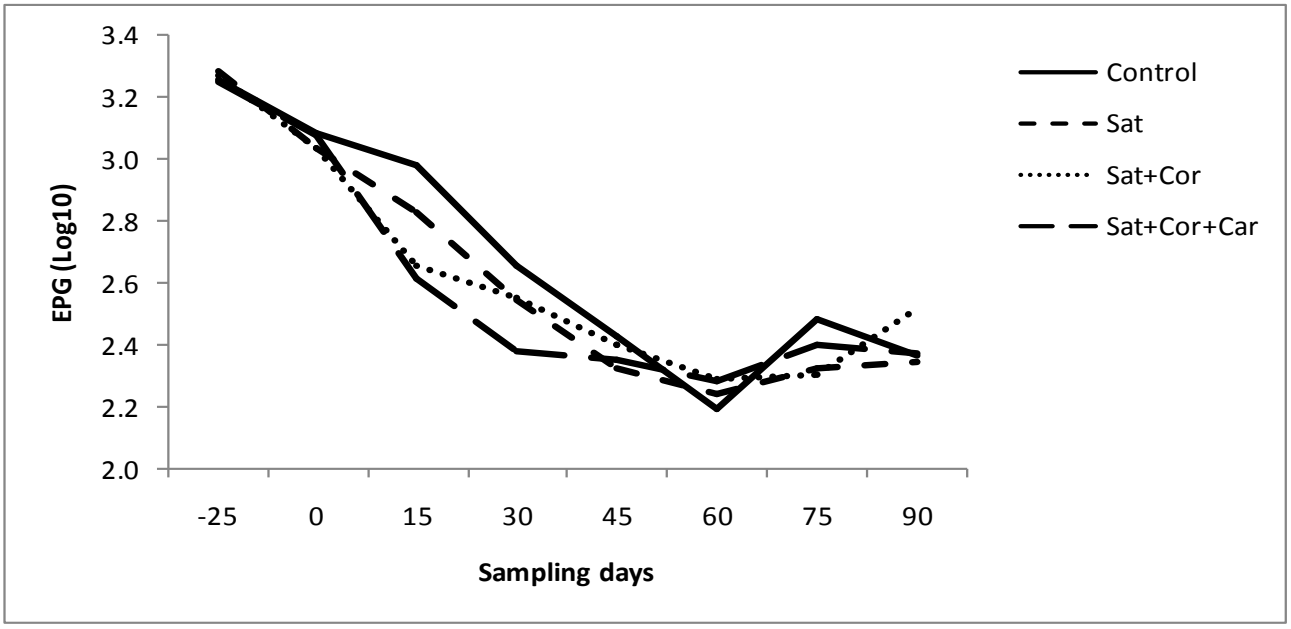

Figure 3. Trend of the egg count per gram of feces (EPG; transformed data) during the trial as affected by dietary treatments. Control = no aromatic plant, Satureja, Sat+Cor = Satureja + Coriandrum, Sat+Cor+Car = Satureja + Coriandrum + Carum.The treatment × time interaction was significant at P<0.0001.On day 15, Control and Sat were significantly higher than Sat+Cor and Sat+Cor+Car. On day 30, Control, Sat and Sat+Cor were significantly higher than Sat+Cor+Car.

Supplementation of blends of aromatic plants to lactating ewes: effects on milk production and quality, blood and rumen parameters

CHAPTER 4

Oscar Boaventura Neto

Supplementation of blends of aromatic plants to lactating ewes: effects on milk production and quality, blood and rumen parameters

1. Introduction

Aromatic plants have diverse blends of secondary compounds (Dudareva and Negre, 2005). Among secondary compounds, essential oils have been studied for their antimicrobial effects- They are secondary metabolites of very diverse composition, usually isolated by stem distillation or solvent extraction, made up mainly by volatile terpenoids and phenyolpropanoids (Calsamiglia et al., 2007; Benchaar et al., 2008a; Patra, 2011). The composition and amount of EO of plant extracts varies with plant parts (e.g. leaf, root, stem, fruit peel or pulp, flower or seed) (Dorman & Deans, 2000) and plant species (Bezic et al., 2005) and they are strongly affected by genetic, age and environmental factors (Cosentino et al., 1999).

Essential oils, as other secondary compounds, can act in additive, synergistic and or antagonistic manner (Burt, 2004). For example, Cardozo et al., (2005) and Castillejos et al., (2005, 2006, 2007) showed interesting results, due to the effects of essential oils (**EO**) used individually or in mixtures, on rumen metabolism in several *in vitro* studies. Several studies have been published in which different combinations of purified EO or EO extracts have been tested *in vivo*, as reviewed by Cannas et al. (2013) and in the Introduction of this dissertation. However, according to our knowledge few studies have tested possible synergistic effects among whole aromatic plants or part of these plants. No one was carried out on lactating sheep. For this reason and based on the results of the experiments described in the Chapter 2, in which three aromatic plants (*Carum* sp., *Coriandrum* sp. and *Satureja* sp.) were studied individually, the aim of this study was to test possible interactions and synergistic effects of blends of the same plants on milk production and composition, rumen function and health status of lactating Sarda ewes.

2. Materials and methods

Location and duration

The trial was conducted in private dairy sheep farm, located in Cuglieri, Province of Oristano, Sardinia, Italy, which was selected for its history of cleanness and careful milking and feeding operations. The trial was carried out in accordance with the principles and specific guidelines on animal care and welfare as required by Italian law (Gazzetta Ufficiale, DL no. 116, January 27, 1992).

Selection of the animals and preliminary period

The trial was carried out between early November 2012 and mid December 2012. During the end of October and the first two weeks of November a group of about 50 ewes was selected and monitored for the first month of lactation, during which the ewes were suckling their lamb. After weaning, the ewes were confined indoors and adapted to the confinement and to the preliminary period diet. Based on milk production, 42 of them were selected and started the preliminary period. In the last days of this period milk yield and composition, body weight (BW) and body condition score (BCS; 0 to 5 scale of Russel et al., 1969) were measured. At the end of the preliminary period, 2 ewes were discarded, whereas the others were divided in 4 experimental groups of 10 ewes each, which were balanced on the basis of their BW (mean±S.D., 41.8±3.5 kg), BCS (2.53±0.11), milk production (1.42±0.39 kg/d) and milk protein (5.56±0.39 %) and fat (5.98±0.69 %) concentration.

The experimental period was divided into a period of adaptation to the experimental treatments, which lasted 2 weeks and a period of measurements of the effects of the treatments being applied, which lasted 9 days. During the preliminary period, the ewes were group-fed a diet made of a commercial concentrate, in the amount of 100 g/d per head at each of the two daily milkings (8:30 and 16:30), and a basal diet, made of dehydrated alfalfa, corn grains, beet pulps, alfalfa hay and soybean meal, supplied in the pen (Table 1). By the end of the preliminary period, all the ewes were subjected to measurements of BW, BCS, milk production and composition.

Experimental period and experimental design

In the first experimental day, each of the 4 groups was randomly assigned to an aromatic plant or a blend to be used throughout the experiment. The three plants used were *Carum* sp., *Coriandrum* sp.and *Satureja* sp., supplied with the following dosages (Table 3):

i) Dose 1: Satureja (**Sat**)

ii) Dose 2: Satureja and Coriandrum (**Sat+Cor**)

iii) Dose 3: Satureja, Coriandrum and Carum (**Sat+Cor+Car**).

