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CONJUGATED LINOLEIC ACID (CLA) CONTENT IN DIFFERENT TISSUES OF RUMINANTS FED WITH CLA SUPPLEMENTATION

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Riassunto

Dal 1987 anno in cui il Dott. Pariza e il suo gruppo di ricerca scoprirono i Coniugati dell'Acido Linoleico (CLA) molti sono stati gli studi che hanno cercato di definire le principali caratteristiche di queste molecole. Caratterizzati da un alto valore biologico gli furono attribuiti molti effetti benefici sulla salute umana, come l'effetto anticancerogeno, la riduzione del rischio di malattie cardiovascolari e la riduzione del rischio di sviluppo dell'aterosclerosi. In seguito gli fu attribuita anche importanza nel miglioramento delle *performance* animali, come l'aumento delle capacità di accrescimento, dell'efficienza alimentare e una riduzione della deposizione di grasso con conseguente aumento della massa magra. I Coniugati dell'Acido Linoleico (CLA) sono un gruppo d'isomeri geometrici e posizionali dell'Acido Linoleico caratterizzati da una catena di 18 atomi di carbonio contenente due doppi legami non in posizione classica (*cis*), ma coniugati dal carbonio 9, 10 o 11. I doppi legami possono presentare diversa disposizione spaziale dando origine a quattro diverse configurazioni: *cis/trans*, *trans/cis*, *cis/cis* e *trans/trans*. Secondo i carboni ai quali sono legati, possono avere diverse posizioni: ([7,9], [8,10], [9,11], [10,12], [11,13] e [12,14]) con un totale d'isomeri identificati pari a 24. I due più presenti e più identificati sono il C18:2*cis*9,*trans*11 (60-85% degli isomeri presenti nella carne e >90% nel latte) e il C18:2*trans*10,*cis*12. Altri isomeri molto presenti nella carne sono anche il C18:2*trans*7,*cis*9 e il C18:2*trans*11,*cis*13. Grazie alla capacità del rumine di produrre acidi grassi e in particolare CLA, si possono trovare soprattutto nei prodotti di origine animale (latte, prodotti lattiero caseari e carne). La presenza del doppio legame rende i CLA delle molecole complesse da identificare, perché può essere facilmente soggetto a fenomeni d'isomerizzazione o epimerizzazione che possono portare a un aumento delle forme di tipo *trans/trans* con conseguente riduzione delle forme *cis/trans* o *trans/cis*. Diversi studi hanno cercato di definire quale sia il metodo più adatto per l'estrazione del grasso (determinazione estratto etereo, EE) e per la successiva trasformazione in composti volatili, ovvero, esteri metilici degli acidi grassi (FAME). Per questo motivo parte della mia tesi è stata improntata su un approccio metodologico allo scopo di capire tra i tanti metodi quale fosse il più idoneo e che differenze i diversi metodi potessero avere. I dati contenuti nei contributi legati all'approccio metodologico sono stati raccolti da vitelloni maschi nati da un incrocio tra vacche da latte di razza Bruna e tori di razza Bianca Blu del Belgio. Questi animali sono stati allevati presso l'azienda sperimentale dell'Università degli Studi di Padova Lucio Toniolo. Durante tutta prova sono stati allevati in azienda e alimentati con una dieta a base di *unifeed* caratterizzata

da tre diverse integrazioni di CLA rumino protetti (*rp*CLA): 0, 8 e 80 g di CLA al giorno per ogni animale. La prova è terminata con la macellazione avvenuta in un macello esterno all'Università situato a Pergine (Provincia di Trento, Trentino Alto Adige). I tessuti utilizzati per le analisi sono stati prelevati in macello ed in laboratorio di Qualità carne durante lo svolgimento delle analisi di qualità. I tessuti prelevati e studiati sono stati tre: muscolo *Longissimus Thoracis*, grasso sottocutaneo e fegato. Nel primo contributo (Chapter 2) i tessuti (*Longissimus Thoracis*, grasso sottocutaneo e fegato) sono stati analizzati allo scopo di determinare il profilo acidico, confrontando tre diversi metodi di estrazione del grasso (Folch (1957), *Acellerated Solvent Extraction* (ASE) e Jenkins (2010)) e utilizzando come tecnica cromatografica la Gas Cromatografia a due dimensioni (GCxGC). La scelta di eseguire un confronto metodologico è legata al fatto che secondo il metodo d'analisi utilizzato i risultati sono diversi. Di conseguenza, uno degli obiettivi era trovare un metodo che fosse in grado di salvaguardare i CLA senza provocare isomerizzazioni. A questo è dovuta la scelta del metodo Folch (1957), uno dei più antichi e più utilizzati in matrici di diversa natura. Nato per essere usato in campioni con grasso molto ricco di fosfolipidi (come il grasso presente nel cervello), lavora a temperatura ambiente utilizzando una miscela di solventi composta da cloroformio:metanolo (2:1, v/v). Il metodo *Acellerated Solvent Extraction* (ASE) è stato utilizzato allo scopo di confrontare una metodica che lavora con alte temperature e pressioni (120°C e 20 MPa) con una miscela di solventi identica a quella usata nel metodo Folch (1957) (cloroformio:metanolo (2:1, v/v)). Grazie alle caratteristiche positive il metodo ASE, negli ultimi anni si è molto diffuso. Esso è caratterizzato da una maggiore velocità d'estrazione, un ridotto utilizzo di solventi e una minore laboriosità da parte degli operatori. Si pensa che le condizioni (temperatura e pressione) a cui lo strumento lavora possano provocare isomerizzazioni incrementando gli isomeri con configurazione *trans/trans*. L'ultimo è un metodo diretto che in seguito ad una fase preparativa (di liofilizzazione del campione fatta con lo scopo di rimuovere l'acqua) permette di ottenere gli esteri metilici degli acidi grassi (FAME) da poter analizzare in GCxGC. Questo metodo richiede un'esterificazione di tipo acido-basico. Il lavoro è stato suddiviso in quattro passaggi in modo da: identificare il potere di risoluzione e l'incidenza di picchi non identificati, lo studio delle fonti di variazione, test di Levene per determinare l'omoscedasticità o eteroscedasticità delle varianze e infine degli studi per valutare la correlazione tra metodi. L'incidenza di valori non identificati è legata al numero di picchi osservati, che dipendono dalla sensibilità del metodo e dal tessuto analizzato: fegato, grasso e muscolo (da 0.04 a 0.08, da 0.05 a 0.06 e da 0.05 a 0.12, rispettivamente). La maggiore incidenza di valori nulli è stata osservata nel fegato e in

particolare negli acidi grassi a corta/media catena (C8:0 e C10:0) e nel muscolo negli acidi grassi C24:0, C20:1t1*t unknown isomers* e nei PUFA*n*3 (C20:3*n*3, C20:4*n*3, C22:5*n*6) sempre con il metodo Jenkins (2010). Tra tutte le fonti di variazione analizzate lo scopo, era considerare le due principali che sono state molto significative (P<0.001) e sono rappresentate dal tessuto, dal metodo e dalla loro interazione. L'effetto della dieta è stato significativo per gli acidi grassi facenti parte dell'integrazione (C18:0, C18:1*cis*9 e C18:2*trans*10,*cis*12). Dai test di Levene è emerso che le varianze sono eteroscedastiche, tranne la dieta che è risultata, omoscedastica.

Il passo successivo è stato considerare una tecnica cromatografica alternativa al GCxGC che fosse più specifica per lo studio degli isomeri dei CLA (Chapter 7) Nonostante, il GCxGC abbia un maggiore potere di risoluzione non è in grado di identificare tutti gli isomeri dei CLA, ma solo i due principali (C18:2*cis*9,*trans*11 e C18:2*trans*10,*cis*12). Per questo motivo sono presenti altre tecniche cromatografiche che permettono un'identificazione più precisa come la cromatografia liquida su colonna d'argento (Ag^+HPLC). Il lavoro è stato svolto in collaborazione con il *Leibniz Insitute for Farm Animal Biology* (Dummerstorf, Germany) e in particolare l'unità di *Muscle and Biology Growth*. L'obiettivo in questo caso era identificare il maggior numero d'isomeri dei CLA presenti nel campione. I tessuti analizzati erano: muscolo (*Longissimus Thoracis*), grasso sottocutaneo e fegato. Il grasso (estratto etereo, EE) è stato ottenuto con il metodo Folch (1957) cui è seguita poi un'esterificazione acido-basica. Le analisi statistiche sono state eseguite considerando gli effetti di dieta, tessuto e ripetizione sulla distribuzione degli isomeri. La prima cosa che differenzia questa metodica dal GCxGC è il numero di picchi identificati che sono molto più elevati (13 isomeri). Questo tipo di tecnica premette un'identificazione precisa di molti isomeri dei CLA con un unico problema nell'identificazione dei picchi che si trovano nella regione dei *cis/cis* (l'ultima a comparire nel cromatogramma), per la quale è difficile identificare l'isomero C18:2*cis*9,*cis*11. Dai risultati è emerso che il tessuto è molto significativo (P<0.001) e la distribuzione degli isomeri è tessuto dipendente, con una concentrazione più alta nel grasso. L'effetto della dieta, in particolare quella con integrazione 8.0 g di CLA al giorno, è risultato significativo per gli isomeri principali (C18:2*cis*9,*trans*11 e C18:2*trans*10,*cis*12).

Nella seconda parte della tesi sono state prese in considerazione le fonti di variazione del profilo acidico, come razza, sesso, dieta, ordine di parto, età e tessuto (Chapter 3 e Chapter 4). Gli animali usati in queste prove appartengono a quattro razze ovine Venete: Alpagota, Brogna, Foza e Lamon che presentano diversa condizione di criticità; valutata in base al numero di capi allevati, di maschi arieti utilizzati per gli accoppiamenti e di allevamenti. Al

fine di salvaguardare e recuperare queste razze, Veneto Agricoltura, su indicazione della Regione Veneto ha creato l'azienda sperimentale di Villiago, un importante centro di conservazione di queste razze, che mira a produrre nuovi giovani riproduttori (agnelle e montoni) per gli allevatori interessati e a organizzare azioni a sostegno dello sviluppo dell'allevamento.

I dati riportati in questa tesi coinvolgono animali che sono stati allevati seguendo un piano di conservazione *in-situ* che prevedeva la collaborazione tra l'azienda sperimentale di Veneto Agricoltura "Villiago" e l'azienda sperimentale dell'Università di Padova "Lucio Toniolo". In totale gli animali utilizzati sono stati 115 e sono stati allevati tra Dicembre 2010 e Luglio 2012. Macellati a età diverse, erano considerati appartenenti a tre categorie: 31 agnelli da latte, 36 agnelli leggeri, 24 agnelloni pesanti e 24 pecore. Le tre prove (agnello leggero, agnellone pesante, agnello da latte-pecora) erano caratterizzate da diete diverse. Nella prima prova "agnello leggero" le diete utilizzate erano: pascolo (PAS), fieno e concentrati con integrazione CLA rumino protetti (*rp*CLA, 8.0 g/d/animale) (CLA+) e dieta con fieno e concentrati senza integrazione di *rp*CLA (CLA-). Nelle prove "agnellone pesante" e "agnello da latte-pecora" le diete erano composte da concentrati con integrazione *rp*CLA (8.0 g/d/animale per gli agnelloni pesanti, 12 g/d/animale per le pecore e 4.0 g/d/animale per gli agnelli da latte) (CLA+) e concentrati senza integrazione *rp*CLA (CLA-). Dai risultati è emerso che diversi fattori possono influenzare il profilo acidico e in particolare dalla prima prova che la dieta può avere un ruolo molto importante ($P<0.001$). La presenza del pascolo è risultata significativa per molti acidi grassi a catena dispari e catena ramificata (forme *iso* e *anteiso*) che tendono a essere meno elevati al pascolo. Il motivo è che con la dieta è possibile alterate il PH ruminale e la flora microbica in esso presente modificando così anche gli acidi grassi che questa può produrre. Il ruolo del pascolo è importante anche per quanto riguarda gli acidi grassi a lunga catena (LC-PUFA). Il pascolo può aumentare gli Ω3 (rispettivamente, PAS=2.70, CLA-=1.46 e CLA+=1.54) riducendo gli Ω6 (rispettivamente, PAS =3.76, CLA-=4.41 e CLA+=4.75) e di conseguenze anche il rapporto Ω6/Ω3. Rispetto alla dieta a base di concentrati con integrazione (CLA+) il pascolo ha portato a un aumento dei CLA (rispettivamente, PAS=0.80, CLA-=0.56 e CLA+=0.71). La dieta si conferma significativa, anche nelle prove "agnellone pesante" e "agnello da latte-pecora" con il confronto tra le diete a base di concentrati (CLA- e CLA+). L'integrazione di *rp*CLA nella dieta è risultata significativa ($P<0.001$ e $P<0.01$) per gli acidi grassi considerati i suoi principali costituenti, per cui il C18:0 e il C18:2*trans*10,*cis*12. Gli agnelli da latte hanno presentato dei risultati diversi dagli altri animali, soprattutto in relazione ai CLA. Sono risultati significativi gli

isomeri C18:2*cis*11,*trans*13 (P<0.01), C18:2*cis*11,*cis*13 (P<0.001) e C18:2*cis*9,*cis*11 (P<0.001). Il motivo di questa diversa distribuzione è stato attribuito al ruolo del latte di pecora, usato come alimento per gli agnelli e che può aver influito sulla composizione acidica dei loro tessuti. La razza in tutte le prove è stata un effetto che non ha portato a delle differenze significative dal punto di vista statistico, come anche il sesso e l'età di macellazione. Dai contrasti effettuati tra tessuti, è emerso, com'era stato osservato per i vitelloni, che la distribuzione degli acidi grassi è tessuto specifica e può cambiare secondo il tessuto analizzato. Differenze sono evidenti in tutti i tessuti ma in particolare tra i magri e quelli grassi. Il fegato in tutte e prove è stato il tessuto che presenta delle caratteristiche particolari perché caratterizzato da una maggiore quantità di acidi grassi polinsaturi (PUFA) e in particolare da una maggiore quantità di Ω3 e CLA. La diversa composizione di questo tessuto è legata alla composizione in termini di tipologia di lipidi che lo costituiscono e dai processi metabolici ai quali quest'organo partecipa che portano alla produzione, oppure all'assorbimento degli acidi grassi assunti con la dieta (Chapter 3 e Chapter 4).

Sempre in animali appartenenti alle razze (Alpagota, Brogna, Foza e Lamon) sono state prese in esame le *performance* animali ed i vari fattori che le possono influenzare. E' noto che l'utilizzo di *rp*CLA può provocare un incremento dell'efficienza alimentare favorendo l'aumento della massa magra con la successiva riduzione di quella grassa. Negli animali in lattazione (vacche, pecore e capre) è dimostrato che l'integrazione di *rp*CLA può provocare una riduzione nel contenuto di grasso presente nel latte. Nella mia tesi è stato considerato l'effetto della dieta sul latte di pecore (Chapter 5) al fine così di valutare quelle che vengono definite proprietà di qualità del latte espresse in termini di composizione chimica e proprietà di coagulazione (MCPs). Gli animali utilizzati in questa prova sono i medesimi utilizzati per lo studio dei profili acidici delle carni. Dal punto di vista della qualità della carne alla macellazione per cercare di definire se dieta, sesso e razza potessero influire sulle rese di macellazione (Chapter 6). Gli animali usati in questa prova sono i medesimi della prova vista in precedenza, ovvero la prova "agnello leggero". I risultati ottenuti in questo lavoro sono dei risultati che comprendono solo parte del lavoro che invece comprenderà anche le prove "agnellone pesante" e "agnello-pecora". Da questo lavoro quello che si vuole esprimere è che gli animali appartenenti a queste razze sono in grado di produrre delle carcasse che presentano caratteristiche ottimali e idonee al mercato alimentare. La caratteristica in più che queste presentano è che le modalità di allevamento, ben lontane da quelle di tipo industriale o più specializzato ci permettono di ottenere un prodotto allevato nel rispetto delle tradizioni e che

può far parte di presidi ampiamente riconosciuti come l'Agnello Alpagoto noto per essere un presidio *Slow Food*.