The ewes were confined in four large indoor pens (one for each group) and group-fed a diet made of dehydrated alfalfa (1.3 kg/d as fed per head), beet pulps (0.30 kg/d as fed per head), alfalfa hay (0.40 kg/d as fed per head) and the experimental concentrate mix (0.50 kg/d as fed per head) (Table 2). Water was always available.

At each milking, the ewes were also fed a commercial concentrate (100 g/d per head). The experimental mixes were supplied in the pens twice per day immediately after milking. Each mix was divided into two doses of 250 g/d per head as fed. Based on the chemical composition of the various ingredients (Table 4), the proportion among corn grains and soybean meal was changed for the various groups and dosages so that all concentrate mixes supplied in the pen were isoproteic. The mixes were prepared in the laboratory of the Dipartimento di Agraria, University of Sassari, and then stored in plastic bags until use.

Measurements and samplings

Individual milk yield of all ewes was measured at each of the two daily milkings during the preliminary (end of the period) and the experimental period (days 14 and 16 days after the beginning of this period). Individual milk samples were taken at each of the two daily milkings. The fresh milk samples were refrigerated and analyzed within 48 h for standard milk composition (fat, protein, lactose, somatic cell count, casein, urea and pH) in the laboratories of the Associazione Regionale Allevatori of Sardinia (Oristano, Sardinia, Italy).

In the last days of the experimental period, blood samples were drawn from each animal early in the morning, before milking, from the jugular vein by using a needle gauge of 18, a holder and two different tubes depending on the analysis required: complete blood count (**CBC**) and biochemical profile. For the analysis of CBC, 3 ml-vacutainer tubes with purple cap, which contained EDTA to

prevent coagulation of the blood sample, were used. For the analysis of biochemical profile, 5 ml-vacutainer tubes with red cap, without anticoagulant substances, were used. Once collected, the samples for CBC were shaken gently for about 10 times to mix the blood with EDTA. After that, the tubes were kept upright ina cooler (4-8 °C). Within 4 hours from collection,allblood samples were delivered to the laboratory of the Istituto Zooprofilattico Sperimentale della Sardegna (Sassari, Sardinia, Italy) for analysis. Just after taking the blood samples, the body weight and BCS of the animals were determined.

In the last experimental day, rumen liquid samples were taken from half of the ewes (20 in total, 5 from each group), 2 hours after the morning milking and meal, using a stomach tube. The first portion (about 30-50 ml) of rumen liquor collected was discarded to avoid or reduce saliva contamination. The samples were immediately filtered, subjected to pH measurement and then divided into two subsamples, one immediately frozen and the other frozen after the addition of sulfuric acid. Then, the body weight and BCS of the animals were determined.

Chemical and microbial analysis

Feed analyses. Samples of each of the aromatic plants were taken weekly during the trial. For each plant species, the samples were pooled, thoroughly mixed and subsampled. The final samples were then stored at room temperature (max 18 °C) until chemical analyses. Half of each sample was then ground with a 1 mm screen for subsequent chemical analysis. Feed samples were analyzed for neutral detergent fiber (**NDF**), acid detergent fiber (**ADF**), acid detergent lignin (**ADL**) (Van Soest et al., 1991), ether extract (AOAC, 1990a), ash and crude protein (**CP**) (AOAC, 1990b). When the samples had more than 4% of ether extract, their subsamples used for NDF analysis were pre-treated with ethane to solubilize the lipids of the feed and avoid interferences with the NDF analyses, as suggested by Van Soest et al. (1991).

Milk analyses. Milk fat, protein, casein and lactose were measured with a infrared method (Milkoscan 4000, Foss Eletric, Hillerød, Denmark) and milk somatic cell count was measured with a flow-citometry method (Fossomatic 5000, Foss Elettric, Hillerød, Denmark). Both the Milkoscan and the Fossomatic equipments were previously calibrated for sheep milk based on the calibration method of the Italian Association of Breeders (AIA, Rome). The reference methods for the

calibration were: for milk fat, the Rose-Gottlieb method (FIL/IDF; 1D:1996); for milk protein, the Kjeldahl method (N x 6.38) (FIL/IDF-20B:1993); for lactose, the differential pH-metry; and for somatic cell count, the fluorometric method with flux cell (FIL/IDF 148 1995, method C). Milk urea was analyzed with an automatic system (Chem spec 150 based on infrared reading, Bentley Instruments, Chaska, Minnesota USA), previously calibrated with an enzymatic-colorimetric method.

Blood analyses. Red blood cells (RBC), hemoglobin (HGB), hematocrit (Hct), mean corpuscular volume of red blood cells (MCV), mean corpuscular hemoglobin concentration (MCHC), total platelets (PLT), and white blood cells (WBCB), neutrophils cells (NEUTS), lymphocytes cells (LYMPHS), monocytes cells (MONOS), eosinophils cells (EOS) and basophils cells (BASOS) were evaluated using LaserCyte Analyzer (IDEXX Laboratories, Mi, Italia). The biochemical parameters: albumin (ALB), alkaline phosphatase (ALP), total bilirubine (BT), creatinine (CRE), gamma glutamil transpeptidase (GGT), glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and total protein (PROT) were determined by a clinical analyzer spectrophotometric method (Dimension RXL Chemistry Analyzer, Dade Behring, Munich, Germany).

Rumen fluid analyses. Rumen liquid samples were subjected to pH measurement immediately after the sampling with a pH meter (Orion Research Inc., model 250A, Boston, MA, USA), equipped with a thermometer and a penetrating glass-electrode (Hamilton Company USA, model 238405, Reno, NV, USA), and then promptly filtrated and collected in a plastic beaker and frozen (-80 °C) for further analysis. Analysis of volatile fatty acids of rumen fluid was performed according to the method described by Kramer et al. (1997). Briefly, rumen fluid was took 1ml and diluted with sulfuric acid H_2SO_4 0.1 N and water bidistilled (1:1), after this, the samples were centrifuged 2 times per 15000 rpm per 10 min to allow the separation of the two phases and then the supernatant were filtered by filter (0.45 µm). Finally the samples were performed on a HPLC system (Varian Inc. Palo Alto, California,USA). The chromatographic system consists of: degasser (Model Varian 9012 Q), autosampler (Model Varian 9300), and UV-Vis detector (Model Varian 906P POLYCHROM) connected in series. The data acquisition and treatment were controlled by Varian Star 3.4.1 Software (Varian Inc. Palo Alto, California, USA). The analytical column used was HyperREZ XP Carbohydrate H^+ (300mm X 7.7mm and 8µm particle size) (Thermo Electron

Corporation, Cheshire, UK). Methane production was estimated based on the following equation of Moss et al. (2000):

$CH_4 = 0.45*C2 – 0.275*C3 + 0.40*C4$,

where CH_4 is methane production, C2 is acetate, C3 is propionate and C4 is butyrate.