Summary

Conjugated Linoleic Acid (CLA) are a group of positional and geometric isomers of Linoleic Acid characterized by a carbon chain containing 18 carbon atoms and two double bonds, not in the classic position (*cis*), but conjugated from the carbons atoms 9, 10 or 11. Double bonds have different position in the carbon chain ([7,9], [8,10], [9,11], [10,12], [11,13] and [12,14]) and four different geometric distribution (*cis/trans*, *trans/cis*, *cis/cis* and *trans/trans*). In total 24 possible isomers are identify, but the two most present and often most identified are: C18:2*cis*9,*trans*11 (60-85% of the isomers identify in meat and >90% in milk) and C18:2*trans*10,*cis*12 isomers. Observing meat samples other isomers are commonly identified, as example, C18:2*trans*7,*cis*9 and C18:2*trans*11,*cis*13. In 1987, CLA have been discovered thanks to the research group of Dr. Pariza that studying some carcinogenic components in grilled meat, identify these molecules with anticancer activity, called Conjugated Linoleic Acid. After the discovery, they still receive many attention because of their biological activities and implication on human health. The biological effect are due to the separate actions of the main isomers (C18:2*cis*9,*trans*11 and C18:2*trans*10,*cis*12) and sometimes by the synergistic action of both. Also, minor isomers have biological effect, as examples, C18:2*trans*9,*trans*11 that inhibits platelet aggregation and has anti-proliferative effect and C18:2*cis*9,*cis*11 that showed anticancer effect. In animal model, CLA not only reduce initiation, promotion and progression steps of cancer development, but also reduce metastasis. Nevertheless, was show that CLA isomers are important because of they have effect on animal *performance* and the principal effects are: prevent chemically-induced tumors, protect against the catabolic effects of immune stimulation, improve feed efficiency, reduce excess body weight gain, reduce body fat, increase lean body mass and lower blood lipids. In human diet, the main sources of CLA are representing by ruminants products, in particular milk, dairy product and beef. The higher concentration of CLA in these products is linked to the presence of rumen that through microbial biohydrogenation (*Butyrivibrio fibrisolvens)* can transform Linoleic Acid to the major isomer C18:2*cis*9,*trans*11 (Rumenic Acid). Conjugated Linoleic Acid (CLA) can be analyze with different methods of lipid extraction and derivatization, but always after transformation in methyl ester derivatives of fatty acids (FAMEs) that is carried out in a simple reaction. This simple reaction becomes more complicated because of conjugated fatty acids are involved and the presence of conjugated double bond makes them unsuitable for the most common techniques employed for fatty acids analysis. The presence of double

bonds may increase isomerization and epimerization of these bonds, increasing CLA with *trans*/*trans* configuration and reducing *cis*/*trans* or *trans*/*cis* configuration. For this reason is very important find a suitable method for lipid extraction (and the resulting determination of crude fat) and subsequently for the transformation in methyl ester derivatives of fatty acids (FAMEs). For this reason at the first part of my thesis was given a methodological approach in order to understand the differences between methods and which is the most suitable (Chapter 2 and Chapter 7). All the data used, belonging to a trial carried out at "Lucio Toniolo", the Experimental Farm of the University of Padova in Legnaro (Padova, Italy). Animals used are a crossbreed between Belgian Blue bulls and Brown Swiss dairy cows fed with one of 3 experimental diets. Diets were composed by *unifeed* and differ depending on the rumen protected CLA supplementation (*rp*CLA), overall, three supplementation are available 0, 8 and 80 g/d/animal. The trial ended in March 2011 when animals were slaughtered outside the faculty in a slaughterhouse located in Pergine province of Trento (Trentino Alto Adige Region). Samples were collected and in particular three tissues were subjected to analysis for fatty acids profile and CLA content: muscle *Longissimus Thoracis*, Subcutaneous Fat and Liver. In the main Chapter (Chapter 2) tissues (*Longissimus Thoracis*, Subcutaneous Fat and Liver) were analyzed for determine fatty acids profile comparing three different methods of extraction (Folch (1957), *Accelerated Solvent Extraction* (ASE) and Jenkins (2010)) using as chromatography technique two dimensional GC (GC×GC). The purpose of this trial was identify among the different methods of extraction, one method that is able to identify CLA isomers without causing isomerization. Folch (1957) is one of the older and most used methods. It was born for analyze samples rich in phospholipids (as lipids of brain). It works at room temperature using a mixture of solvents composed by chloroform/methanol (2:1, v/v). *Accelerated Solvent Extraction* (ASE) was used with the purpose to compare a room temperature method with a method that works at high temperature and pressure (120°C and 20MPa) with the same mixture of solvents (chloroform/methanol, 2:1, v/v). This method, which have recently been introduced; reduce the use of solvent and saving time at work, giving results that were similar or better if compared with the conventional Folch (1957) extraction. On the contrary, it is expected to increase the isomerization of double bonds and the isomers with *trans*/*trans* configuration. The last method, Jenkins (2010), is a direct method, which reduces the length of the total procedure, saving time at work, reducing the sample amount, reducing the use of solvents, giving analysis less expensive and easier. The main characteristic is that lipid extraction step is avoided and fatty acids are extracted and *trans*-esterified in the same time. The statistical

analysis was performed in four steps: resolution power and assessment of the number of undetected FA, study the main sources of variation, using Levene's test explored the variances homogeneity for the main sources of variations and relationships between methods. The incidence of undetectable values on the total number of expected observation, which depend on the sensitivity of the method used, for liver, fat and muscle, ranged 0.04 to 0.08, 0.05 to 0.06, and 0.05 to 0.12, respectively, with incidences greater for the Jenkins method compared to the other two for liver and muscle samples but not for subcutaneous fat. In liver the highest incidence of null values with the Jenkins method was mainly observed for short chain FA (C8:0 and C10:0), whereas in the case of muscle the highest incidence of undetectable values was mainly observed for the C24:0, for two C20:1*t* unknown isomers, and for Ω3 (C20:3*n*3, C20:4*n*3, C22:5*n*6). Many sources of variation result high significant (P<0.001). Diet was significant in particular for the FA that are the main components of the supplementation (C18:0, C18:1*cis*9, C18:2*cis*9,*trans*11 and C18:2*trans*10,*cis*12). The results of the Levene's test evidenced, except for diet, as the variances among levels within tissue, method or method×tissue were not homoscedastic for the large majority of the FA. The second part of the methodological approach is reported in Chapter 7 (*Minor Chapter*) and considers the effect of the chromatography technique on CLA isomers content. Despite the high resolution power of GC×GC this technique allows to identify all the FA and the CLA isomers recognized by internal standard. However, it is not able to identify the other isomers and for this reason is necessary to find a new method that allows a clear and complete identification of CLA. Silver Ion High Performance Liquid Chromotography (Ag^+HPLC) is currently the most effective way to separate and quantitate individual isomers of CLA in beef. This part was performed with the contribution of the Leibniz Institute for Farm Animal Biology (Dummerstorf, Germany) and in particular the Muscle and Biology Growth Unit. Tissue analyze in this trial were *Longissimus Thoracis*, Subcutaneous Fat and Liver. Lipids were extracted using Folch (1957) and methylated using and acid-base catalysis. Data were analyzed considering as main sources of variation: diet, tissue and repetition. Tissue resulted always significant (P<0.001) with a tissue depending distribution of the isomers and a higher concentration in subcutaneous fat. Diet was significant for the main isomers (C18:2*cis*9,*trans*11 and C18:2*trans*10,*cis*12) because of constituents *rp*CLA supplementation.

In the second part of my thesis are considered the effects that can modify FA profile, such as, breed, gender, diet, type of birth, age and tissue. Animals used belonging to four native alpine sheep breeds: Alpagota, Brogna, Foza and Lamon. All these breeds represent

an important genetic resources and the purpose is created a program of conservation for increase the number of animals in these populations. For this reason Veneto Agricoltura created the experimental farm "Villiago" (Belluno province, Veneto Region, North Italy). Animals used for this research belongs to two flocks undergoing an *in situ* conservation program between "Lucio Toniolo" Experimental Farm of the University of Padova and the Experimental Farm of Veneto Agricoltura located in Villiago. In total 115 animals were used and reared in the period between December 2010 and July 2012. Animals are slaughter at different age and they are considered belonging to three different categories: 31 suckling lambs, 36 lambs, 24 heavy lambs and 24 ewes. The different trial were characterized by different diets: pasture (PAS), penned in the open barn and fed with hay, concentrate and supplemented with rumen protected Conjugated Linoleic Acid (*rp*CLA) product (CLA+) and penned in an open barn and fed with hay and concentrate (CLA-). In trials with "lambs" and "heavy lambs" animals were supplement with 8.0 g/d/animal of rpCLA. In "suckling lambs" and "ewes" animals were supplement with 4.0 g/d/animal of *rp*CLA and 12 g/d/animal of *rp*CLA, respectively. From the results, is possible observe that diet is highly significant ($P<0.001$) and in particular pasture. Fatty acids are statistically influenced by pasture and mainly fatty acids with odd chain and branched fatty acids (*iso* and *anteiso*) that tend to be lower in diet at pasture. The reason is that the diet can influence ruminal Ph and microorganisms that consequently change reactions and final products. Pasture is also important because it can influence the amount of long chain fatty acids (LC-PUFA), increasing Ω3 (respectively, PAS=2.70, CLA-=1.46 and CLA+=1.54), reducing Ω6 (respectively, PAS=3.76, CLA-=4.41 and CLA+=4.75) and Ω6/Ω3 ratio. CLA isomers content is higher in pasture than in diet with concentrate supplement with *rp*CLA (respectively, PAS=0.80, CLA-=0.56 and CLA+=0.71). In the other two trials characterized by CLA+ and CLA- diet was considered an important and significant effect. *rp*CLA supplementation reported significant effect ($P<0.001$ and $P<0.01$) for FA constituents of supplementation, such as C18:0 and C18:2*trans*10,*cis1*2. In suckling lambs results were different from other trials, in particular for CLA isomers, such as, C18:2*cis*11,*trans*13 ($P<0.01$), C18:2*cis*11,*cis*13 ($P<0.001$) and C18:2*cis*9,*cis*11 ($P<0.001$). The reason is the different distribution is ewe's milk that was used in suckling lambs diet and could have influence their fatty acids profile. Breed was not a significant effect, such as, gender and age at slaughter. Observing data about orthogonal contrast FA and CLA isomers distribution is tissue specific and there are many differences between lean tissues (muscle) and fat tissues. Liver has particular characteristics, in fact, its content of PUFA and particular Ω3 and CLA

is higher than in the others tissues. Differences of this tissue are linked to its specific lipid composition and metabolic process which lead to the production and absorption of fatty acids.

In Chapter 5 and Chapter 6 were evaluate effects that CLA supplementation can have on animal *performance* because of many research have been conduct *in vitro*, but other research are needed.

In Chapter 5 was examined the effect of *rp*CLA supplementation in lactating ewes on their milk composition and Milk Coagulation Properties (MCPs) of sheep's milk. Animal used in this trial are the same of the trial describe above and in particular of the trial call "suckling lambs" and "ewes".

This study allowed to know that *rp*CLA supplementation in sheep can change the composition and cheese-making properties of milk, as example, delaying gelation, slowing curd firming and accelerated syneresis and future studies are necessary to know the effect of CLA on cheese yield/ quality. *rp*CLA supplementation affect milk composition, reducing protein content, solid non-fat content, casein index and increasing SCS. *rp*CLA supplementation had negative effects on parameters of coagulation and curd firming.

In Chapter 6 was evaluate the effect of breed and sex on growth rate, slaughter traits and meat quality traits of lambs of Alpagota, Brogna and Foza breeds. Animals used in this trial are the same of the trial describe above and in particular of the trial call "lambs". These results are part of a bigger study which comprises also growth rate, slaughter traits and meat quality of the trial call "heavy lambs", "suckling lambs" and "ewes". Observing the results of this trial is possible obtain lamb carcasses and meat with valuable characteristics that can be exploited through typical products and food preparation in local markets and gastronomy, according to tradition. The valorisation of these productions can be an important tool for the *in situ* conservation of these breeds. As example, *Slow Food* organization has recognized "Agnello Alpagoto" (lambs of Alpagota breeds) as a *Slow Food* Presidium.

CHAPTHER 1: General Introduction

PART I: LIPID GENERAL INTRODUCTION

1.1 Lipid classification and major sources

The first study carried out on the chemistry of lipids begin from pioneering researcher, such as Robert Boyle, Poulletier de la Salle and Antoine François de Fourcroy from the 17th and 18th century. In the 19th century the chemist Chevreul, identified several fatty acids (FA) called "cholesterine" and for the substance identify in gallstones coined the word "glycerine" showing that fats are made by glycerol and fatty acids. In 20th century many discoveries are made in term of understanding lipids structures and functions, studying the relationship between lipids and health diseases. Lipids include waxes, oils, fats, steroids and related compounds ranging from soap to petrochemicals, triacylglycerols and phospholipids. Triacylglycerols (TAG), which are neutral lipids, are made up of three fatty acids attached to a molecule of glycerol and they vary in their physical properties according to the chemical structure of these fatty acids. Phospholipids (PL) are lipids that contain phosphoric acid as mono o diester and are the main components of cell membranes. Fatty acids are "amphiphilic" molecules that have a carboxyl group at the polar end (hydrophilic) and hydrocarbon chain at the non polar tail (hydrophobic). In relation to the double bond composition, they are divided in two groups: Saturated Fatty Acid (SFA) characterized by a carbon chain with only single bonds and Unsaturated Fatty Acid (UFA) characterized by carbon-carbon double bonds in the carbon chain. Unsaturated Fatty Acid are divided in: Monounsaturated Fatty Acid (MUFA), such as Oleic Acid (C18:1*cis*9) that contain one double bond in the carbon chain and Polyunsaturated Fatty Acid (PUFA) that contains more than one double bonds. Normally PUFA are derived from vegetables oil but Polyunsaturated Fatty Acids with twelve or more double bonds in their carbon chain (called Long-Chain Polyunsaturated Fatty Acids, LC-PUFA) are typical of fats from animals origins. According to the geometric configuration and the position of the double bond in the space, fatty acids have two different configuration: *cis* and *trans* (Figure 1) (Webb & O'Neill, 2008).

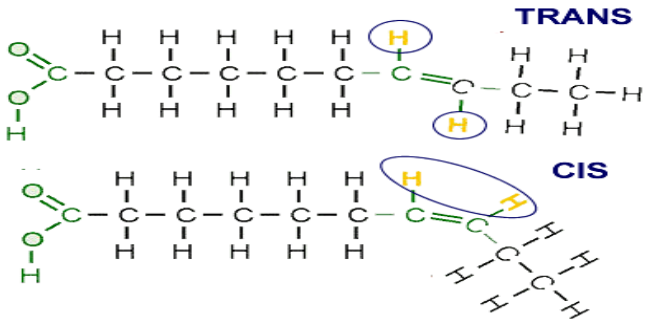


Figure 1: *Cis* and *Trans* double bonds configuration in the carbon chain.