Rumen microbiota

DNA isolation

Genomic DNA was isolated from 300 µl homogenized rumen liquid sample using a PowerSoil® DNA Isolation Kit (MOBIO Carlsbad, CA, USA) following the manufacturer instructions. The yield and the purity of the extracted DNA samples were assessed by optical density measurements using a LVisPLATE on a SPECTROSTAR-NANO spectrophotometer (BMG LABTECH GmbH, Germany).

Temporal Temperature Gradient Electrophoresis (TTGE)

Bacteria

The microbiome in each of the cultures was examined by using temporal temperature gradient gel electrophoresis (**TTGE**). The V3 region of the 16S rRNA gene of bacteria was amplified by using bacterium-specific primers (V3P3clamp CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGG GCCTACGGGAGGCAGCAG V3P2, ATTACCGCGGCTGCTGG,) with a 40-bp GC clamp attached to the 5’ end of the forward primers. The polymerase chain reaction (**PCR**) mixture (50 µl) contained: Taq polymerase buffer (Euroclone), 200 µM of desoxynucleoside triphosphate mix, 1 µ M each primer, 2 U Taq polymerase (Euroclone) and 1 µl of template DNA (~ 40 ng). The PCR amplification was performed on a iCyclerTherman Cycler (BIO-RAD Laboratories, Hercules, CA, USA). The amplification program was 95 °C for 2 min; 30 cycles of 95 °C for 30 s, 63 °C for 30 s, and 72 ° C for 1 min. To eliminate artifactual double TTGE bands, a final elongation step at 72°C for 30 min was included at the end of the PCR. This is a simple remedy against artifactual double bands in denaturing gradient gel electrophoresis (Janse et al., 2004).

Archaea

The microbiome in each of the cultures was examined by using TTGE. The V3 region of the 16S rRNA gene of Archaea was amplified using bacterium-specific primers (GC-ARC344fc: CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACGGGGYGCAGCAGGCGCGA;

519r,GWATTACCGCGGCKGCTG,) with a 40-bp GC clamp attached to the 5' end of the forward primers. The PCR mixture (50 µ l) contained: 5X Green GoTaq® Reaction Buffer (Promega), 200 µM of desoxynucleoside triphosphate mix, 0,5 µM each primer, 1,5 U GoTaq® DNA Polymerase (Promega) and 2 µl of template DNA (~ 80 ng). PCR amplification was performed on iCycler Therman Cycler (BIO-RAD Laboratories, Hercules, CA, USA) according to Yu et al. (2004). After an initial denaturation at 94°C for 5 min; 10 cycles of touchdown PCR were performed (denaturation at 94 °C for 30 s, annealing at 61°C with an 0,5°C/cycle decrement at 56°C for 30 s, and extension at 72 ° C for 1 min) followed by 25 cycles of regular PCR (94°C for 30 s, 56°C for 30 s and 72°C for 1 min). To eliminate artifactual double TTGE bands, a final elongation step at 72°C for 30 min was included at the end of the PCR (Janse et al.2004).

Finally, for both bacteria and Archaea the PCR products were confirmed using agarose (1.5%) gels and resolved using polyacrylamide gels (10%) and a migration at 130 V for 6 h at constant temperature of 70°C. Following staining with SYBR safe (Invitrogen), the images were captured using a CHEMIDOC XRS (BIO-RAD Laboratories, Hercules, CA, USA) and analyzed with INFOQUEST software (BIO-RAD Laboratories, Hercules, CA, USA).

Statistical analysis

All data were analyzed by using the PROC MIXED procedure of SAS (2002; SAS Institute Inc., Cary, NC). Nutrient intake and digestibility data were analyzed by a completely randomized ANOVA design. The model used was as follows:

$Y_{ij} = \mu + T_i + e_{ij}$,

where Y_{ij} is the dependent variable, µ is the overall mean, T_i is the fixed effect of treatment (i= 4) and e_{ij} is theresidual error assumed to be normally distributed, with mean = 0 and constant variance. Means were separated by pairwise *t*-test (PDIFF option of PROC MIXED).

Rumen microbiota was studied with a cluster analysis, performed by using the Cluster comparison tools of INFOQUEST software (BIO-RAD Laboratories, Hercules, CA, USA). The principal component analysis was also performed, with the STATGRAPHICS XV software(STATPOINT Technologies, Warrenton, VA, USA) by using the correlation matrix imported from the INFOQUEST software.

Statistical differences were declared at $P<0.05$. Differences between treatments at $0.05<P< 0.10$ were considered as a trend toward significance.

3. Results and Discussion

Characteristics of the diets

In the period of adaptation, the ewes were group-fed dehydrated alfalfa, alfalfa hay, beet pulps, corn grains and soybean meal (Table 1), without the aromatic plants. The ewes adapted quickly to the confinement and to the diets supplied during this period.

At the beginning of the experimental period, the ewes were group-fed a basal diet (Table 2) composed of dehydrated alfalfa, alfalfa hay, beet pulps and a mix, made of corn grains, soybean meal and different mixtures of aromatic plants (Table 3).

The three aromatic plants used in the mixtures differed notably for all parameters considered. In particular, the CP concentration was 11.57% in Satureja, 16.19% in Coriandrum and 26.25% in Carum. The NDF concentration was 34.14% in Satureja, 53.88% in Carum and 75.72% in Coriandrum. The EE concentration was 2.75% in Satureja, 8.16% in Coriandrum and 8.67% Carum (Table 4).

The experimental mixes were composed of corn grains and soybean meal, added with aromatic plants for all, except for the Control group. Therefore, the amount of mix supplied daily was the same during all the experimental period, but its composition varied among the 4 groups depending on the treatment. In addition, even if the aromatic plants had a very different content of CP (Table 4), the mixes were planned to be isoproteic. The animals adapted quickly to the experimental mixes containing the aromatic plants, eating almost all the amount of mix supplied daily after only 2 days from their inclusion in the diet (Table 5). Similarly, the intake of the basal diet was high and similar among the 4 groups (Table 5).

Body weight and body condition score

The body weight of the ewes was not influenced by dietary treatments ($P>0.2$). It increased in all groups during the experiment, being on average equal to 41.8 kg in the period of adaptation, 42.8 kg at the middle of the experimental period and 43.8 kg at its end (Table 6). Similarly, the BCS was not influenced by the treatments ($P>0.2$), being on average 2.55 at the period of adaptation and in the middle of trial, and 2.53 at the end of the trial (Table 6). The lack of differences in BW and BCS might have been a result of the short length (16 days) of the experimental period. Accordingly,

Khiaosa-ard and Zebeli (2013) reported that these traits were not affected in short-term experiments in which essential oils were used.

Blood composition

Our trial showed that the aromatic plants tested did not affect, in comparison to the Control, the variables considered in the hemogram (P>0.20 for all treatments; Table 7), as well as the differential white blood cells (P>0.35; Table 8). All measured blood values were within or very close to the reference ranges, suggesting that the health status of the ewes was good and not affected by the treatments. Only red blood cells were slightly lower than the reference values, indicating a possible, even though limited, iron deficiency.