Fatty acids described above are associated to human health benefits or diseases. Many studies have indicated that meat and its content in SFA, especially Miristic Acid (C14:0), Palmitic Acid (C16:0) and *trans*-fatty acids (TFA), are responsible of many diseases, such as cardiovascular diseases, atheroscelosis diseases and colorectal cancer (De Smet et al., 2004; Turk and Smith, 2009). In next twenty years, and in line with an increasing of world population, the consumption of meat and milk is predicted to increase in all the world. These two products are found to supply 25% of total dietary energy intake, contributing also to half of the Saturated Fatty Acids (SFA) intake and the largest MUFA intake if referred to meat products (Woods and Fearon, 2009). As consequence, International Medical Institution recommend to pay attention at the dietary fat intake, the type of fats and daily amount of calories. In most developed countries is recommended to the population a decrease in the total fat, SFA and TFA intake and an increase in the consumption of Long Chain Polyunsaturated Fatty Acids (LC-PUFA) in particular of the Ω3 family, such as C20:5*n*3 and C22:6*n*3 (Chizzolini et al., 1999, Shingfield et al., 2013; Givens, 2010). Regarding these potential effects of red meat and its products, the German Nutrition Society (DGE) recommends the restriction of its consumption to 600 g per week and a daily fat intake of up on 30% of the total daily energy intake (<10% should be SFA, approximately 7-10% should be PUFA and the remaining 10% should be MUFA). Red meat is a sources of high biological value protein a significant source of important trace elements (iron, zinc, selenium), water, fat soluble vitamins (A, B_6, B_{12}, D and E) and phosphatidylcholine (Dannenberger et al., 2013). It is also a sources of essential fatty acids, which have important biological activities: Omega 3 (Ω3), Omega 6 (Ω6) and Conjugated Linoleic Acid (CLA). Omega 3 (Ω3) and Omega 6 (Ω6) are require with the diet because they cannot be synthesized *de novo* and they play an important role in the immune response and as carriers of fat-soluble vitamins (vitamins A, D, E and K). Other essential fatty acids are Linoliec Acid (C18:2*n*6), α-Linolenic Acid (C18:3*n*3), Arachidonic Acid (C20:4*n*6) and

Eicosapentaenoic Acid (EPA-C20:5*n*3). The C20:4*n*6 and EPA-C20:5*n*3 are formed respectively by desaturation and elongation of Linoleic Acid and desaturation and elongation of α-Linolenic Acid. Also, Conjugated Linoleic Acid are responsible of many biological effects on human health and in particular the two major isomers (C18:2*cis*9,*trans*11 and C18:2*trans*10,*cis*12) (Webb & O'Neill, 2008). Many factors can influence this fat and fatty acids composition and we shall see below the main.

1.2 Factors affecting meat fatty acid profile

As well as the fat level could be up to 15% depending on the breed, age and diet also the fatty acids profile can be modified by many factors. Beef fat contains 25-30% of the C16 carbon fatty acid palmitate, known for his hypercholesterolemic affect. On the other hand, C18 carbon chain fatty acids (C18:0) has a lower atherogenic effect, very similar to short chain fatty acids. Furthermore, Stearic Acid in liver is subjected to a conversion in Oliec Acid by Δ^9desaturase. For this reason has prompted attempts to modify the fatty acids composition on beef increasing C18 carbon fatty acid at the expense of palmitate. Fatty acids composition can be changed by altering the diet, fatness, the selective breeding, age, body weight, gender, application of hormones, tissue, type of lipids and post harvest related factors. These factors can be classified into different groups: intrinsic to the animal (breed, gender, age, fatness and tissue) and extrinsic (diet). Below, the main factors are briefly described:

- **Diet:** This effect is strictly connected with the assumption that lipids in various tissues (adipose and skeletal) strongly reflect the major dietary fatty acids (FA). This potential effect is greater on FA composition of monogastric animals depots than in ruminants. It's because of Saturated (SFA) and Unsaturated Fatty (UFA) acids from the diet pass through the digestive system without changing and after they are deposited. The possible ways in which the diet can act are the addiction of particular supplementation, as example, linseed or linseed oil that increase the concentration of C18:3*n*3 in tissue with a decrease in the Ω6/Ω3 ratio. On the contrary, sunflower oil can increase the concentration of C18:3*n*6 in tissue with an increase in the Ω6/Ω3 ratio. The inclusion of C18:3*n*3 generally increase the concentration of EPA but not of DHA (Scollan et al., 2006). Consumption of rumen protected lipids and in particular rumen protected CLA (*rp*CLA) is possible and it is responsible to the increase of their content in tissues. In particular the two main CLA isomers, that normally are part of the

supplementation (C18:2*cis*9,*trans*11 and C18:2*trans*10,*cis*12). The CLA supplementation is another possibility to change the FA profile, but it dramatically reduces milk fat yield (Banni et al., 1996; Schiavon et al., 2011; Chouinard et al.,1999). Pasture can influence FA profile but the nutritional improvement is dependent on the duration of grazing. Many studies report that pasture has been shown to increase the CLA concentration and decrease the Ω6/Ω3 ratio. The C18:2*cis*9,*trans*11 isomer percentage concentration increase significantly and was higher in grass-based system (0.87% *vs* 0.72% in German Simmental bulls and 0.84% *vs* 0.75% in German Holstein) (Noci et al., 2007; Nuernberg et al., 2005). However, pasture can increase Stearic Acid (C18:0), Vaccenic Acid (C18:1*trans*11) and reduce Linoleic Acid (C18:2*n*6) as part of the family of Ω6 (Noci et al., 2007; Pordomingo et al., 2012a). Grass based diet showed an higher lipid oxidation capacity than the indoor-fed animals and the growth performance of animals are lower, producing leaner animals with lower intramuscular fat content (and different color characteristic) (Pordomingo et al., 2012b);

- **Carcass fatness:** not many study are reported, but Wood et al. (2008) report an increase of fatness with the increase of CLA supplementation;
- **Age and body weight:** the amount of adipose tissue increases with age and the effect of age on fatty acids profile is also related to body fatness and the capacity of fatty acids to change during the life of animals. As example, in lambs the accumulation of Saturated Fatty Acids (SFA) increase with age and growth of animals but PUFA decrease (Matsushita et al., 2010). Slaughter weight results significant in fatty acids profile because of an increase of it correspond a reduction in the percentage of phospholipids (PL) and monoglycerides (MG) and a reduction of Cholosterol (C) and Cholesterol Esters (CE). Changing in the FA composition correspond to an increase of SFA and MUFA percentage and the decrease of PUFA with increasing of slaughter weight was observed and this is likely due to the increasing importance of neutral lipids in total lipids as fattening proceeds (Indurain, Beriain; Sarries and Insausti, 2010). Lambs slaughter at different body weight have different CLA isomers content, in fact, animals slaughter at 17 months contain an higher amount total FA in compare to animals slaughter at 11 months (1422.97 mg/100 g of total lipids *vs* 1689.87 mg/100 g of total lipids) (Serra et al., 2009);
- **Gender:** important mainly for the effect on carcass fatness because at equal slaughter weights male are leaner than gilts which in turn are leaner than male castrates. As example, PUFA and Linoleic Acid content in cover fat decrease in order of: males >

females > male castrates but on the contrary SFA increased. Borys et al. (2007) confirm this result reporting that gender not influence SFA content but MUFA content is higher in rams compared to ewes. In lambs no differences were found in mean CLA content according to sex of lambs;

- **Breed:** this effect significantly affect FA profile and it is connect with the carcass fatness (Demirel et al., 2006). In cattle, there are genetic-based differences in intramuscular fatty acid composition of *Longissimus* muscle. An example of genetic effect is give by the double muscle genotype (mh/mh) within the Belgian Blue Breed that has low proportions of C18:1*cis*9 and high proportion of C18:2*n*6 in muscle lipid compared with normal genotype. De Smet at al. (2004) report that CLA content was positively related to the total intramuscular fatty acid content due to the fact that CLA are mainly found in the triacylglicerol fraction. Also in dairy cow is suggested that Holstein-Friesian have a greater activity and expression of Δ^5 and Δ^6 desaturase enzymes. Belgian Blue genotype converts a higher proportion of C18:3*n*3 to C20:5*n*3(EPA) and C22:5*n*3 but not C22:6*n*3 (Wood et al., 2008). Smith et al. (2009) suggest that breeds type differ in the ability to accumulate MUFA in their adipose tissues and for this reason Brahman cows and steers contain a greater proportion of MUFA and less SFA than adipose tissue from Hereford steers when cattle are raised under identical condition;

- **Applications of hormones:** A variety of hormonal and neural influences allowing the hydrolysis of adipose tissue triacylglycerols, stored in adipose tissue, releasing their energy. The key enzymes of lipolysis are two lipases: lipoprotein lipase and hormone sensitive-lipase (Nürnberg, Wegner and Ender, 1998).

- **Type of lipids:** the major lipid class in adipose tissue are neutral lipid. In muscle an high amount is given by phospholipids which has a high amount of PUFA in order to perform their function as constituent of cellular membranes. Wood et al. (2008) reported that in different species analyzed (pigs, sheep and cattle) Oleic Acid is much more present in neutral lipid. On the contrary, Linoleic Acid and Linolenic Acid (both C18:3*n*3 and C18:3*n*6) are much higher in phospholipids. In pigs and sheep muscles Linoleic Acid and Linolenic Acid are detected also in neutral lipids. CLA are higher in neutral lipids than in phospholipids. As confirm by Raes et al. (2004) phospholipids content is constant and not influenced by species, breed, nutrition and age. The only factor that can influence their content are the metabolic fibre type of muscle: if they are

more oxidative muscle contain a higher phospholipids proportion due to the higher amount of mitochondria

- **Tissue:** FA composition change in relation to the anatomical location and the tissue. As example, muscle contains an higher concentrations of the long chain Ω6 and Ω3 fatty acids. Many studies report an important effect of tissue, Garcia et al. (2008) studying FA composition of *Longissimus Dorsi* and leg muscle from Patagonia lambs reports the existence of differences also between muscles (leg muscle has higher content of FA if compare to *Longissimus Dorsi).* Also Schiavon et al. (2011) report a different composition of tissues between muscle and fat tissues and in particular between different fat tissue (inter-muscular fat and cover fat).

Finally, CLA content can be influences by post-harvest related factors, such as processing conditions, storage, cooking, aging and converting one product to another (milk into cheese or yogurt). Therefore, many studies found that CLA content in this case depend largely on the CLA content of the original milk or meat (Khanal and Olson, 2004). Regarding meat products, for example, studies conducted on cooking methods and temperature methods concluding that neither the method and the temperature has significant effects on CLA composition (Fritsche and Steinhart, 1998).

1.3 Conjugated Linoliec Acid (CLA) and Biosynthesis

The usual configuration of fatty acids which contain double bonds is the *cis* configuration and the double bonds are normally positioned at the 3rd, 6th or 9th carbons atom from the terminal methyl group, as example, Oleic Acid (C18:1*cis*9) and Linoleic Acid (C18:2*cis*9,*cis*12 or C18:2*n*6). However, some fatty acids have one or more double bonds in the *trans* configuration and they are called *Trans* Fatty Acids (TFA) and Conjugated Linoleic Acid (CLA). The term Conjugated Linoleic Acid and its acronym CLA represent a group of positional and geometric isomers of the Ω6 essential fatty acid, Linoleic Acid (Fritsche and Steinhart, 1998; Kelly, 2001). CLA would have 24 possible isomers, because they have been reported to contain conjugated double bonds in different positions in the carbon chain and in particular at the positions: [7,9], [8,10], [9,11], [10,12], [11,13] and [12,14]. These double bonds could have four different geometric configurations in relation to the position of bonds: *cis*/*trans*, *trans*/*cis*, *cis*/*cis* and *trans*/*trans*.

They were firstly discovered (1987) by Dr. Pariza and his group at the University of Wisconsin-Madison when studying the carcinogenic component of grilled beef, surprisingly

and for the first time, were found one of their principal effect, the anticancer capacity (Whale et al., 2004; Park, 2009). Not all the isomers are identify though gas chromatographic analysis but it was assume that the two main isomers, in which have focused the early study, are C18:2*cis*9,*trans*11 and C18:2*trans*10,*cis*12. A number of study reported the presence of CLA isomers in human adipose tissue, bile, blood and milk. CLA are incorporated into both neutral lipids and phospholipids (Kramer et al., 1998). Two main isomers are the most common found in meat from ruminant species and bovine dairy food products, but minor components, such as the [*t*7,*c*9], [*t*8,*c*10], [*t*11,*c*13], [*c*11,*t*13] and [*t*12,*t*14] isomers and their *cis*/*cis* and *trans*/*trans* isomers were also present in these products. As reported by Dhiman et al. (1999), the high presence of CLA in milk and meat products from ruminants is because there have two different ways of production:

- **Ruminal Biohydrogenation:** in which, lipids that come from the diet are transform as a result of rumen microbial biohydrogenation. Lipids composition of forage consist in glycolipids and phospholipids and the major fatty acids are the unsaturated fatty acids, as example, Linoleic and Linolenic Acid (C18:2*n*6 and C18:3*n*3). On the contrary, lipids composition of feedstuffs are predominantly triglycerides containing Linoleic and Oleic Acid (C18:2*n*6 and C18:1*cis*9). The main isomer C18:2*cis*9,*trans*11 (Rumenic Acid) is formed by Linoleic Acid isomerase enzyme activity generated by *Butyrivibrio fibrisolvens* and other endogenous bacteria found in the rumen of cattle and dairy cows. Rumen bacteria are divided in two main groups, based on the reactions and end products of biohydrogenation: Group A or bacteria that are able to hydrogenate Linoleic and Linolenic Acid to Vaccenic Acid (C18:1*trans*11). Group B: bacteria that utilize Vaccenic Acid (C18:1*trans*11) transforming this in Stearic Acid (C18:0). As reported in Figure 2, ruminal biohydrogenation is divided into two important transformation: the initial that is a prerequisite for the second transformation, and consist in an hydrolysis of the esters linkages catalyzed by microbial lipase. The second transformation or biohydrogenation of unsaturated fatty acid that consist in a reduction of C18:2*cis*9,*trans*11 to C18:1*trans*11. Other studies, have isolate also a *Propionibacter* that converts Linoleic Acid to C18:2*trans*10,*cis*12, confirming that certain rumen bacteria has the possibility to produce this isomer as the main Rumenic Acid (Bauman et al., 1999);

- **Endogenous synthesis of CLA (*De novo* synthesis):** was introduced by the precursor C18:1*trans*11 (Vaccenic Acid) that is converted to C18:2*cis*9,*trans*11 by

oxidation at the ninth carbon of the fatty acid by the enzyme Δ^9desaturase, that is encoded by the Stearoyl-CoA-desaturase (SCD) gene, present in mammary gland, in muscle, duodenal mucosa and liver (Mir et al., 2010; Corl et al.,2001). Adipose tissue seems to be the major site of endogenous synthesis of C18:2*cis*9,*trans*11 in growing ruminants and mammary glands in lactating animals (Bauman et al., 1999; Duffy et al., 2006). Regarding this synthesis that occurs in the tissues there are three different fatty desaturases (Δ^5,Δ^6 and Δ^9) and one elongase. The elongase adds 2 carbon unit of fatty acid chain and thus converts C16 to C18. Δ^5,Δ^6desaturase were involved in the conversion of C18 PUFA to their long chain derivatives. Δ^9desaturase insert a double bond at the ninth carbon atom of the fatty acid chain and in this way is responsible to the conversion of saturated to monounsaturated. The activity of this enzyme is far greater for C18 fatty acids than for C16 fatty acids (Malau-Aduli et al.,1997; Mele et al., 2007). On the contrary, mammary gland doesn't possess Δ^{12}desaturase, so this tissue could not convert C18:1*trans*10 to C18:2*trans*10,*cis*12, that is absorbed from the gastrointestinal tracts;

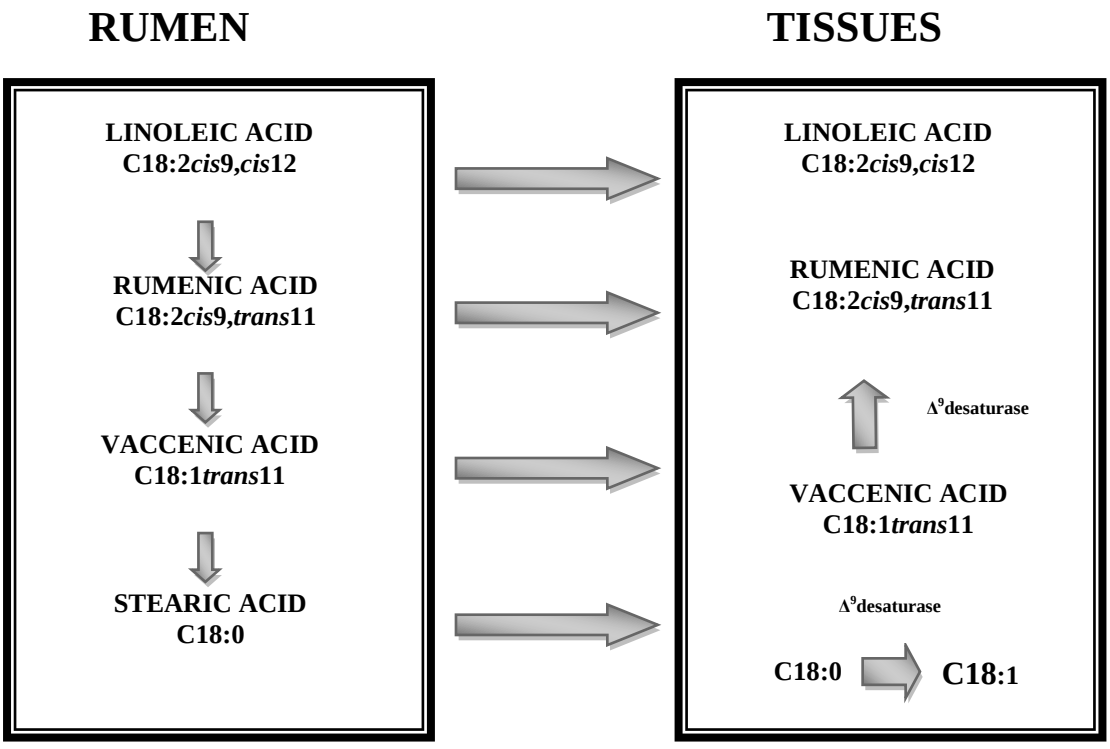


Figure 2: Role of rumen biohydrogenation and tissue Δ^9desaturase in the production of C18:2*cis*9,*trans*11 (**Modified by:** Bauman et al., 1999).