Small but significant differences occurred for some biochemical blood components (Table 9). In particular, the values affected were those of albumin (P=0.061), lower in Sat than in Sat+Cor. The total bilirubine of Sat+Cor+Car decreased (P=0.025) of about 38% in respect to the Control. The creatinine concentration of Sat+Cor was lower (P=0.041) by 15% than that of the Control, and the total protein of Sat+Cor was higher (P=0.027) by 6.5% than that of the other three groups. Even if these values were still within the normal reference ranges, these differences among treatments suggest that the aromatic plants and their blends might have affected protein metabolism and that clearly affected bilirubine, decreasing it proportionally to the level of inclusion of aromatic plants in the diets. Low levels of bilirubine are indicative of good gallbladder and liver function, suggesting that the aromatic plants might have had a beneficial effect in this sense. In the Chapter 2 of this dissertation, the same aromatic plants were used but as single plants, without any specific effect associated to protein metabolism. Thus, it is possible that the blend exerted a synergic effect on the animals.

We are not aware of other studies on possible health effects of Carum, Coriandrum , Satureja and their mixture on blood parameters of ruminants. With other plant species, Ramírez-Restrepo et al. (2010) observed in young ewes grazing a mixed pasture that the supplementation of willow (Salix spp.) fodder blocks, containing condensed tannins, increased the values of white blood cells and lymphocytes tannins, improving the immune system. In our study, the aromatic plants (rich in essential oils) tested did not cause any detrimental neither benefit effects on immune system of ewes.

Rumen analyses

Rumen pH tended to be positively affected by the aromatic plants (P=0.081; Table 10). The pH of rumen fluid was numerically higher for Sat+Cor+Car (6.57) and Sat+Cor (6.47) than for the Control (6.32) and Sat (6.30) groups (Table 10). Interestingly, rumen pH was significantly higher in Coriandrum than Control groups in the long term trial (Chapter 2 of this dissertation), suggesting a possible positive effect of Coriandrum (present in the two mixes with the highest rumen pH of this trial) on rumen pH. However, as the amount of aromatic plants in the mix increased, the amount of corn, a feed very rich in starch, decreased, which might have favoured higher rumen pH. No effects on pH of aromatic plants were reported by most literature, as reported in Chapter 1 and by Khiaosa-ard and Zebeli (2013).

The total and the individual volatile fatty acids (**VFA**) were not affected by the treatment (P>0.35; Table 10). In contrast, the review of the literature of Chapter 1 suggested that in sheep and goats, one study only reported a decrease of rumen pH, whereas several experiments reported an increase in total VFA and a decrease of the acetate to propionate ratio.

In accordance with the results of Giannenas et al. (2011), Tekippe et al. (2011) and Hristov et al. (2013). In this sense, Khiaosa-ard and Zebeli (2013) in their review observed a reduction of the acetate to propionate ratio in beef cattle, an almost significant tendency for reduction in small ruminants, and no effects in lactating cows.

The lack of variations of VFA in our experimentare in contrast with the marked higher NDF digestibility of the ewes supplied with aromatic plants in the long term studies of this dissertation (Chapter 2). It is possible that long periods of feedings are necessary to observe significant effects of the aromatic plants.

Milk yield and composition

Milk yield was not affected (Table 11) by the feeding treatments. Milk fat, protein, casein, urea and lactose concentrations were also not affected by the treatments. Somehow surprising is the low fat concentration and the high protein concentration observed in all groups, including the Control, with a clear inversion of the concentration of the two main milk components. The low fat concentration was probably due to the fact that the ewes were in positive energy balance, which in dairy ewes tend to markedly reduce milk fat concentration (Pulina et al., 2006). In addition, the feeds used in the basal diet had a small particle size, reducing the rumination time of the ewes and

thus inducing potential conditions of reduced milk fat synthesis. Even though sheep can intensively ruminate even small feed particles, the utilization of diets with small particles size can have a negative effects on their milk fat concentration (Cannas, 2004).

The high total milk protein and casein concentration observed in all groups was probably induced by the high dietary energy concentration, which probably favored rumen energy availability and microbial protein synthesis (Pulina et al., 2006). Milk lactose was high and the somatic cell count was low in all groups, without significant differences among them, suggesting a good mammary health status.

Rumen microbiota

A small proportion of the total rumen microorganism species have been so far identified (Ward et al., 1992), probably because a poor knowledge regarding the real microorganisms growth conditions and by the interactions among them. New techniques based on molecular biology have been used in the last years to improve the identification and characterization of rumen microbiota (Muyer and Smalla, 1998).

The banding of the TTGE profiles indicated that inclusion of aromatic plants in the diets of dairy sheep resulted in apparently different bacteria communities in the ruminal liquid (Figure1). Considering the experimental groups with regard to the difference of the ruminal bacteria, it can be seen that there are *virtually* two different groups, in which the Control group and the group that contained the Sat were moved to the same side, while on the other side are the groups that contained mixtures of two (Sat+Cor) or three (Sat+Cor+Car) aromatic plants. So, the groups that were moved to the same side probably have the same bacteria species, because according to Muyer and Smalla (1998) when the bands are separated with TTGE, the same methodology used in our trial, the samples reveal phylogenetic information. In this case, the blends contained Sat+Cor and Sat+Cor+Car had a synergistic effect, possibly due to the essential oils contained on Coriandrum (mainly linalool) and Carum (mainly carvone). According to Davidson and Parish (1989), synergism occurs when the effect of the blend is greater than the single effect.

It is important to note that the Control group and the Sat+Cor+Car group showed high variability, as also confirmed by the PCA analysis of the TTGE profiles (Figure2), where the two groups are separated from the first principal component and have a similar score in the second principal components analysis. This may mean that the two sheep groups have in common a set of species,

while a component of the microbiota is absent in one and present in the other. In contrast, the Sat group and Sat+Cor group were separated only by the first principal component, suggesting that in these two groups occurs a transition in the microbiota with the loss of certain bacterial groups.

This analysis of rumen liquid clearly showed that aromatic plants can affect the rumen bacteria population, but further analyses and studies are needed to confirm this and to identify the species involved in these changes. In addition, it is not clear why the changed observed in rumen bacteria were not associated to changes in rumen volatile fatty acids production (Table 10).

In ruminants enteric methane emissions represent a loss of dietary energy (Czerkawski, 1969). In recent years, more attention has been focused in a possible environmental effect of this gas produced by archaea (Leslie et al., 2008). In our trial, regarding archaea population, we analized 5 ewes per treatment (20 in total). However, in the TTGE profiles (Figure 3), the 16s rDNA of archaea was amplified for the Sat+Cor and Sat+Cor+Car groups (n=5 per each one), but regarding Control and Sat groups only 2 and 3 samples, respectively, were amplified, despite the reactions were repeated 3 times, suggesting that in these samples archea were not present ort present in very low numbers. Based on these results was not possible to find a marked difference among treatments regarding archea species (Figure 3 and Figure 4).

In our trial, the ruminal pH tended (P=0.081, Table 10) to be lower in the Control and Sat groups. This could explain the fact that for some animals of the treatments Control and Sat the 16s rDNA gene of archaea did not amplify. At the same time, for the group Sat+Cor+Car it was possible to amplify the 16s rDNA gene of archaea in all animals (n=5) and the ruminal pH tended to be higher (P=0.081, Table 10). However it should be considered that the pH values of our trial were within the optimal range (6-8) for the growth of methanogens (Jones et al. 1987). Finally, this group (Sat+Cor+Car) was the only that had a similar sequences on DGGE profiles (Figure 3and Figure 4), perhaps due to addition of Carum. In summary, the effect of aromatic plants on the archaea population is less marked than that recorded on bacterial communities.