Some study, report that C18:2*trans*10,*cis*12 isomer, present in food in low amount, is considered "man-made" and may not have much relevance if CLA is obtained from natural

sources (Park, 2009). Recent studies, investigated the effect of the diet and in particular the supplementation of lipid and mainly rumen protected lipid. As reported by Schiavon et al. (2011), ruminants CLA content can be influences also with the addition of commercial rumen protected CLA (*rp*CLA) preparations containing the two main isomers C18:2*cis*9,*trans*11 and C18:2*trans*10,*cis*12 and able to increase the content of these CLA in tissues. In non-ruminants (rats) was investigated the capability to produce CLA and it was discovered that the intestinal bacterial flora of rats is capable to convert Linoleic Acid to C18:2*cis*9,*trans*11 and C18:2*trans*9,*cis*11 (Fritsche and Steinhart, 1998).

According to the sources of origin, food products derived from ruminant animals are the major source of CLA in human diet, in particular, dairy products and beef fat. The CLA content can change in relationship to the kind of food analyzed, as it is possible to observe in Table 1. Therefore, the amount found in milk and meat are small if compared to the recommended daily intake for appreciation of health benefits in humans (3500 mg/d). Many studies summarize the amount of CLA really obtained from the diet and the amount is from 1.2 to 12.5 mg/g fat, much lower than expected amount (Mir et al., 2010). The most abundant CLA isomer in dairy and beef fats is C18:2*cis*9,*trans*11 (60-85% of the CLA isomers in beef and >90% in milk), with smaller concentrations of C18:2*trans7,cis*9 and C18:2*trans*11,*cis*13 (Nuernberg et al., 2007).

As reported by Fritsche and Steinhart (1998), the concentration of CLA in milk fat ranged from 0.24% to 1.77%, in dairy products range from 2.9 to 8.92 mg CLA/g fat and as saw before the C18:2*cis*9,*trans*11 isomer is the most abundant and makes up between 73-93% of the total CLA. Conjugated Linoleic Acid in cheeses typically range from 3.59 to 7.96 mg CLA/g fat and some cheeses as Blue, Brie, Edam and Swiss cheeses have been found to have significantly higher CLA content than other cheeses. It was reported that C18:2*cis*9,*trans*11 values for sheep and goat cheeses range 0.4 to 0.5%, according to cheese type and aging period (Weiss et al., 2004). The wide variability is caused by the range of CLA content in raw milk, in turn influences by different dairy cow breeds, feeding system, content of PUFA in fed and processing parameters (heat treatment, composition of starter cultures, storage and ageing).

In meat and meat products, the content is higher in ruminants than non-ruminants because of the lack of rumen and because of the main source in non-ruminants is mainly determined by feedstuffs that they eat. Comparing different meat breeds, it was shown that lambs contain the highest amount of CLA that is 5.6 mg/g if compared to other breeds, as example, beef (2.9-4.3 mg/g) and veal (2.7 mg/g). Non ruminant meats such as chicken and

pork contain 0.9 mg/g and 0.6 mg/g respectively and the levels of C18:2*cis*9,*trans*11 in chicken fat ranging from 0.120-0.130 mg/g (Hur et al., 2007). Therefore in small amount was found C18:2*trans*10,*cis*12. Reports suggest that CLA concentration in beef fat varies among countries: Australian beef had the highest values, with CLA representing 1% of total fatty acids, German values are intermediate with concentration at an average of 0.65% of total fat and USA beef had the lowest values with CLA representing 0.3-0.5% of total fat (Griinari & Bauman 1999).

Not all the non-ruminants are unable to produce CLA isomers, in rats it was found that CLA synthesis (C18:2*cis*9,*trans*11 and C18:2*trans*9,*cis*11) may occur because of the contribution of intestinal bacteria (Fritsche et al., 1999).

Food	mg/g fat	Food	mg/g fat
Dairy Product		**Meats/Fish**	
Condensed milk	7.0	Lamb	5.8
Colby	6.1	Fresh Ground Beef	4.3
Butter Fat	6.1	Veal	2.7
		Fresh Ground	
Ricotta	5.6	Turkey	2.6
Homogenized Milk	5.5	Chicken	0.9
Cultured Buttermilk	5.4	Pork	0.8
American Processed Cheese	5.0	Egg yolk	0.6
Mozzarella	4.9	Salmon	0.3
Plain Yogurth	4.8		
Custard Style Yogurth	4.8		
Butter	4.7	**Vegetable oils**	
Sour Cream	4.6		
Cottage	4.5	Safflower oil	0.7
Low Fat Yogurth	4.4	Sunflower oil	0.4
2% milk	4.1	Peanut	0.2
Mediam Cheddar	4.1	Olive	0.0
Ice Cream	3.6		
Parmesan	3.0		
Frozen Youghrt	2.8		

Table 1: CLA content of various food (**Modified by:** Evans et al. 2002)

1.4 Biological activities on human health and animal performance

The fatty acids composition of meat has long been studied and still receives a lot of attention in research because of its implication on human health. Many studies confirm that an alteration of the level of fatty acids in blood can affect immune function in both physiological and pathological conditions. FAs added with the diet are the factors that most influence fatty acid composition of blood and cell membrane and in particular the effect of saturated fatty acids (SFA) and monounsaturated fatty acid (MUFA) on the inflammatory processes has been widely studied.

Some guidelines report the importance to reduce fat intake, but increase intake of polyunsaturated fatty acids (PUFA) especially Ω3 fatty acids, bringing the polyunsaturated fatty acid/saturated fatty acid ratio (PUFA/SFA) of meat closer to the recommended value, as well as for the Ω6/Ω3 ratio. The CLA isomers have received much attention for their health promoting effects. However, few and controversial information are found about the effect of CLA isomers on human health. Many studies reported that, CLA isomers present numerous biological effects, due to the separate actions of the main C18:2*cis*9,*trans*11 and C18:2*trans*10,*cis*12 isomers and in some case by the synergistic action of both isomers. Also minor isomers has biological effect, as examples, C18:2*trans*9,*trans*11 isomer inhibits platelet aggregation and has an anti-proliferative effect, C18:2*cis*9,*cis*11 have show anti-cancer effect. In contrast, C18:2*cis*11,*trans*13 incorporating into mitochondrial cardiolipin could adversely affect the activity of keys enzymes in the cellular energetic economy (De La Fuente et al., 2006; Park, 2009). The multiple beneficial effects of CLA could be a results of multiple interactions of the biologically active CLA isomers with numerous metabolic signaling pathways. However, studies focused on the two major isomers, report that there are three possible interactions between CLA isomers: additive, independent or antagonistic effects. Therefore, studies to define the beneficial effect of each CLA isomers are necessary and should be undertaken. Regarding the biological effects of CLA isomers and the different interaction:

- both C18:2*cis*9,*trans*11 and C18:2*trans*10,*cis*12 isomers have anti-cancer effect;
- independent effects of C18:2*trans*10,*cis*12 is the body fat reduction, inhibition of Stearoyl-CoA desaturase and reduction of hepatic alipoprotein B secretion;

- independent effect of C18:2*cis*9,*trans*11 improves growing performance in rodents;
- C18:2*cis*9,*trans*11 and C18:2*trans*10,*cis*12 can work against each other because of C18:2*trans*10,*cis*12 isomer increase insulin resistance but C18:2*cis*9,*trans*11 would compensate for this (Park & Pariza, 2007).

These research has relied entirely on animal models and cell culture system employing isomeric mixtures of CLA. Recent studies in human subjected has been carried out with contrasting results (Watkins & Li, 2002). Nevertheless, the potential biological activities of CLA isomers, discovered in the last years, can be summarized as follow:

- **Anti-cancer effect:** this effect became known as a consequence of isolation and identification experiments from cooked beef that was screened for bacterial mutagens. It was identify in many animal models, such as skin, forestomach, colon, mammary gland and liver with a dose-dependent effect. CLA not only reduce initiation, promotion and progression steps of cancer development, but also reduce metastasis of cancer. In rodents, CLA induce reduction in colon cancer incidents, probably through mechanisms involving apoptosis (Khanal, 2004). This effect, was exclusively tested in animal model and in cell culture system and there is no direct evidence that this fatty acids protect against carcinogenesis in human (Pariza et al., 2001). Afterwards, anti-cancer effect was tested in various human cell culture model and the results was that this properties might be limited to certain types of cancers and may not be effectives under some experimental conditions. Study on Finnish women found a significant effect of CLA supplementation in the reduction of mammary cancer when the intake was >200 mg/d (Weiss et al., 2004). Smith et al. (2009) reported that C18:2*trans*10,*cis*12 strongly depresses SCD gene expression in hepatic and human breast cancer cell lines. C18:2*cis*9,*trans*11 is without effect except at the highest concentrations;
- **Prevention of cardiovascular disease:** CLA has been reported to reduce atherosclerotic lesions in rabbits and hamsters, reducing total cholesterol, triacylglycerides, LDL-cholesterol (Low Density Lipoprotein-cholesterol) and increasing HDL-cholesterol (high density lipoprotein-cholesterol) in a number of animal model. Using rabbit model, diet that contain 1% of CLA mixture showed 30% regression of the experimentally induces atherosclerosis;
- **Body fat reduction:** reported for the first time in 1995 when C18:2*trans*10,*cis*12 was recognize as the isomer responsible of this activity. It is currently the most studied of the reported physiological effects of CLA. This is the effect of multiple

mechanism that increase energy expenditure by reducing lipid accumulation in adipose tissues and/or adipocytes differentiation by increasing adipocyte apoptosis, by modulating adipokines and cytokines (Evans et al., 2002);

- **Effects on skeletal muscle:** this possible effect is poorly understood if compared with the effect on adipocityes. This is known as a "possible effect" that enhanced CPT activity (phosphocholine cytidylyltranserase) in skeletal muscle in CLA-fed mice, increasing lean body mass gain relative to fat mass gain;
- **Reduce the risk of diabetes:** this capacity is confused and related to the differences in species response. Park (2009) and Park and Pariza, (2007), reported that not only in animal model the CLA effect was study, but also in human clinical trial, using a commercial CLA preparation used for human and composed by >90% of the two biologically active isomers in equal amount (C18:2*cis*9,*trans*11 and C18:2*trans*10,*cis*12). From these studies were reported that the duration of CLA supplementation can influence the benefit effects and an improving glucose metabolism as show by decreasing glucose and increasing insulin concentration. Khanal (2004) report that the role of CLA in regulating type-2-diabetes is linked to obesity. The effect of C18:2*trans*10,*cis*12 linked to body fat reduction is implicated also as an antidiabetic;
- **Immune and inflammatory responses:** The activity of CLA isomers is not only enhances immune response, but also protects tissues from collateral damage. The effect of CLA is connect with the capacity of modulate eicosanoic and immunoglobulin production and it is usually associated with anorexia and wasting;
- **Bone health:** Finally research indicates that CLA may play a role in bone health. It was demonstrate that through altered fatty acid composition and PGE2 (Prostaglandine E2) production in bone organs cultures, CLA isomers have the potential to influence bone formation and resorption (McGuire and McGiure, 2000);
- **Antioxidant Action:** It is recognized for first time in 1990 from in vitro experimental result. Ha et al. (1990) report that CLA are an effective antioxidant more potent than α-tocopherol an butylated hydroxytoluene (BHT) and it may be partly responsible for the anticarcinogenic effect of CLA. Dietary CLA reduces Arachidonic Acid, Linoleic Acid and Oleic Acid content in fat and shifts the whole fatty acid composition to more saturated side. Therefore, meat from animal fed CLA may be less susceptible to lipid oxidation, colour changes and volatile production than those from control diet (Hur et al., 2007);

Until now little have been reported about the effect on humans by CLA isomers, important information have been found in animal models where they prevent chemically-induced tumors, protected against the catabolic effects of immune stimulation, improved feed efficiency, reduced excess body weight gain, reduced body fat, increased lean body mass and lowered blood lipids. Bisosnauth et al. (2006) reported that C18:2*trans*10,*cis*12 rather than C18:2*cis*9,*trans*11 is responsible of the biological effects on plasma lipids and body condition, increasing LDL-C. There is evidence that feeding CLA (C18:2*trans*10,*cis*12) may affect liver metabolism in mice, increasing its weight independently of body weight. The biological action of the C18:2*trans*10,*cis*12 depress the SCD enzyme activity during growth, it may decrease carcass adiposity, reducing body fat accretion (Pariza, 2004; Archibeque et al., 2005). Many study with beef cattle and dairy cows, confirm this capacity to reduce body fatness in growing animals. Low protein (LP) diets supplement with *rp*CLA, in DMB bulls, are thought to increase feed efficiency, increasing the lean tissue deposition and reducing fat deposition (Schiavon et al., 2012). Besides *rp*CLA has been proposed to exert some protein-sparring effects that can be better exploited using DMB bulls under condition of protein restriction (Park et al., 1997; Pariza et al., 2001; Schiavon et al., 2011). The importance of *rp*CLA has been recognize also by other authors: in dairy cows, during lactation, abomasal infusion of CLA or feeding *rp*CLA result in a strong reduction of milk fat content (Baumgard, Sangster, & Bauman, 2001). Perfield II et al (2002) identified a relationship between the supplementation of *rp*CLA and the reduction of milk fat content in lactating cows and demonstrated that C18:2*trans*10,*cis*12 inhibited milk fat synthesis. In contrast Sinclar et al. (2007) reported no significant effect of C18:2*trans*10,*cis*12 supplementation on milk performance but supplemented animals were found to be in a greater positive energy balance compared to control. At the same time supplementing dairy cows with C18:2*trans*10,*cis*12 has been associated with increases in milk and milk protein yields and in early lactation was observed beneficial effects on reproduction performance. All this effects are seen to be dose–dependent (Weerasinghe et al., 2011; De Veth et al., 2009). Park et al. (1997) and Pariza et al. (2001) also suggested that muscle mass may be preserved or enhanced as a result of CLA induced changes in the regulation of some cytokines that profoundly affect skeletal muscle catabolism and immune function.