In a recent study, Jeyanathan et al. (2011) did not observe differences on denaturating gradient gel electrophoresis (**DGGE**) fingerprints of methanogens comparing different ruminant species (cattle, red deer and sheep) fed different diets (pasture, silage, concentrated-based diet and willow) in different season (autumn, winter, and summer). In agreement, Ouwerkerk et al. (2008) did not observe differences on DGGE patterns of methanogen communities at different species (cattle and sheep) fed different forages types (hay from *Leucaena* spp., Lucerne pellets and fresh

grass), however the same authors observed difference in methanogen communities when animals fed barley-based diet, probably because the percentage of grain content in both trials (75% w/w in Ouwerkerk et al., 2008 and 45% w/w in Jeyanathan et al., 2011). In fact, Lana et al. (1998) affirms that in diets with high grain contents the ruminal pH values is low and affects methanogen species.

4. Conclusions

The inclusion of blends of aromatic plants Carum, Coriandrum and Satureja in the diet of lactating ewes in early lactation produced the following main results:

- the ewes adapted in few days to the mixes with aromatic plants, despite they were totally unknown by the ewes and had marked aroma and taste;
- the aromatic plants did not cause any negative effect on the health of the animals;
- the aromatic plants did not induce negative effects on milk yield and composition;
- the aromatic plants tended to increase the ruminal pH, while did not influence rumen volatile fatty acid production and proportion;
- the analysis of rumen microbiota clearly showed that aromatic plants affected the ruminal bacteria of the liquid phase, but further analyses and studies are needed to confirm this hypothesis and to identify the species involved in these changes;
- unclear results were observed for the archaea rumen population.

Based on these results, it appears that these aromatic plants can be used to exploit the antimicrobial properties demonstrated by other groups.

5. Acknowledgments

I thank the members of the Sezione di Scienze Zootecniche of the University of Sassari: Dr. Ana Helena Dias Francesconi for editing the English of the chapter, Mr. Gesumino Spanu, Mr. Antonio Fenu, Mr. Antonio Mazza and Mrs. Alessandra Marzano for their technical assistance.

I gratefully acknowledge Dr. Paola Nicolussi, Dr. Piero Bonelli and the staff of Istituto Zooprofilattico Sperimentale della Sardegna (Sassari, Sardinia, Italy) for the assistance in the blood analyses.

I would thank to Mr. Antonello Meles for making his farm available for the experiment and for the generous daily collaboration.

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Table 1. Basal diet (kg/d of DM per head) supplied to the 42 ewes group-fed during pre-experimental period.

Feed	Morning kg/d	Afternoon kg/d	Total kg/d
Dehydrated alfalfa	0.527	0.527	1.054
Alfalfa hay	0.091	0.091	0.182
Beet pulps	0.123	0.123	0.246
Corn grains	0.165	0.165	0.329
Soybean meal	0.047	0.047	0.094
Total	0.953	0.953	1.906

Table 2. Basal diet (kg/d of DM per head) supplied to the ewes during the experimental period.

Feed	Morning kg/d	Afternoon kg/d	Total kg/d
Dehydrated alfalfa	0.554	0.554	1.107
Alfalfa hay	0.174	0.174	0.347
Beet pulps	0.129	0.129	0.258
Experimental mix[1]	0.222	0.222	0.444
Total	1.079	1.079	2.158

[1] Detailed composition reported in Table 5.

Table 3. Scheme of the individual dosages of aromatic plants supplied during the trial.

Treatment	Ewes	Adaptation g/d	Experiment g/d
Control	10	0	0
Sat	10	0	Dose 1
Sat+Cor	10	0	Dose 2
Sat+Cor+Car	10	0	Dose 3

[1]Control = no aromatic plant, Sat = Satureja, Sat+Cor = Satureja + Coriandrum, Sat+Cor+Car = Satureja + Coriandrum + Carum.

Table 4. Chemical compositionof the feeds used in the experiment.

Feed	Chemical composition				
	DM % as fed	Ash % DM	CP % DM	EE % DM	NDF % DM
Dehydrated alfalfa	85.16	10.04	23.70	2.1	31.99
Alfalfa hay	86.79	11.76	20.16	-	-
Beet pulps	86.13	4.96	11.70	-	50.69
Corn grains	88.63	1.30	9.28	3.70	15.86
Soybean meal	90.00	6.70	48.20	1.20	15.80
Commercial pellets	87.49	8.86	18.15	-	-
Carum	87.49	8.14	26.25	8.67	53.88
Coriandrum	89.15	6.35	16.19	8.16	75.72
Satureja	87.43	9.11	11.57	2.75	34.14

Table 5. Daily average individual intake (DM basis) during the experiment.

Parameter	Treatment[1]			
	Control	Sat	Sat+Cor	Sat+Cor+Car
Basal diet intake[3], kg/d	1.44	1.56	1.39	1.49
Mix intake[4], kg/d	0.45	0.44	0.44	0.44
Total intake[5], kg/d	1.89	2.00	1.83	1.93

[1] Control = no aromatic plant, Sat = Satureja, Sat+Cor = Satureja + Coriandrum, Sat+Cor+Car = Satureja + Coriandrum + Carum. [2] SED = standard error of the difference, [3] Included alfalfa hay, alfalfa dehydrated and beet pulps, [4] Included corn grains, soybean meal and aromatic plants, [5] Included forage and mix, ns = not significant, P>0.4.

Table 6. Body weight and body condition score (BCS) during the trial.

Variable	Treatment[1]					
	Control	Sat	Sat+Cor	Sat+Cor+Car	SED[2]	P value
Body weight[3], kg	41.85	43.35	41.35	40.70	1.582	ns
Body weight[4], kg	42.15	44.80	42.05	42.10	2.015	ns
Body weight[5], kg	43.85	45.45	42.90	43.00	2.058	ns
BCS[3]	2.55	2.60	2.48	2.55	0.088	ns
BCS[4]	2.55	2.60	2.58	2.48	0.081	ns
BCS[5]	2.50	2.48	2.60	2.53	0.095	ns

[1] Control = no aromatic plant, Sat = Satureja, Sat+Cor = Satureja + Coriandrum, Sat+Cor+Car = Satureja + Coriandrum + Carum.[2] SED = standard error of the difference, [3] Period of adaptation, [4] Middle of trial, [5] End of trial, ns = not significant, P>0.20.

Table 7. Hemogram of the ewes during the experimental period.

Variable	Normal range	Treatment[1]				SED[2]	P value
		Control	Sat	Sat+Cor	Sat+Cor+Car		
RBC, x 10^6 cells/µl	8.85-16.0	8.81	9.07	8.79	8.70	0.311	ns
HGB, g/dl	8.9-15.4	10.50	10.50	10.33	10.18	0.356	ns
HCT, %	25.8-44.0	30.10	30.01	29.70	29.41	1.001	ns
MCV, fl	21.6-34.9	34.20	33.10	33.83	33.93	0.674	ns
MCH, pg	8.3-12.3	11.92	11.60	11.80	11.73	0.279	ns
MCHC, g/dl	32.7-37.3	34.90	35.00	34.85	34.60	0.556	ns
PLT, x 10^3 cells/µl	247-765	560.00	548.70	615.70	575.70	68.404	ns

[1] Control = no aromatic plant, Sat = Satureja, Sat+Cor = Satureja + Coriandrum, Sat+Cor+Car = Satureja + Coriandrum + Carum. [2] SED = standard error of the difference, ns = not significant, P>0.20. RBC = Red Blood Cells, HGB = Hemoglobin, HCT = Hematocrit, MCV = Mean Corpuscular Volume of Red Blood Cells, MCHC = Mean Corpuscular Hemoglobin Concentration, PLT= Total platelets.

Table 8. Total and differential white blood cells count of the ewes during the experimental period.

Variable	Normal range	Treatment[1]					
		Control	Sat	Sat+Cor	Sat+Cor+Car	SED[2]	P value
WBCB, x 10^3 cells/µl	4.0-13.0	7.76	8.37	8.15	7.95	0.917	ns
NEUTS, x 10^3 cells/µl	1.4-6.0	2.44	3.15	2.78	2.60	0.592	ns
LYMPHS, x 10^3 cells/µl	2.0-9.5	4.49	4.44	4.56	4.57	0.505	ns
MONOS, x 10^3 cells/µl	0-0.9	0.38	0.22	0.27	0.25	0.093	ns
EOS, x 10^3 cells/µl	0-1.3	0.37	0.45	0.46	0.44	0.114	ns
BASOS, x 10^3 cells/µl	0-0.2	0.06	0.06	0.06	0.04	0.009	ns

[1] Control = no aromatic plant, Sat = Satureja, Sat+Cor = Satureja + Coriandrum, Sat+Cor+Car = Satureja + Coriandrum + Carum, [2] SED = standard error of the difference, ns = not significant, P>0.357, WBCB = White blood cells, NEUTS = Neutrophil cells, LYMPHS = Lymphocytes cells, MONOS = Monocytes cells, EOS = Eosinophils cells, BASOS = Basophils cells.

Table 9. Blood serum biochemistry during the experimental period.

Variable	Normal range	Treatment[1]				SED[2]	P value
		Control	Sat	Sat+Cor	Sat+Cor+Car		
ALB, g/dl	2.0-3.5	2.73 [ab]	2.65 [b]	2.81 [a]	2.71 [ab]	0.057	0.061
ALP, U/l	45-250	193.7	182.5	193.9	227.7	36.370	ns
BT, mg/dl	0.15-0.65	0.26 [a]	0.21 [ab]	0.20 [ab]	0.16 [b]	0.031	0.025
CRE, mg/dl	0.3-0.9	0.64 [a]	0.66 [a]	0.54 [b]	0.59 [ab]	0.044	0.041
GGT, U/l	60-120	87.5	87.1	93.9	88.1	4.887	ns
GOT, U/l	70-200	159.5	154.5	147.0	161.8	14.329	ns
GPT, U/l	15-45	32.4	33.20	33.6	36.0	2.640	ns
PROT, g/dl	6.0-8.5	7.55 [b]	7.46 [b]	8.03 [a]	7.61 [b]	0.193	0.027
UREA, mg/dl	25-60	74.30	73.30	77.00	73.20	4.555	ns

ALB = Albumin, ALP = Alkaline phosphatase, BT = Total bilirubine, CRE = Creatinine, GGT = Gamma glutamil transpeptidase, GOT = Glutamic oxaloacetic transaminase, GPT = Glutamicpyruvictransaminase, PROT = Total protein, [1] Control = no aromatic plant, Sat = Satureja, Sat+Cor = Satureja + Coriandrum, Sat+Cor+Car = Satureja + Coriandrum + Carum. [2] SED = standard error of the difference, [a, b] in the same row differ statistically, $P<0.05$, ns = not significant, $P>0.4$.

Table 10. Means of pH values and volatile fatty acid (VFA) concentration of the rumen fluid sampled a t the end of the experiment.

Variable	Treatment[1]					
	Control	Sat	Sat+Cor	Sat+Cor+Car	SED[2]	P value
pH	6.32	6.30	6.47	6.57	0.110	0.081
VFA, m*M/L*						
Total	64.42	71.52	70.68	64.09	9.058	ns
Acetate	43.88	48.35	47.88	44.48	6.140	ns
Propionate	12.40	13.76	12.69	12.15	1.800	ns
Butyrate	8.14	9.41	10.11	7.45	1.588	ns
Acetate:Propionate	3.51	3.60	3.89	3.62	0.345	ns
Methane*	19.59	21.74	22.10	19.66	2.886	ns

[1] Control = no aromatic plant, Sat = Satureja, Sat+Cor = Satureja + Coriandrum, Sat+Cor+Car = Satureja + Coriandrum + Carum, [2] SED = standard error of the difference, *Estimated by Moss et al. (2000); ns = not significant, P>0.20.

Table 11. Milk yield and composition. Mean of days 14 and 16 of the experimental period.

Variable	Treatment[1]				SED[2]	P value
	Control	Sat	Sat+Cor	Sat+Cor+Car		
Milk						
Yield, kg/d	1.54	1.50	1.49	1.54	0.206	ns
FPCM, kg/d	1.42	1.38	1.41	1.42	0.188	ns
FCM, kg/d	1.41	1.36	1.39	1.39	0.189	ns
Fat, %	5.64	5.52	5.70	5.55	0.265	ns
Protein, %	5.73	5.80	5.76	5.78	0.155	ns
Lactose, %	4.96	4.96	4.86	4.99	0.126	ns
Casein, %	4.50	4.56	4.51	4.54	0.130	ns
Urea, mg/dl	35.09	43.43	45.31	42.74	4.736	ns
pH	6.76	6.78	6.80	6.78	0.042	ns
CCS, $\log_{10}$ cell/ml	2.45	2.48	2.41	2.30	0.297	ns

[1] Control = no aromatic plant, Sat = Satureja, Sat+Cor = Satureja + Coriandrum, Sat+Cor+Car = Satureja + Coriandrum + Carum., [2] SED = standard error of the difference, [a, b] in the same row differ statistically, P<0.06, ns = not significant, P>0.15.