PART II: CLA ISOMERS ANALYSIS

Fatty acids may be found in the free form, but in general they are combined in more complex molecules through ester or amide bonds. As reported in Figure 3, the most common methods used to analyzed fatty acids profile and CLA isomers consist in multiple-steps method which include:

- **Sample preparation**, carried out in order to homogenize the sample before analysis. This step includes drying, size reduction or hydrolysis and the purpose is obtain an ideal sample that should be identical in all of its intrinsic properties to the bulk of the material from which is taken. This preparation for lipids analysis depends on the type of food and the nature of its lipids and it is not possible to devise a single standard method for extraction of all kinds of lipids in different foods;
- **Extraction procedure** to extract lipids from the samples carried out using organic solvents and generally based on Folch (1957), Hara & Radin (1978) or modified Folch and Hara & Radin methods. Lipids extraction use organic solvents to avoid interference in the next step or in the FAME synthesis;
- **Derivatization of fatty acids (FA)** to methyl esters (FAMEs), more volatile compounds that can analyzed by chromatography analysis;
- **Chromatography analysis**. Establish mainly by gas chromatographic analysis (GC), two-dimensional gas chromatographic analysis (GCxGC) and silver ion high performance liquid chromatographic (Ag^{+}HPLC).

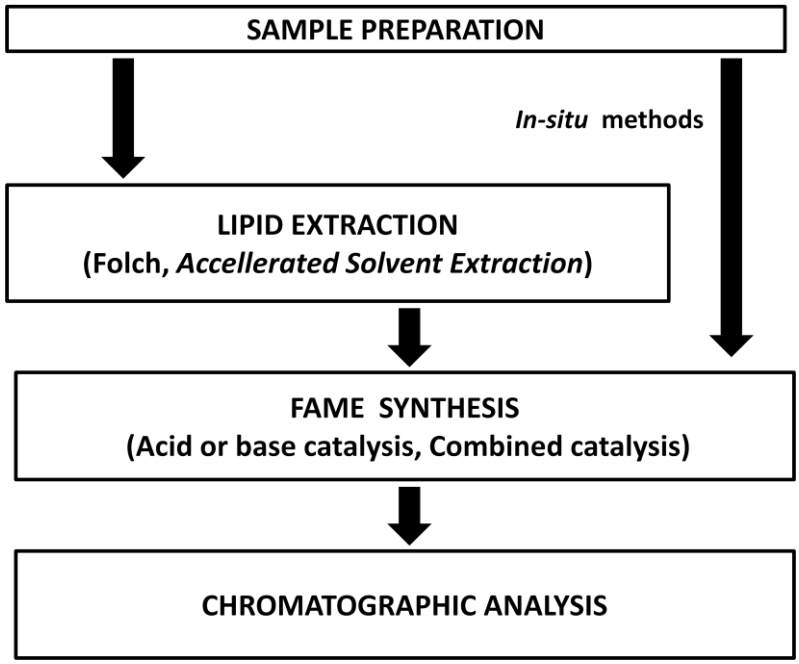


Figure 3: Different steps of lipid extraction (**Modified by:** Carrapiso & Garcia, 2000).

2.1 Fat extraction procedures

Actually a large number of methods are available for extract lipids from biological materials and an accurate determination of fatty acids composition is the main problem in total fatty extraction. A large number of methods are currently used for this purpose and they are divided in relation to the type of solvent used. There are:

- Method that use **single organic solvent** as Soxhlet (which is an AOAC recommended method) and Goldfish;
- Method that use a **combination of organic solvents**, in which, the most important are: Folch (1957), Bligh & Dyer (1959), Hara & Radin (1978);
- **Other methods**, that use non-organic solvents, such as microwave, supercritical fluid extraction (SFE), acid digestion method, detergent method, and physical method (percolation, maceration, digestion and stream distillation).

The use of combination of organic solvents is because a single non polar solvent may not extract the polar lipids from the tissues and to ensure a complete recovery of tissue lipids a solvent system composed of varying proportions of polar and non polar components may be used. The type of solvent and the method depend both on the chemical nature of the sample and the type of lipid extract desired. The main characteristics of the ideal solvent for lipid extraction is the high solubility of lipids, this solvent may also to prevent enzymatic hydrolysis of lipid and should penetrate in sample particles having a low boiling point. Solvents used for isolation of lipids are: alcohols (methanol, ethanol, isopropanol, *n*-butanol), acetone, acetonitrile, ethers (diethyl ether, isopropyl ether, dioxane, tetrahydrofuran), halocarbons (chloroform, dichloromethane), hydrocarbons (hexane, benzene, cyclohexane, isooctane) or their mixtures. The results of these extractions is the "fat" content, sometimes called ether extract, neutral fat or crude fat, that is refers to "free" lipid constituents that can be extracted into less polar solvents, such as petroleum ether or diethyl ether (Akonh and Min, 2002).

Between methods see above, the most important is the traditional or modified Folch (1957) procedures which employs a mixture of organic solvents, chloroform/methanol (2:1, v/v). This is one of the oldest methods to extract lipids from brain and biological samples rich in phospholipids. Bligh and Dyer method was created with the purpose to separated lipids from non-lipids in samples containing 80% of water (as fresh tissues) and use a different combination composed by chloroform/methanol/water. Bligh and Dyer method

thought to yield recovery of >95% of total lipids but samples with an high lipid content (>2%) were greatly underestimated (Iverson, Lang and Cooper, 2001).

Many research about fat extraction on biological samples recognized that the method choose is related to the composition of samples: Lee et al (1996) reported a modified version of Folch were the proportion of solvents use were different in relation to the fat content (chloroform/methanol (2:1, v/v) or chloroform/methanol (1:1, v/v)). Biochemists in the last year have paid more attention to the fat extraction procedures and the potential health hazard of these techniques, as example, chloroform can produce tumors in animal and methanol is known for its damage to the visual system. For these reasons, studies on the various available solvents led to the conclusion that hexane/isopropanol (HIP) shows significant advantages and a low toxicity. Hara & Radin (1978) created a new technique that use this solvents mixture in particular for milk and cheese samples. Causes of the toxicity of solvent, others procedures were employed to extract lipid reducing solvent use. One of these is, *Pressurized Liquid Extraction* (PLE) or *Accelerated Solvent Extraction* (ASE), an automated extraction techniques that have been introduced. This use the same solvents of the current extraction methods but in smaller amount, with the minimal analyst exposure and under varying extraction parameters such as temperature, pressure and volume process (Dionex-Application note n. 334 and 345; Toschi et al. (2003)). Solid sample are packed into an extraction vessel. Temperatures used, where above the boiling point of the solvent, due to the elevate pressure used in the process and for enhances solubilization and diffusion of lipids from samples to the solvent (Richter et al., 1996). According to Schafer (1998), content of fatty acids of the lipid extracted from muscles using ASE was similar or better in comparison with the conventional Folch extraction. As confirmed by many comparative study, it has been demonstrated that the use of different methods results in different lipid recoveries and the results varied widely due to the different extraction methodology. Pérez-Palacious et al. (2008), reported an higher capacity of Folch to extract lipid if compared with Soxhlet method. As consequence Folch can be considered suitable for meat and meat products with low, intermediate, high or very high lipid content and on the contrary Soxhlet was not adapt for lipid extraction in meat with very high lipid content. Also, FA content in cereal and yolk lipids extracted by ASE are highest when isopropanol/hexane is used. However, in muscle lipids results higher FA content when chloroform/methanol was used (Ruiz-Rodriguez et al.2010).

2.2 Fatty acids derivatization (FAME)

Lipid extract from animal sources are a complex mixture of individual class of compounds (triglycerides, free fatty acids, steryl esters, free sterols, phospholipids, glyceroglycolipids, gangliosides, ceramides and sphingolipids) with a complex structures that require further separation to purify components. For example fatty acids found in meat lipids are bond to an alcohol (glycerol) via ester bonds (triacylglycerols, phospholipids and sterol esters) or to a long-chain base (sphingosines) via amide bonds (sphingolipids). For these reasons, FA are converted to more non-polar and volatile derivatives by different derivatization methods.

Between the different type of formation of alkyl-esters (methyl, ethyl, propyl or butyl esters), fatty acids methyl ester preparation is the most common and esters formed with higher molecular weight alcohols (ethanol, propanol, isopropanol, butanol) are useful in determining volatile short chain fatty acids as found in dairy products and milk. Derivatization with isopropanol separates the low molecular analytes from the solvent front and improves the determination of the C4–C10 group of fatty acids, reducing the loss of such acids during the preparation procedure. The preparation of the methyl ester derivatives of fatty acids is carried out in a simple reaction, but it becomes more complicated when derivatization of conjugated fatty acids are involved, because of the conjugated double bond makes them unsuitable for the most common techniques employed for fatty acids analysis (Aldai et al., 2005 and 2006). Various methods are employed and everyone have advantages and disadvantages. For this reason is necessary optimize the derivatization procedure in order to obtain accurate quantitative and qualitative results. The most common problems include:

- Incomplete conversion of the fatty acids to their methyl esters homologues (often as result of the presence of water);
- Alteration of the original FA profile during esterification, because of chemical mediated changes in the proportion of different positional and/or geometric isomers;
- Formation of artifacts which can be wrongly identify as fatty acids;
- Contamination and subsequent damage of the GC column resulting from unclean samples.

For milk and rumen lipids, several mehtylation methods are available and included the following catalysts in anhydrous condition: HCl/methanol, BF_3/methanol, acetyl

chloride/methanol, $NaCOH_3$/methanol, tetramethylguanidine/methanol, H_2SO_4/isopropanol and diazomethane in diethyl ether/methanol (Kramer et al., 1997).

These mechanism may be divide in two groups:

- **Base-catalyzed:** performed using $NaOCH_3$ or KOH in methanol at room temperature. It is considered as a reference procedure to derivatize milk fat because of it reduce loss in short chain fatty acids. It is not use for matrices such as ruminal liquid and for tissues with high concentration of phospholipids, because it does not esterify free fatty acids (FFA) and phospholipids (PL) are not completely methylated. However, it is considered the most reliable for determining CLA isomers distribution because avoid migration and isomerization of double bonds;
- **Acid-catalyzed:** is very common and performed using BF_3, HCl or H_2SO_4. Esterify all complex and simple forms of fatty acids but may causer isomerization of conjugated double bonds and contribute to forming allylic methoxy artifacts. (De la Fuente et al., 2006). One of the many disadvantages of BF_3/methanol is the limited shelf-life of the reagents (Nuernberg et al., 2007).

There is a need to investigate exactly the concentration of CLA isomers in all the different lipids fractions, including free fatty acids, N-acyl lipids and plasmalogens of ruminant meat and meat products because of their different functions and effects on metabolism. The determination of CLA is complex because of their unstable nature, due to the presence of unsaturated double bonds that are subjected to epimerization and isomerization (Fuchs et al., 2011; Dance et al., 2010). As saw before, this step of methylation was found to be a critical step for the determination of CLA content in various lipids samples, and to overcome this problem of the methylation Jenkins (2010) proposed a modification of Sukhija and Palmquist (1988) method given by a combination of acid and base catalyst for shorter incubations times minimizing the problems given in other methods (epimerization and isomerization of double bonds). This method was give by a combination of basic catalysis with Sodium Metoxide (0.5M in methanol) and acid catalysis with Methanolic HCl (5%) at 50°C and 80°C for short time (respectively 5 and 8 minutes). Nuernberg et al. (2006) reported a comparison between four different methylation methods for quantified CLA isomers content and coming to the conclusion that temperature can produced artifacts if they are high (80-100°C) and if the exposure time are high. Also Park et al. (2001) and (2002) measure up to 13% of total artifacts and impurities at the reaction temperatures of 80°C and 20-60 minutes of reaction time with BF_3/MeOH. Many studies on CLA isomers, report that from 50 to 60% of the main isomers is given by C18:2*cis*9,*trans*11

and C18:2*trans*10,*cis*12 and only <10% of the C18:2*trans*9,*trans*11. Artifacts product by acid-catalyzed on CLA isomers can hinder the chromatography analysis, because of increasing temperature and/or incubation time decrease Rumenic Acid (RA, C18:2*cis*9,*trans*11) and C18:2*trans*10,*cis*12 but increase levels of C18:2*trans*9,*trans*11 and C18:2*trans*10,*trans*12. When HCl and BF_3 are used is possible to observe an increase of *trans/trans* isomers and a decrease of *cis/trans* and *trans/cis* isomers (Jenkins, 2010; Murrieta et al., 2003). Sometimes all these methods involve in a loss during the singles extraction steps, for these reasons and for increase the efficiency of the method, Internal Standard (IS) is added. Internal Standard is added to quantify the single fatty acid according to a known amount of a given fatty acid used as standard. Many fatty acids can use as IS and the requirements for the most suitable are:

- It be unique and not present in the sample. If it is present in the sample separate runs with and without the standard must be run;
- It must have chemical characteristics that are similar to the unknowns;
- Readly available;
- Economical.

The choice of the Internal standard may sometimes be important for obtain accurate results. The most commonly standard FA were indicated to be the C17 and C19. As example, applying direct trans-esterification procedure to a fat supplement high in saturated fatty acids yielded 613 mg/g of total fatty acids when C17:0 was used as IS compared with 930 mg/g of total fatty acids when C19:0 was used as IS. However, fatty acids content increased to 952 mg/g when a unique unsaturated fatty acid (C13:1) was used as IS (Palmquist & Jenkins, 2003; Jenkins, 2010).

2.3 In-situ method

As reported in Figure 3 methods with the previous fat extraction are not the only possibility. In fact, a very large number of analytical approaches based on initial extraction have been employed, but many critical condition and limitation of their application were found, in particular during the isolation of lipids and methylation of FAME. To avoid this problem most attention was paid to *in-situ* trans-esterification methods. In this case the derivatization technique is mainly a trans-esterification because of FA in biological samples are included in triacylglycerols (TAG) and phospholipids (PL). The main characteristics is that the lipid extraction step is avoided and fatty acid are extracted and *trans*-esterified in the

same time. This method reduce the length of the total procedure, saving time at work, reducing the sample amount, reducing the use of solvents, giving analysis less expensive and easier. The two rules to follow in this method are: lipid solubilization in solvents and prevention of the interference of water or other compounds. Samples must be submitted to a previous step to remove water. Bibliographical references, report that is necessary a pretreatment for removing water and facilitate the penetration of solvents. If water is present, it must also be integrated into the system and if it is present above specific levels, triacylglycerols, particularly those with long-chain saturated, tend to precipitate and react much more slowly. Water can also interfere because it is a strong electron donor compared to methanol. Regarding the other rule or the lipid solubilization, the choose of catalytic reagent is very important and is made in relation to free fatty acids (FFA), type of bond of the fatty acids linked and the feasibility of using strong heating conditions (Carrapiso & García, 2000).

In the study of O'Fallon et al. (2007) an alternative method was reported. In this study the researcher of Dr. O'Fallon's group present a method that is based on a surprising concept that the addition of water was considered part of the method and not an antagonist. Also Ulberth and Henninger (1994) found that a small amount of water in samples did not interfere with the formation of methyl esters using methanol-HCl/toluene.