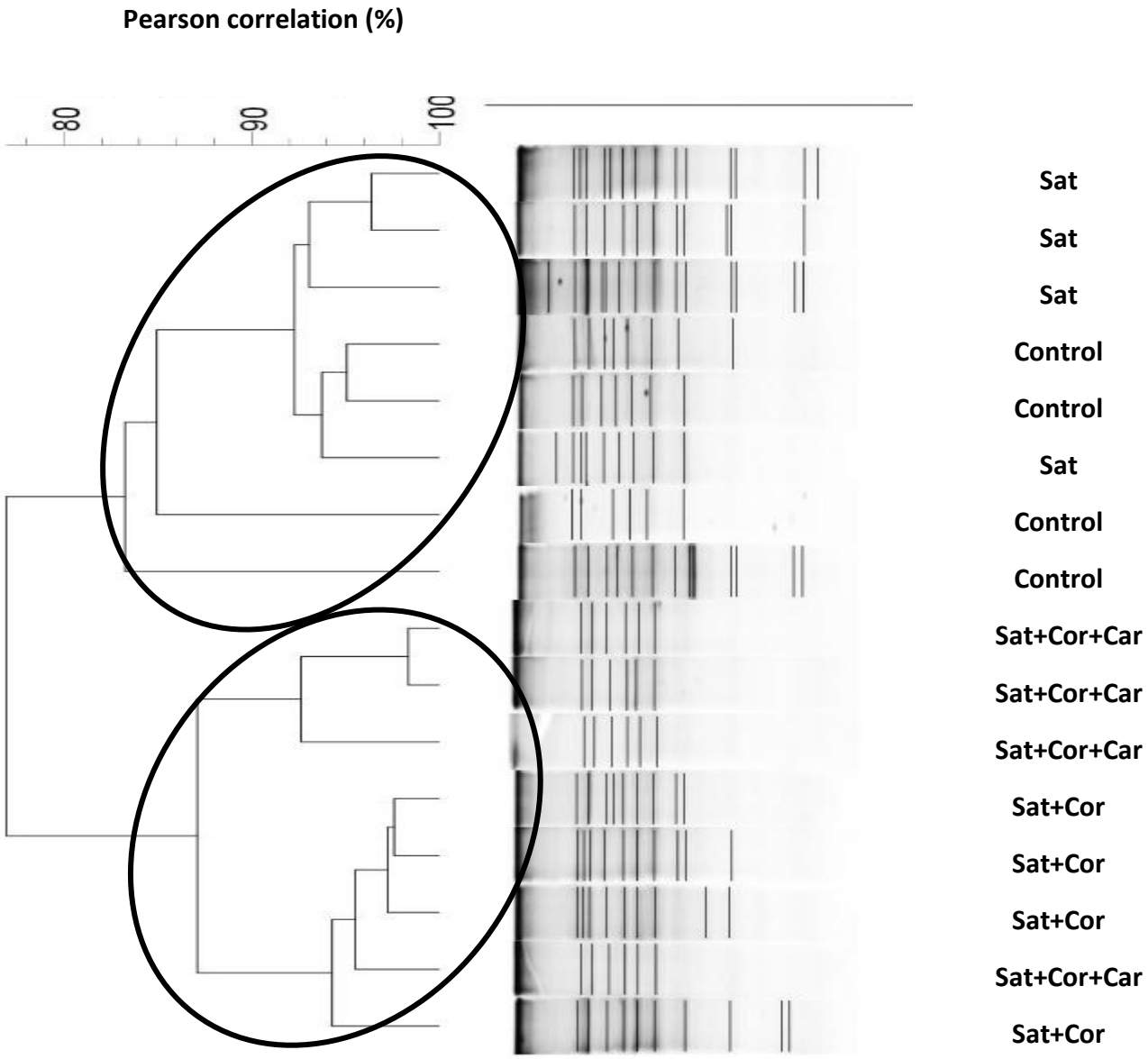

Figure 1. Dendrogram of bacteria community obtained from the DGGE banding profile analysis of the liquid phase of the rumen in four different groups of ewes (4 each one) fed aromatic plants. Control = no aromatic plant, Sat = Satureja, Sat+Cor = Satureja + Coriandrum, Sat+Cor+Car = Satureja + Coriandrum + Carum.

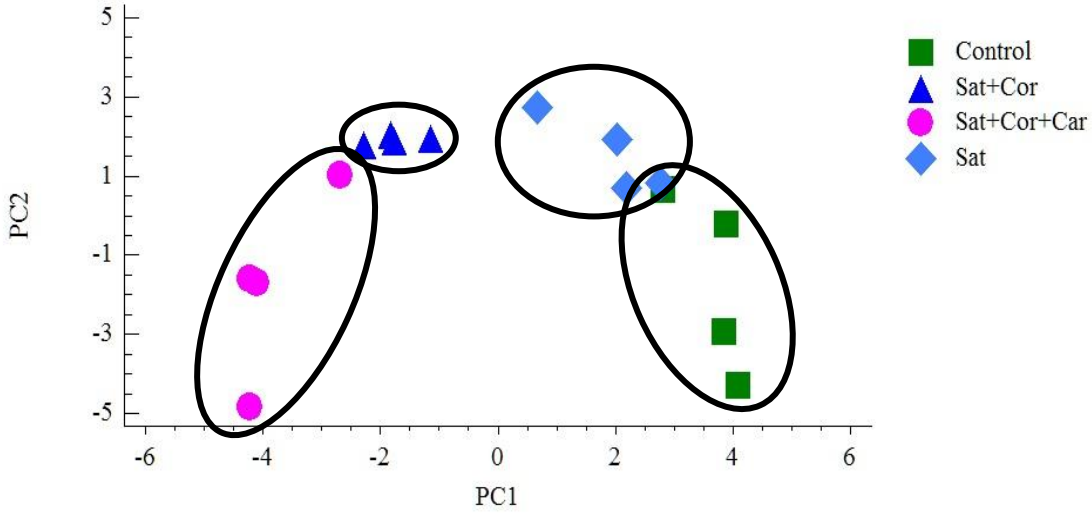

Figure 2. Ordination biplot for the principal component analysis of DGGE banding patterns for 16S rRNA gene of amplicons of bacteria from the liquid phase of the rumen. Control = no aromatic plant, Sat = Satureja, Sat+Cor = Satureja + Coriandrum, Sat+Cor+Car = Satureja + Coriandrum + Carum.

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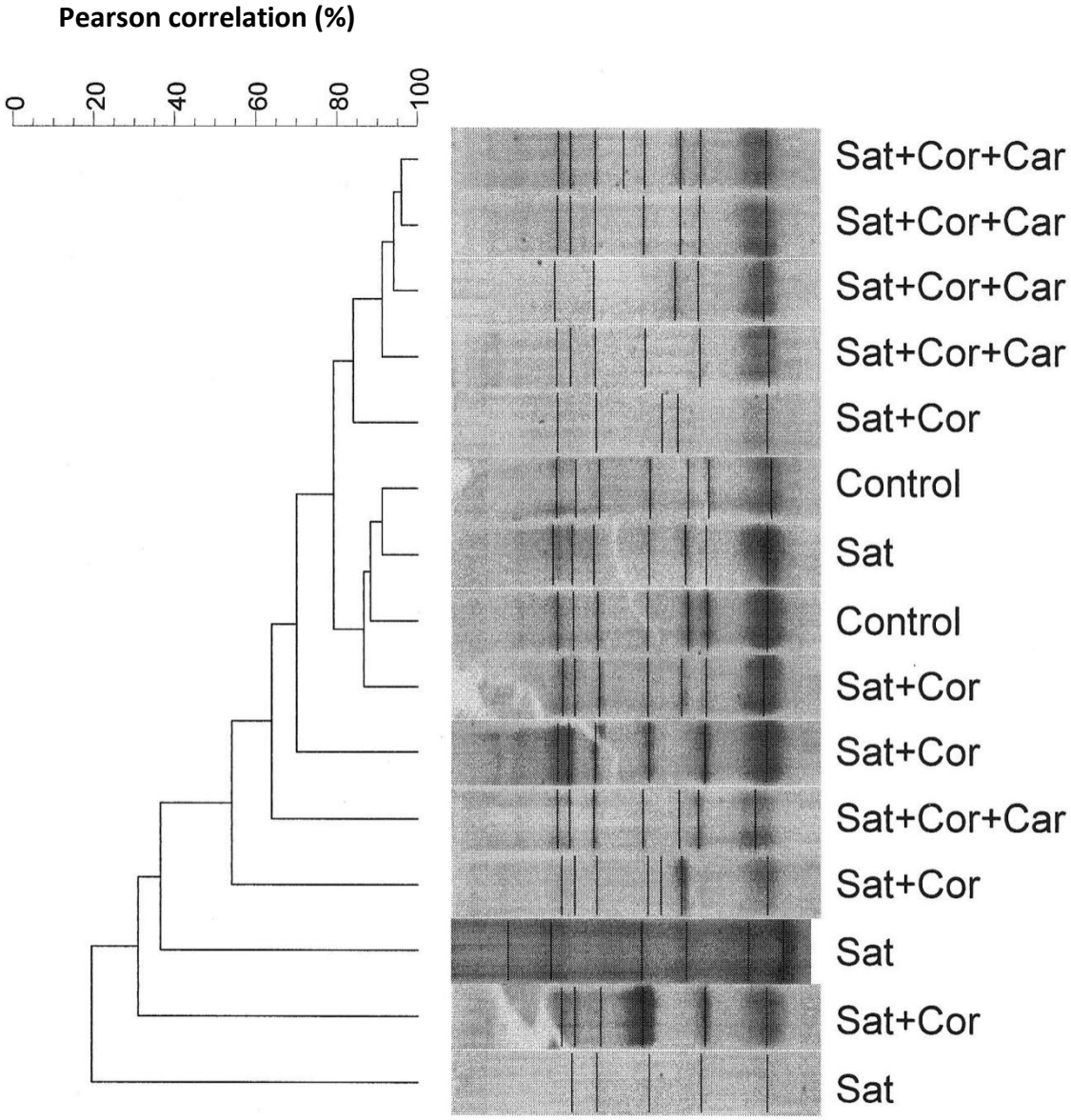

Figure 3. Dendrogram of archaea community obtained from the DGGE banding profile analysis of the liquid phase of the rumen in four different groups of ewes (4 each one) fed aromatic plants. Control = no aromatic plant, Sat = Satureja, Sat+Cor = Satureja + Coriandrum, Sat+Cor+Car = Satureja + Coriandrum + Carum.

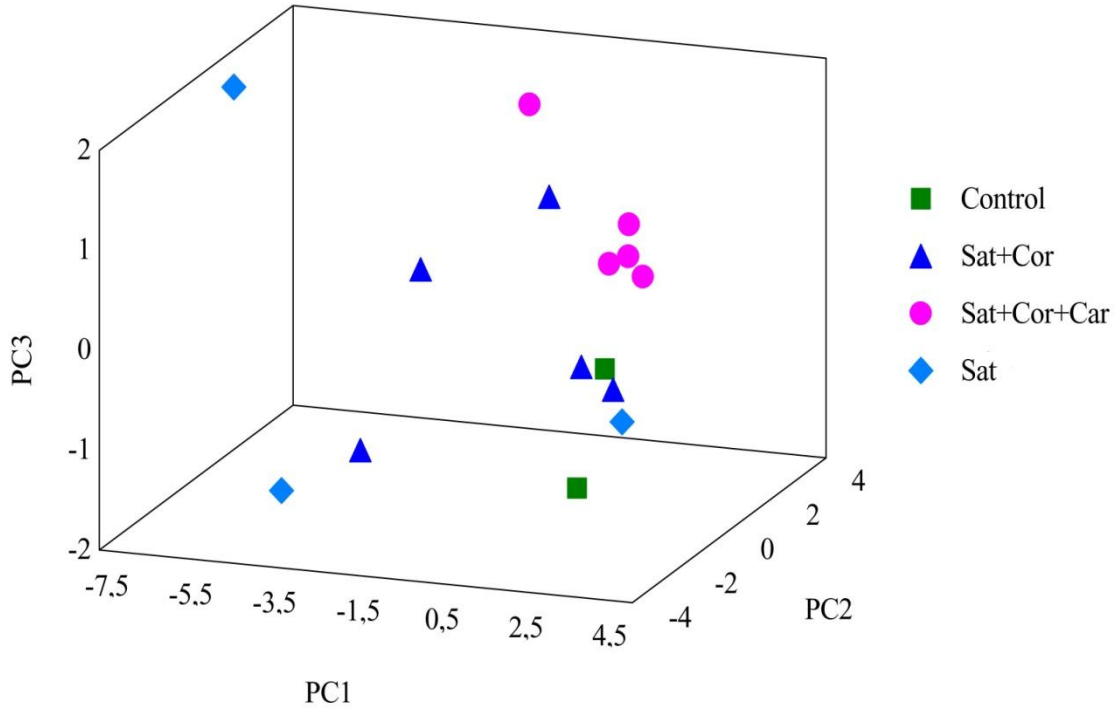

Figure 4. Ordination biplot for the principal component analysis of DGGE banding patterns for 16S rRNA gene of amplicons of the archea from the liquid phase of the rumen. Control = no aromatic plant, Sat = Satureja, Sat+Cor = Satureja + Coriandrum, Sat+Cor+Car = Satureja + Coriandrum + Carum.

General Conclusions

CHAPTER 5

Oscar Boaventura Neto

1. General Conclusions

- milk yield did not differ among aromatic plant species, even if the Carum group showed numerically higher milk yields than the other groups, whereas milk yield was affected by the effect of dose;
- milk lactose was higher for all three dosages of aromatic plants than for dosage zero;
- milk urea was in general higher in the control dose than in the treated groups, suggesting a certain effect of aromatic plants in reducing the degradation rate of proteins;
- the high dose of aromatic plant supply markedly modified milk fatty acids;
- there were no adverse effects of the utilization of aromatic plants on DM digestibility, whereas OM digestibility was the highest for the Satureja group;
- NDF digestibility was markedly higher (by 22%) in the three treated groups, despite the high level of lignification of the aromatic plants, compared to the Control group;
- the Satureja group had the highest numerical or statistical digestibility values for all nutrients considered, with the exception of EE, for which it had the lowest values. These results suggest that, among the four groups studied, Satureja ensured the best ruminal conditions;
- the presence of aromatic plants in the ration of pregnant ewes did not cause any negative effect on the animals;
- the addition of aromatic plants such as *Satureja* sp., *Coriandrum* sp. and *Carum* sp. to the diet showed quantitative anthelmintic effects (fecal egg count reduction) in the treated Sarda sheep;
- the ewes adapted in few days to the mixes with aromatic plants, despite they were totally unknown by the ewes and had marked aroma and taste;
- the aromatic plants did not cause any negative effect on the health of the animals;
- the aromatic plants tended to increase the ruminal pH, while did not influence rumen volatile fatty acid production and proportion;
- the analysis of rumen microbiota clearly showed that aromatic plants affected the ruminal bacteria of the liquid phase.