Many studies have focused on this one-step method: MacGee and Allen (1974) in biological tissues, Outen et al. (1974, 1976) in feeds, Shimasaki et al. (1977) in mammalian tissues, Haan et al.(1979) in human tissues, Lepage and Roy (1986, 1988) in plasma, Browse et al. (1986) in leaves and Sukhija & Palmquist (1988) in feed, forages and feces. Among all the *in-situ* existing methods, in this thesis is descript and use only one. This method is very popular in many laboratories and is described in Sukhija and Palmquist (1988) with changes made by Jenkins (2010). This is used for the first time to analyze fatty acids of feed and digesta and is based on a mild acid-base methylation. As reported in Cesaro et al. (2011, 2013) this method was found to be similar or better than others for determining the FA profile in feces, which is a complex matrix for presence of un-esterificable fractions such as soaps. As reported by Meier et al. (2006) in the analysis of marine sample's FA the challenge was relate to the high content of PUFA and in 1985 was reported the first one-step procedures for FA determination. The most used reagent for this methylation is methanolic HCl applied with a large variation in reaction parameters, such as type of catalysis, polarity of solvent, water content of sample, reaction temperature and time. The condition of reaction can influence results, for example regarding CLA isomers, the most common adjustments

needed to the Sukhija and Palmquist (1988) and reported by Jenkins (2010) are alteration in methylating conditions and the selection of an appropriate Internal Standard (IS) suitable for the samples test. Modification were made to prevention CLA isomerization and were based on the work done by Kramer et al.(1997) that considered the effects of acid and base catalysts on CLA isomerization during methylation. As reported by Juárez et al. (2008) *in-situ* method had low variation values, show high precision, repeatability and reproducibility. To our knowledge this method was not tested to evaluate the FA profile of meat samples.

PART III: CHROMATOGRAPHY ANALISYS

3.1 Chromatographic separation and identification of fatty acids profile and CLA isomers

In addition to esterification and methylation another problem is given by the chromatography technique used, because often the most common is not the one that give the most optimal results. Considering past studies, the first references on chromatography process come from the Old Testament, after that other informations come from Venice in the Middle Ages but the real discovery was made by a russian botanist called Michail Semënovič Cvet (Tswett). In 1903 and 1906 he presents his experiments at the First International Petroleum Conference and named the process "chromatography" after the Greek words "chromos" meaning "color" and "graphy" which means "to write". After this discovery the development of chromatography until the early 1930s was very slow and Tswett's experiments were forgotten. Chromatography was reborn with the work of Kuhn et al. (1931) and after that a significant development is seen in the early 1940s by the Nobel Prize winner, Martin. Martin and Synge (1941) revitalized chromatography by developing the theory for liquid–liquid chromatography. Also gas chromatography was improve, in fact, GC was divided into two different analytical methods: gas–solid chromatography (GSC) and gas–liquid chromatography (GLC) (Brondz, 2001). Actually, a number of methods have been developed for analysis of fatty acids and octadecenoic fatty acids in food are many and the main are infrared spectroscopy (FTIR), gas chromatography (GC) combined with flame ionization detector (FID) or mass spectrometry (MS) and silver ion high performance liquid chromatography (Ag^+HPLC) or reverse phase HPLC (Villegas et al., 2010).

In the last two decades, Infrared Spectroscopy (IR) was the classical and routinely method used to determine *trans* fatty acids and conjugated *trans* double bonds in food. Generally, IR measurements are carried out using FAMEs and the main principle of this techniques is isolated *trans* double bonds that show absorbption at 966cm^{-1} deriving from the C-H out-plane deformation band for *trans* R_1-HC=CH-R_2, groups accompanied by the CH_3 in-plane rocking band for saturated fatty acid methyl esters (FAMEs). The major problem with IR is that samples analyzed, as methyl esters, produce *trans* levels which are 1.5-3.0% lower for trans values from 1% to 15%. As consequence, correction factors to compensate this lower absorption were proposed by the Association of Official Analytical Chemists (AOAC). Conjugated *trans* double bonds can interfere with the isolate *trans* isomers measurement because they are absorb very closely to the isolate *trans* bond. The overlap of the *trans*

absorption by other bands in the spectrum produces a strongly sloping background that converts the trans band into a shoulder levels below 2% and reduces the accuracy of determination. (Fritsche and Streinhart, 1998). Another technique called Attenuated Total Reflection (ATR) is created to avoid the problem of IR and give results that are close to those found with Gas Chromatography (GC). For the minor unknown fatty acids, as example CLA isomers, GC techniques are associated with others. One example is when Gas Chromatography (GC) is coupled with IR spectroscopic creating the Gas Chromatography-*Furier* Transformation Infrared Spectrometry (GC-FTIR) or when for analyze the structures of fatty acids a mass spectrometer is connected to a GC (GC-MS). As reported by Manzano et al. (2009) GC-FTIR and GC-MS are the most employed techniques to study fatty acids and the conjunction with FID and MS detector permit more fatty acids and isomers separation as happened with linoleic acid. Regarding CLA isomers and their study, in the second half of 1990s a lot of research was undertaken and at the present the most important for determining the CLA isomers profile are GC-MS and Ag^{+}HPLC with columns in series and a UV detector of FAME. However, GC-FTIR can contribute to confirm the geometric configuration (*cis* or *trans*) of the double bonds as nuclear magnetic resonance (NMR) spectroscopy (De la Fuente, 2006).

All the chromatography analysis of CLA isomers are hindered by a lack of well-characterized references material. Most commercial CLA mixtures contain only four major positional isomers (C18:2*trans*8,*cis*10, C18:2*cis*9,*trans*11, C18:2*trans*10,*cis*12 and C18:2*cis*11,*trans*13) with smaller amount of the corresponding *cis/cis* and *trans/trans* isomers. Moreover, only few pure isomers are available in the marked (C18:2*cis*9,*trans*11, C18:2*trans*10,*cis*12, C18:2*cis*9,*cis*11, C18:2*cis*11,*trans*13 and C18:2*trans*9,*trans*11) (De la Fuente, 2006). Below, are shown some of the chromatography techniques mentioned above and used in my thesis for the high ability to represent the fatty acids profile and CLA isomers.

3.2a One Dimensional Gas-Chromatographic Analysis

Gas chromatography (GC) is the routine procedure and the most important to investigate fatty acid composition. Compared to the past, GC has many important presupposes:

- Good resolution as show by the symmetric peaks;
- High repeatability and reproducibility of retentions times;
- High precision and accuracy in quantitation based on peak area measurements;

- Minimal termal and catalytic decomposition of sensitive samples components (Seppänen-Laakso, Laakso and Hiltunen, 2002).

GC separation is based on partitioning and/or adsorption of the lipid components between solid and liquid (mobile) phases. The most common stationary phases for column are silica, alluminia and ion exchange resins, whereas the preferred column material for lipid analysis are silicic acid and magnesium silicate (Akonh and Min, 2002). The separation requires volatile analytes as fatty acids methyl esters (FAMEs). Identification is limited to the comparison of retention time (RT) with a limited number of references standards (IS). FAME analysis demand an high power resolution especially to provide evidence of positional and geometrical isomers of unsaturated fatty acids in complex mixtures. GC should be equipped for a capillary column with a detected by a flame ionization detector (FID), a split/splitless injection system, an autosampler and a computer software to collect, integrated and transform the results data. In GC important is the column used and in particular the main parameters involved, as example: the weight of the absorbent, conditioning of the absorbent (moisture content), polarity, column size (in particular length because is able to influence its capacity and elution order). Regarding the column size, is known that long narrow column give the best resolution, but a large diameter columns increase sample capacity. However, elution order is strongly influences by the different stationary phases and polarities, as well as the temperature program (Roach et al., 2002). For this reason temperature programs are chosen in order to obtain the best resolution of the major FAMEs with emphasis on the octadecadecenoate *cis* and *trans* isomers and non-conjugated C18:2 and C18:3 isomers. Concerning the length of column, shorter columns are more prone to interferences than 100m column. The capillary column length and diameter are, also, the major determinants of the time of analysis. With this column the elution order of CLA isomers is: first the *cis/trans* and *trans/cis*, followed by *cis/cis* and finally all the *trans/trans* positional isomers. As reported by Aldai et al. (2006) GC yields satisfactory results on analysis of major and minor fatty acid in a single run. On the contrary, Fritsche and Steinhart (1998) and the Americal Oil Chemists' Society (AOCS) have found a difficulty in identifying C18:1 *cis* and *trans* isomers and the total *trans* unsaturated fatty acids, however it seemed possible improve the separation increasing the length of the column. The column can influence FA profile also through the order and the resolution power of branched-chain with odd-chain and even-chain lengths, that is distinctive depending and where *iso* and *anteiso* branched-chain configuration elute prior to the corresponding saturated FAME. For example C15:0$_{iso}$ and C15:0$_{anteiso}$, C16:0$_{iso}$ and C16:0$_{anteiso}$, C17:0$_{iso}$ and C17:0$_{anteiso}$, C18:0$_{iso}$ and C18:0$_{anteiso}$ have shorter retention time

compare to C15:0, C16:0, C17:0, C18:0 respectively. Also in the determination of CLA isomers many problems are presents and in particular some CLA are co-elutes with others:

- C18:2*trans*8,*cis*10 co-elutes with C18:2*cis*9,*trans*11;
- C18:2*trans*9,*cis*11 can be separated by C18:2*cis*9,*trans*11;
- C18:2*cis*10,*trans*12 elutes between both the 9,11 CLA;
- C18:2*cis*11,*trans*13 co-elutes between 10,12 CLA.

In the last case, in many samples, when the amount of C18:2*trans*10,*cis*12 is high C18:2*cis*11,*trans*13 may be overestimated due to the inappropriate resolution power (Alves and Bessa, 2004; Ruiz-Rodriguez et al.2010). Also Christie (2001) reported the low capacity of GC to give distinct peaks of CLA isomers, confirming a well separation of the two main isomers (C18:2*cis*9,*trans*11 and C18:2*trans*10,*cis*12) but not from the other isomer. Other important parameter is the type of carrier gas used (frequently, hydrogen and helium) and the velocity of this gas. In conclusion, considering the gas chromatography (GC), often the single GC using the currently available columns is not always the best option to identify components from natural samples.

3.2b Two Dimension Gas-Chromatographic Analysis

Recently gained much more attention, especially in relation to food analysis, the two-dimensional gas chromatography (GC×GC) a multidimensional technique that may resolve some limitation in FAMEs analysis due to the increased power separation in comparison with one-dimensional GC according to carbon length and the number of double bonds. It allow a great versatility in separating complex mixture in a single run and were very reproducible in retention times in both dimensions. In GC×GC the first column effluent is separated into small fractions according to the resolution of the first-dimension and then effluent is subjected to a second dimensional study using another column. The interface between the two columns is a modulator that has like main function increase the amplitude of the signal and to facilitate its transfer to the second dimension. Normally the various types of geometrical isomers give distinct peaks but whiting these groups, positional isomers are not always completely resolved, for these reasons a single run give an approximate idea of the total content of CLA relative to other components. In order to establish a complete isomeric distribution it is essential use a combination of analytical methods, as example GC×GC and Ag^{+}HPLC (Zabala et al., 2007).

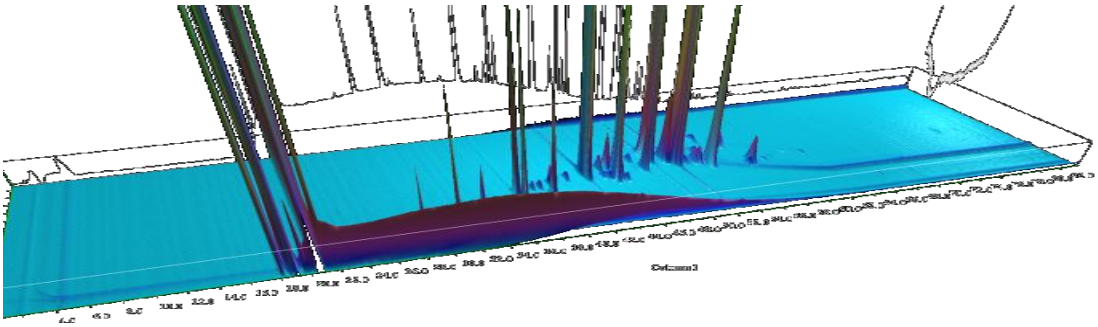


Figure 4: Example of two dimensional chromatogram (GCxGC).

3.3 Silver Ion High Performance Liquid Chromatographic (Ag^+HPLC)

A suitable separation of all CLA isomers present in biological tissues is not possible by GC, for this reason the complementary use of Ag^+HPLC is currently the most effective way to separate and quantitate individual isomers of CLA in beef (Roach et al., 2002; Nuernberg et al., 2007). Ag^+HPLC has been one of the most important technique available to lipid analyst for the separation of molecular species of lipids since its introduction by Morris in 1963. It can separate FA according to the configuration and the number of their double bonds and also according to the position of the double bonds (Fritsche and Steinhart, 1998; Fritsche et al., 2000). CLA FAME are detect thanks to their characteristic UV absorbance at 233nm. The identities of the isomers in HPLC chromatograms are based on co-injections of known reference materials obtained from commercial sources or synthesized. The main proprieties of this techniques are:

- The retention increases rapidly with increasing number of double bonds in the molecule;
- *cis*-(Z) isomers are retained stronger than *trans*-(E) isomers;
- Compounds with conjugated double bonds are retained less strongly than compounds with methylene interrupted double bonds;
- FA with longer chain eluite ahead of shorter-chain components of the same unsaturation;
- RT of dienes with separated double bonds depends on the distance between the double bond passing through a maximum at 1.5-diene system;
- RT decreases with the increasing of chain-length.

Regarding, CLA isomers quantification, the problem of this technique as saw for GC analysis, is that only a limited number of CLA isomers were available and pure isomers are C18:2*cis*9,*trans*11, C18:2*trans*10,*cis*12, C18:2*cis*9,*cis*11 and C18:2*trans*9,trans11. In opposite

to GCxGC, Ag^{+}HPLC profile is show to separate first *trans/trans* compounds followed by *cis/trans* and *trans/cis* regions and in the end the *cis/cis* region. However in some samples, as example milk fat, a wide peak can mask the *cis/cis* area and as reported by Luna et al. (2005), in ewes milk 12 CLA isomers have been identified. The existence of other minor interference in CLA area have also been reported. Experiments show that retention order of the positional isomers of each group of configurationally isomeric CLA is the same and is determined by the position of the double bonds in the carbon chain:

- *trans*: C18:2 -12,14- < 11,13- < 10,12- < 9,11 < 8,10- < 7,9;
- *cis/trans*: C18:2 - 11*t*,13*c*-<10*t*,12*c*-<9*c*,11*t*- <~9*t*11*c*-+8*c*,10*t*-<8*t*,10*c*;
- *cis*: C18:2 - 11,13- < 10,12- < 9,11- < 8,10 (Nikolova-Damyanova, 2009).

Also in this technique the column is very important, operating from one to six columns in series progressively improve the resolution of methyl esters of CLA isomers both from natural and commercial products (De La Fuente, 2006). However Roach et al. (2002), report that the use of three columns in the best compromise because increase the resolution of the peaks but in the contrary more than three columns in series provide a decrease of the benefits. The presence of silver ions, like the ions of other transition metals, interact specifically with unsaturated compounds to form weak charge transfer complexes with olefinic double bonds. Regarding the mobile phase, it plays an important rule, because it will be affect the Retention Time (RT) and the elution of CLA. Depending to the mixture of solvents use for the mobile phase the retention time shift can be totally eliminated (the addition of diethylether in hexane can stabilized solvents mixtures). Many studies try to evaluate which is the best solvent systems with the best stability in retention times and resolution. They found that 0.2% propionitrile in hexane showed the highest stability compared to the reference acetonitrile system, even though it give not a good resolution in CLA isomers determination if compared to acetonitrile (Ruiz-Rodriguez et al.2010).

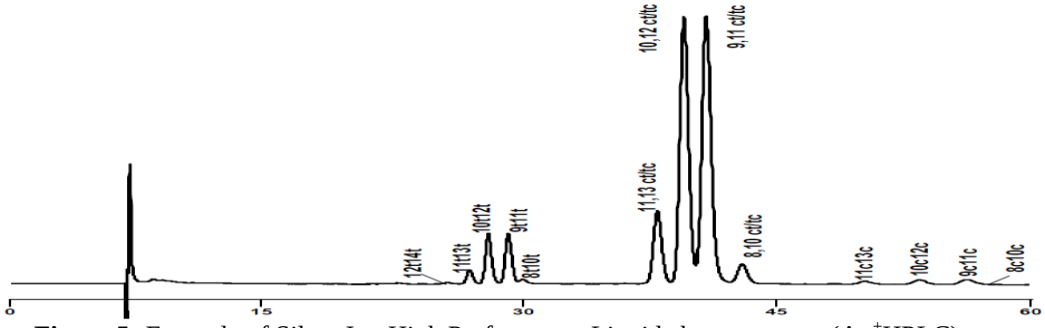


Figure 5: Example of Silver Ion High Performance Liquid chromatogram (Ag^{+}HPLC).

3.4 Thin Layer Chromatography (TLC)

Thin Layer Chromatography (TLC) is one of the main analytical tools used for lipid analysis. It is used for fractionation of lipid complex mixtures, assay of purity, identification, information on the structure, as well as for monitoring extraction and separation of components via preparative column chromatography for routine and experimental purposes. Regarding the fractionation of lipid complex mixtures, lipid extract are a mixture of individual class of compounds: triglycerides, free fatty acids, steryl esters, free sterols, phospholipids, glyceroglycolipids, gangliosides, ceramides and sphingolipids and TLC is used to identify some of these components, as example, triglycerides, free fatty acids and phospholipids. The main components of TLC are the stationary phase, mobile phase and it is based on the difference in the affinity of a component toward a stationary and mobile phase. Analysis, need a prior extraction of fat and after are carried out in different steps: (1) samples lipids extract are applied as spots or as narrow streaks 1.5-2 cm from the bottom of the plate; (2) plate is developed in a chamber containing a solvent or a mixture of solvents (mobile phase); (3) in the chamber mobile phase moves up the plate by capillary action, taking the various components with at different rates, depending on their polarity; (4) after that, when solvents approaches the top, plate is removed from the chamber and dried under a flow of nitrogen and a specific reagent, such as, 2,7-dichlorofluorescin in 95% methanol (1, w/v %); (5) lipids exhibit a yellow color and produce a different color under UV light; (6) the different lipid fractions are separated from the plate (Akonh and Min, 2002). As reported by Pérez-Palacious, Ruiz and Antequera (2007), TLC is used for phospholipids (PL) separation and it is associated to High Performance Liquid Chromatography (HPLC) due to the excellent quantification and resolution.

General Aim

Fatty acids profile of meat, as saw in the general introduction is very important for the effect that fatty acids can have on human health. Diseases and potential negative effect were connect with meat consumption, but its rule is controversial because of it is an important source of trace elements (minerals), fat soluble vitamins and other molecules with and high biological value, as example, Conjugated Linoleic Acid (CLA) and Long Chain Fatty Acids (LC-PUFA). In this thesis, the principal aim is the study of Conjugated Linoleic Acid (CLA) according to different point of view. CLA can have many important effects but also many problems in their identification and analysis. The different aims of this study regarding, firstly, methods of analysis (in terms of extraction procedure and chromatographic techniques), effects that can affected the FA profile and CLA content and effects of CLA supplementation on animal performance.

Summarizing the purposes of this thesis can be divided as follow:

Methodological Approach:

- use the two-dimensional gas chromatography (GC×GC) to compare the effects of three different procedures of fat extraction in terms of mean, repeatability, and variance homoscedasticity, of the measures of single and groups of FA in three tissues collected from young growing bulls (liver, subcutaneous fat and muscle);
- use the Ag^{+}HPLC to analyze the CLA isomers content in three tissues collected from young growing bulls fed increasing *rp*CLA supplementation (liver, subcutaneous fat and muscle).

Effects of *rp*CLA supplementation and source of variation on FA profile:

- study the effect of feeding system (pasture, penned in an open barn and fed with hay and concentrate and penned in the open barn and fed with hay and concentrate added with *rp*CLA supplement), breed (Foza, Brogna, Alpagota and Lamon), gender (raw and ewe) and tissue (liver, round, rib eye, other muscle, cover fat and kidney fat) on fatty acids profile composition in lambs slaughtered at different age;
- Study the effect of feeding system, paying attention to the role of ewe's milk as one of the main component of suckling lamb's diet.

Effects of *rp*CLA supplementation on animals performance in term of:

- Milk composition of lactating ewes and their milk coagulation proprieties (MCPs);
- Growth rate, slaughter traits and meat quality of lambs belonging to three Alpine sheep breeds.

CHAPTER 2

Two-dimensional gas chromatography to evaluate the effect of different procedures of fat extraction on fatty acid profile and repeatability of the measures on liver, subcutaneous fat and muscle of beef cattle.

2.1 ABSTRACT

Aim of this study was to use the two-dimensional GC to compare the effects of 3 different procedures of fat extraction in term of mean, repeatability and variance homoscedasticity, of the measures of single and groups of FA in 3 tissues collected from young growing bulls (liver, subcutaneous fat and muscle). The first method (F) used a mixture of chloroform/methanol (2:1, v/v) at room temperature. The second (A) accelerated solvent equipment, used a mixture of chloroform/methanol (2:1, v/v) associated to these conditions of extraction: 120 °C of temperature and 10 MPa of pressure. The third method (J) was based on a one-step mild acid-base methylation treatment performed directly on sample, firstly, subjected to a treatment of freeze-drying. The experimental design involved 9 crossbred young bulls fed with a supplementation of 0, 8 or 80 g/d of rumen protected CLA (*rp*CLA). Samples analyzed were collected from these animal and came from 3 tissues (subcutaneous fat, muscle and liver). Data were analyzed in different phases: the first, was an analysis of the resolution power and the number of undetected FA for each tissue and for each method. The second, was the analysis of the main sources of variation carried out using a MIXED procedure that considered as fixed effect diet, tissue, diet×tissue, method and method×tissue. Considering the hierarchical structure of the model the fixed effects were tested on different error line: animal (diet), animal (diet×tissue) and residual. The third phase, was an analysis of the variances homogeneity for the main sources of variations using Levene's test. In the fourth phase, linear regression was used to explore the relationship between FA values obtained using the three methods. The incidence of undetectable values depend on the sensitivity of the method used; liver has the highest incidence of null values with the J method in short chain FA (C8:0 and C10:0). In muscle the highest incidence of undetectable values with the J method was mainly observed for the C24:0, for two C20:1*t* unknown isomers, C20:3*n*3, C20:4*n*3 and C22:5*n*6. Among the sources of variation the effect of tissue was highly significant, the interaction diet×tissue was significant ($P < 0.001$) only in the case of the CLA*t*10,*c*12 isomer and both the method and the interaction method×tissue for the majority of the FA. The results of the Levene's test evidenced, except for diet, as the variances among levels within tissue, method or method tissue were not homoscedastic for the large majority of the FA.

2.2 INTRODUCTION

The complexity of meat fatty acid profile and the current interest in the health effects of some fatty acids (Ruiz-Rodriguez et al., 2010) are increasing the research effort to improve the methods of analysis. In recent years, comprehensive two-dimensional gas chromatography (GC×GC) has proven to be a powerful separation method for different substances in many types of samples (Hyötyläinen et al., 2004; Adahchour et al., 2008; Cesaro et al., 2013). To our knowledge, little or no application of GC×GC for the analysis of meat fatty acids (FA) have been reported so far. The availability of this technique allow to achieve a good precision and accuracy in the separation of different FA and this permits to achieve a major detail about the effect of different sample preparation methods on the resulting FA profile.

Comparisons among GC methods for measuring FA profile were mainly focused to evaluate the effects of different combined extraction and methylation procedures and reagents (Kramer et al., 1997; Yamasaki et al., 1999; Fritsche et al., 2000; Park et al., 2002; Aldai et al., 2005; Ficarra et al., 2010). In general, a major emphasis was put on methylation and less on the extraction. Methylation was found to be a critical step for a contextual determination of FA and CLA contents in various lipid samples (Park et al., 2002). Base catalysts avoid migration and isomerization of double bonds but do not esterify free FA (Kramer et al., 1997). On the opposite, acid catalysis esterifies all complex and simple forms of FA but cause isomerization of conjugated double bonds (Kramer et al., 1997; Christie et al., 2007). Much less is known about the effect of different extraction procedures when the same methylation procedure is applied. Cold extraction methods, using different organic solvents to quantify the solvent extract contents of feeds and foods, are popular but time consuming and often uneconomical (Sukhija and Palmquist, 1988). To reduce the analytical times, the labor, the use of solvent and the analytical cost the extraction could be completed with an accelerated solvent extraction technique (ASE). Schafer (1998) evidenced as the content of FA extracted with ASE from muscle matrices was similar or better in comparison to the conventional extraction methods (Folch et al., 1957).

Meat contains a high number of FA, and many of these have been found or are supposed to exert considerable effects in biological system at low or very low concentrations, such as the conjugated linoleic acid isomers (CLA), C20:5*n*3 (EPA), C22:6*n*3 (DHA) and various Ω3 and Ω6 polyunsaturated FA (PUFA). Thus, the interest for determining a high number of FA, even those present in trace is increasing. The quantification of unsaturated FA is complicated by their unstable nature, due to the presence of unsaturated double bonds

which makes these isomers easily subjected to epimerization and isomerization (Park et al., 2002; Jenkins and Lee, 2007; Nuernberg et al., 2007). To overcome the extraction and methylation shortcomings, Jenkins (2010) proposed a one-step method based on a mild acid-base methylation. This method was found similar or better than others for determining the FA profile even when applied in feces, which is complex matrix for presence of un-esterificable fractions such as soaps (Cesaro et al., 2011, 2013). To our knowledge this method was not tested to evaluate the FA profile of meat samples. The aim of current experiment was to use the two-dimensional gas chromatography to compare the effects of 3 different procedures of fat extraction in terms of mean, repeatability, and variance homoscedasticity, of the measures of single and groups of FA in 3 tissues collected from young growing bulls (liver, subcutaneous fat and muscle).

2.3 MATERIAL AND METHODS

Animals, diets, tissues collection and experimental design

All experimental procedures were approved by the Ethical Committee for the Care and Use of Experimental Animals of the University of Padova (CEASA).

Fifty-four crossbred young bulls and heifers were fed ad libitum a total mixed ration containing 108 g/kg DM of CP, 35 g/kg of FA and supplemented with 0, 8 or 80 g/d of rumen protected CLA (*rp*CLA) supplement from 5 to 16 months of age (18 animals for each *rp*CLA dose) and they consumed 9.3 kg/d of DM on average. The total mixed ration was composed, on DM basis, of corn meal (400 g/kg), corn silage (276 g/kg), soybean meal (33 g/kg), dried sugar beet pulp (113 g/kg), wheat bran (70 g/kg), wheat straw (66 g/kg), vitamin and mineral mixture (26 g/kg), calcium soap (9 g/kg), and hydrogenated soybean oil (7 g/kg). The *rp*CLA supplement consisted of methyl esters of CLA bound to a silica matrix and coated with hydrogenated soybean oil. The lipid-coated *rp*CLA was composed of 800, 178, and 22 g/kg of lipid, ash, and moisture, respectively, and 456 g/kg of palmitic and stearic acids, 79.2 and 76.8 g/kg of CLA*c*9,*t*11 and CLA*t*10,*c*12, respectively, and 91 g/kg of other FA. A detailed description of the chemical composition of the *rp*CLA used is given in Schiavon et al. (2011).

At the end of fattening the calves were fasted for one day and then slaughtered. According to the reference meat market, heifers or bulls were slaughtered when they reached an estimated *in vivo* fatness score around 3 or 2 points on a scale from 1 (very lean) to 5 (very fat), respectively (Schiavon et al., 2013). Immediately after slaughter the liver was collected

from each animal. Twenty-four hours after slaughter from the left half part of the carcass the whole cut of the 5th rib was collected (from the cranial edge of the 5th rib to the cranial edge of the 6th rib). The entire rib was vacuum packed, moved to the laboratory, and aged at 4 °C in a chilling room for 10 days. At the end of ageing, the rib cut was dissected into muscles (*Longissimus Thoracis*: LT, and other muscles), fat (subcutaneous fat) and bones. Each fraction was weighted.

In current work, after an anticipated power analysis, 3 tissues (liver, LT and subcutaneous fat) collected from 3 young bulls randomly chosen within each *rp*CLA dose groups were used to be analyzed according to the following experimental design: 3 bulls × 3 *rp*CLA doses × 3 methods × 3 tissues × 2 replications for a total of 162 subsamples analyzed.

Sample preparation methods

The left lobe of liver was cutted in slices which were grinded, mixed and homogenized for 10 sec at 4500 *g* (Grindomix GM200 - Retsch, Haan, Düsseldorf, Germany). From the final mixture 2 subsamples of about 50 to 60 g were collected to be analysed. A slice of LT (around 100 g), and all the subcutaneous fat collected from each rib (40 to 90 g) were homogenized with the same equipment and 2 subsamples (20 to 30 g) were collected for each tissue. All the samples were packed and conserved at -20°C till the analysis.

Three methods for fat extraction and FAME preparation were applied. The first two methods differed from the third because they included a fat extraction step and methylation was performed on the solvent extracted fat, while in third method extraction and methylation were performed contextually and directly on the freeze-dried sample. With all methods methylation was performed with the mild acid-base treatment proposed by Jenkins (2010).

With the first method (F) fat extraction was performed according to Folch (1957) using as a solvent chloroform/methanol (2:1, v/v) at room temperature. Fresh weighted samples (3.0 ± 0.22 g for liver, 1.0 ± 0.18 g for fat and 3.0 ± 0.04 g for LT) were blended 3 times with the solvent (20 ml/ g tissue), by filtering 3 times with 250 ml Hollow glassware (Duran Group GmbH, Mainz, Germany), and by adding saline solution (0.88% KCl wt/vol, 12 ml/g tissue) to allow the phases separation. After one night of resting, the two solution phases were separated, the upper methanol aqueous fraction was collected a part, and the remaining lipid chloroform solution was transferred into another ml Hollow glassware (Duran Group GmbH, Mainz, Germany). To recover additional residues of fat, a distilled water/methanol (1:1, v/v) solution was added to lipid chloroform solution (7 ml/g tissue/g

tissue) and the resulting solution was agitated, rested for 30 min. After this step the upper methanol-water phase was separated from lipid chloroform phase which was collected and transferred into the flat bottom flask (Duran Group GmbH, Mainz, Germany).This final solution was heated at 50 °C under N_2 stream for about 20 min to complete solvent evaporation and the resulting extracted fat material was weighted. About 44 mg of fat were transferred into culture tubes to be immediately methylated according to Jenkins (2010), as later described. The resulting FAME solution was stored in GC vials at −20 °C prior the GC×GC analysis.

With the second method (A), fat extraction was performed using an accelerated solvent equipment (ASE, Dionex, Sunnyvale, USA). The weighted fresh samples (3.0 ± 0.06 g for liver, 0.9 ± 0.07 g for fat and 3.0 ± 0.07 g for LT) were homogenized with Hydromatrix (Phenomenex, Castel Maggiore, Bologna, Italy) and transferred into 10 mL stainless steel extraction cells for ASE (Dionex ASE 350, Thermo Fisher Scientific Inc., MA, USA) with chloroform/methanol (2:1, v/v) as solvent. The conditions of extraction were (Schafer, 1998; Toschi et al., 2003): 120 °C of temperature, 10 MPa of pressure, 1 min of static time, 3 static cycles, rinse 100%, purge 60 s, with about 8 mL/sample of re-flushing volume of fresh solvent (giving a total solvent consumption of <20 mL/sample). Like in the method described above (F), after the extraction procedure was added saline solution (0.88% KCl, 7 ml/g tissue). After one night of resting, the two solution phases were separated, the upper methanol aqueous fraction was discarded, and the remaining lipid chloroform solution was transferred into another 250 ml Hollow glassware (Duran Group GmbH, Mainz, Germany). A distilled water/methanol (1:1, v/v) solution was added to lipid chloroform solution (6 ml/g tissue), to recover additional residues of fat. The resulting solution was agitated, rested for 30 min. After this step the upper methanol-water phase was separated from the remaining lipid chloroform solution which was collected and transferred into the flat bottom flask (Duran Group GmbH, Mainz, Germany). Thereafter, the solution was heated at 50 °C under N_2 stream for about 20 min to complete solvent evaporation, placed in an oven at 60 °C for 15 min, cooled in a desiccator and the resulting EE was weighted. About 44 mg of extracted fat were transferred into culture tubes to be methylated according to Jenkins (2010), as later described. The resulting FAME solution was stored in GC vials at −20 °C prior the analysis.

The third method (J), detailed by Jenkins (2010), was based on a one-step mild acid-base methylation treatment performed on sample (2.0 ± 0.005 g for liver, 0.07 ± 0.001 g for fat and 2.0 ± 0.04 g for LT, in order to treat approximately 40 mg of fat in all tissues)

subjected to a treatment of freeze-drying (CooSafe 90-80, Scanvac, Stockholm, Sweden), as suggested by Jenkins (2010).

Methylation

The J dried tissue samples, as well as the F and the A fat extracted samples, were placed in culture tubes with 2 mL of sodium methoxide (0.5 M in methanol) and 2 mL of toluene, containing 2 mg/mL of methyl 12-tridecenoate as internal standard (# U-35 M, Nu-chek prep inc., MN, USA). Each sample was incubated in a 50 °C water bath for 10 min, removed from the bath and cooled for 5 min. After the addition of 3 mL of freshly prepared methanolic HCl (1.37 M) the sample was incubated again in an 80 °C water bath for 10 min, removed from the bath and cooled for 7 min. Thereafter, 5 mL of K_2CO_3 (0.43 M) and 2 mL of toluene were added to each tube. The tube was vortexed for 30 sec and centrifuged for 5 min at 400 *g* and 4 °C. The organic phase (the upper layer) of the tube was transferred into a screw-capped tube, and 0.5 g of anhydrous sodium sulphate and 0.5 g of active charcoal (Sigma-Aldrich, MO, USA) were added. The solution was vortexed for 5 min and rested for 1 h. After centrifugation for 5 min at 400 *g* at 4 °C the clear upper layer containing the FAME was transferred in a GC vial and stored at −20 °C prior the GC analysis.

Gas chromatographic analysis

The samples obtained with the 3 different methods were analyzed for their FA profile using a GC×GC instrument (Agilent Technologies 7890A, CA, USA) with two columns in series, equipped with a modulator (Agilent G3486A CFT, CA, USA), an automatic sampler (Agilent 7693A, Agilent Technologies, CA, USA) and a flame ionization detector (FID) connected with a chromatography data system software (Agilent Chem Station, Agilent Technologies, CA, USA). Between the two columns the modulator unit collects in a fixed volume channel the analyte bands of the first column and these are successively launched into the short second column in narrow bands. The operative conditions of the GC apparatus were:

- first column of 75 m × 180 µm (internal diameter) × 0.14 µm of film thickness [23348U (polar), Supelco, PA, USA], H_2 carrier flow of 0.2 mL/min increased to 0.3 mL/min at a rate of 0.002 mL/min;
- second column of 3.5 m × 250 µm (internal diameter) × 0.14 µm of film thickness [J&W 19091-L431 (nonpolar), Agilent Technologies,CA, USA], H_2 carrier flow of 22 mL/min held for 2 min and then increased to 30 mL/min at a rate of 0.08 mL/min.

- Planned oven temperature variation: increase from 50 °C (held for 2 min) to 150 °C (held for 15 min) at 2 °C/min and then increased to 240 °C (held for 20 min) at 2 °C/min.
- Valves: modulation delay, 1 min; modulation period, 2.9 s; sample time, 2.77 s.
- Gas flows: hydrogen, 20 mL/min; air, 450 mL/min.
- Sample injection: 0.8 µL (pulsed split mode, injection pressure 0.172 MPa × 0.3 min, split ratio 150:1).
- Splitless Inlet: temperature 270°C, pressure 20.804 MPa, Septum Purge 3mL/min, Split Ratio 35.2mL/min.
- The resulting three-dimensional chromatograms were analyzed with the comprehensive GC×GC software (Zoex Corp., TX, USA) to evaluate the cone volume of each fatty acid.

Identification of fatty acids

Fifty-eight FA were identified by comparison of the cone position in the bi-dimensional chromatogram with the cone position of FA contained in GC reference standards. The reference standards used were mixtures of pure FA [(#674, Nu-chek prep inc., MN, USA), (#463, Nu-chek prep inc., MN, USA), (47080-U Bacterial Acid Methyl Esters - BAMEs, Sigma-Aldrich, MO, USA), (47085-U PUFA-3 Menhaden Oil, Supelco, PA, USA)] plus CLA*c*9,*t*11 (#UC-60M, Nu-chek Prep Inc., MN, USA), CLA*t*10,c12 (#UC-61M, Nu-Chek Prep, Inc. MN, USA), CLA*c*9,*c*11 (#1256, Matreya LLC, PA, USA), CLA*t*9,*t*11 (#1257, Matreya LLC, PA, USA) and CLA*c*11,*t*13 (#1259, Matreya LLC, PA, USA). The identification of other 9 FA: C14:0$_{iso}$, C18:0$_{iso}$, C19:0$_{iso}$, C19:0$_{anteiso}$, C18:1*n*6, C19:1*c*9, C19:1*c* (uncertain position of the double bound), C20:1*n*7, C18:5*n*3, was made considering their elution order and their position in the bi-dimensional chromatogram on the basis of comprehensive GC×GC software (GC Imagine Software, Zoex Corporation, TX, USA).

Other 15 unidentified FA were detected in specific regions of the bi-dimensional chromatogram grouping FA with similar characteristics (i.e. region of the 18:1, 18:2 or 20:1 isomers). In the region of the C18:1 isomers were detected 5 peaks (2 unknown isomers, two isomers partially co-eluted, likely the C18:1*c*4 and the C18:1*t*2, and a fifth isomer, likely the C18:1*c*3). In the region of the C18:2 isomers other 5 peaks (4 unknown isomers and another one likely the C18:2*c*11,*c*14, were detected. In the region of the C19:1 one peak indicated as C19:1*t* was also detected. In the region of the C20:1 isomers other 4 unknown peaks, of which

2 C20:1*t* isomers in the region of the *trans* forms were detected. As the identification of these molecule was uncertain, these FA were indicated as unidentified FA isomers.

Quantification of Fatty Acid Methyl Esters

Quantification of FAME was based on the internal standard technique and the cone volume of each FA was corrected using FID response factors (RF). These RF values were computed on the basis of a calibration based on 5 dilution points of the solution containing the standard FA (1, 0.5, 0.25, 0.125, 0.062). All the calibration curves were linear with an $R^2 > 0.998$. The RF factor was computed as:

$$RF_{FA} = (FA_{conc}/STD_{conc}) \times (STD_{volume}/FA_{volume})$$

where RF_{FA} is the response factor for a given standard FA, FA_{conc} is the concentration (mg/ml) of the standard FA in the solution, STD_{conc} is the concentration (mg/ml) of the internal standard, STD_{volume} is the chromatogram volume of the internal standard and FA_{volume} is chromatogram volume of the standard FA. For the FA identified by position the RF values were assumed to be the mean of the RF factors found for the standard FA located in the same region of the bi-dimensional chromatogram. The cone volumes of each FA, adjusted for $RF_{FA,}$ were summed excluding the volume of the methyl 12-tridecenoate (internal standard), and the relative proportion of each FA was expressed in terms of mg/g total FA.

Fatty acid groups and indexes

The various FA were summed according to various criteria as follow.

- Saturated fatty acids (SFA) category were the sum of: C8:0, C10:0, C12:0, C13:0, C14:0, C14:0$_{iso}$, C15:0, C15:0$_{iso}$, C15:0$_{anteiso}$, C16:0, C16:0$_{iso}$, C16:0$_{anteiso}$, C17:0, C17:0$_{iso}$, C17:0$_{anteiso}$, C18:0, C18:0$_{iso}$, C18:0$_{anteiso}$, C19:0, C19:0$_{iso}$, C19:0$_{anteiso}$, C20:0, C21:0, C22:0, C23:0 and C24:0.
- Monounsaturated fatty acids (MUFA) were the sum of C14:1*n*5, C14:1*t*, C15:1, C16:1*c*7, C16:1*c*9, C16:1*t*7, C17:1, C17:1*t*7, C18:1*c*9, C18:1*c*7, C18:1*t*7, C18:1*t*9, C18:1*n*6, 5 unidentified C18:1 isomers, C19:1*c*9, C19:1*t*9, C19:1*t*12, C19:1*c* (uncertain),C19:1*t* (uncertain), C20:1*n*7, C20:1*n*9, C20:1*n*11, C20:1*t*9, 2 uncertain C20:1*t* isomers, 2 uncertain C20:1 isomers, C22:1*n*9, C22:1*n*11).
- Polyunsaturated fatty acids (PUFA) were the sum of C18:2*n*6, C18:2*t*9,*t*12, 4 C18:2 unknown isomers (C18:2), C18:2*c*11,*c*14 (uncertain), C20:2, C20:2*n*3, C18:3*n*3, C18:3*n*6, C20:3*n*3, C20:3*n*6, C18:4, C20:4*n*3, C20:4*n*6, C22:4*n*6, C18:5*n*3, C20:5*n*3(EPA),

C22:5*n*3, C22:5*n*6, C22:6*n*3 (DHA), CLA*c*9,*t*1,1 CLA*t*10,*c*12, CLA*c*11,*t*13, CLA*c*9,*c*11 and CLA*t*9,*t*11.

- Branched fatty acids were calculated as the sum of C13:0$_{anteiso}$, C14:0$_{iso}$, C15:0$_{iso}$, C15:0$_{anteiso}$,C16:0$_{iso}$, C16:0$_{anteiso}$, C17:0$_{iso}$, C17:0$_{anteiso}$, C18:0$_{iso}$, C18:0$_{anteiso}$, C19:0$_{iso}$ and C19:0$_{anteiso}$ according to Raes et al.(2004).
- Odd Chain fatty acids were calculated according to Or-Rashid et al.(2007).
- The sum of the identified CLA isomers (CLA*c*9,*t*11, CLA*t*10,*c*12, CLA*c*11,*t*13, CLA*c*9,*c*11 and CLA*t*9,*t*11) was indicated as CLA. Other CLA isomers, such as the CLA*t*7,*c*9 which has been found in beef meat (Nuernberg et al., 2007), were not identified.
- The sum of Ω3PUFA or Ω6PUFA were calculated according to Givens et al. (2006) and Connor (2000), as example Ω3PUFA was computed as the sum of C18:5*n*3, C20:2*n*3, C20:3*n*3, C20:4*n*3, C20:5*n*3(EPA), C22:5*n*3, C22:6*n*3 (DHA).
- The Δ^9-desaturase indices for C14, C16, C18, CLA*c*9,*t*11, CLA t10,c12, and total Δ^9-desaturase index were also calculated according to Kelsey et al. (2003) and Capoprese et al. (2010) as product/substrates ratios, as example the Δ^9-desaturase C14 index was computed as C14:1*n*5/(C14:1*n*5 + C14:0).
- The atherogenic index (AI) and thrombogenic index (TI) were calculated according to Ulbricht and Southgate's (1991) as: AI = (C12:0 + 4 × C14:0 + C16:0)/(Ω3PUFA + Ω6PUFA + MUFA); and TI = (C14:0 + C16:0 + C18:0)/(0.5 × MUFA + 0.5 × Ω6PUFA + 3 × Ω3PUFA/Ω6PUFA).

Statistical Analysis

The statistical analysis was performed in the following steps.

First phase: resolution power. An analysis was carried out in order to assess the number of undetected FA related values for each tissue and for each method. Basically, in the cases where for a given FA all the methods provided more than 9 undetectable values in one or more tissues, the values for those tissues were considered missing.

Second phase: main sources of variation. Data of FA, FA category and FA indices (18 data for each tissue and each method) were analyzed using the MIXED procedure (SAS Institute Inc., Cary, NC) according to the following linear model:

$$y_{ijklmn} = \mu + D_i + Bull(D)_{i:j} + Tissue_k + D\times Tissue_{ik} + Bull(D\times Tissue)_{i:jk} + M_l + M\times Tissue_{lm} + e_{ijklmn}$$

where y_{ijklmn} is the observed trait; μ is the overall intercept of the model; D_i is the fixed effect of the *i*th diet (i = 1,…,3); $Bull(D)_{i:j}$ is the random effect of the *j*th animal within diet (j = 1,…,9); $Tissue_k$ is the fixed effect of *k*th tissue (k = 1,…,3); $D\times Tissue_{ik}$ is the fixed effect of the *ik*th diet×tissue interaction; $Bull(D\times Tissue)_{i:jk}$ is the random effect of the sample, expressed as bull within the D×Tissue interaction; M_l is the fixed effect of *l*th method of fat extraction (l = 1,…,3); $M\times Tissue_{lm}$ is the fixed effect of the *lm*the method×tissue interaction; and e_{ijklmn} is the random residual. $Bull(D)_{i:j}$, $Bull(D\times Tissue)_{i:jk}$ and residuals were assumed to be independently and normally distributed with a mean of zero and variance , and ,respectively.

Considering the hierarchical structure of the experimental design, the hypothesis of testing for fixed effects was accomplished considering different error lines. Basically the effect of D was tested using Bull(diet) as error line, the effects of Tissue and of D×Tissue interaction were tested using the Bull(D×tissue) as error line, whereas the effects of M and M×Tissue interaction were tested on the residual error. When in 2 of the 3 tissues analyzed the presence of a given FA was undetectable, the model was simplified by omitting the Tissue and its corresponding interactions as sources of variations. Least square means for each source of variation, the root of the residual error (RMSE), considered as an index of repeatability, and the 162 residual values for each individual FA, FA category and FA index were computed.

Third phase: homogeneity of variances Variances homogeneity for the main sources of variations was explored using Levene's test (Milliken and Johnson, 1984). As the Levene's test showed that variances were generally homoscedastic among different levels of diets and heteroschedastic among different tissues, methods and their interaction, the residual variance was calculated for each group of 18 analytical values (9 groups) within each tissue and method. This was done for each of the 76 individual FA and the 27 categories and indices (927 analyses) with a simplified linear model with the effect of sample/animal (8 df) and the residual (9 df). The root mean square error (RMSE) was considered a measure of repeatability for each tissue and method relatively to each FA, category or index.

Fourth phase: relationships between methods. As the large majority of FA evidenced heteroscedasticity of variances for Methods, Tissue, and Method×Tissue interaction, linear regression (SAS Institute Inc., Cary, NC) was used to explore the relationship between FA values obtained using different methods (i.e., A or the J method were regressed against the values obtained with the F method which was assumed to be the reference) in each tissue. The *F*-test was used to test the significance of any slope that deviated from unity and any intercept that was not zero ($P < 0.05$).

2.4 RESULTS

The comparison among different analytical methods was performed in terms of: 1) resolution power, i.e. the incidence of samples with non-detectable amount of the substance searched; 2) fixed effect of method (bias) and of other sources of variation; 3) homoscedasticity or heteroscedasticity of residual variances (equal or different repeatability); and, in case of heteroscedasticity, 4) correlations between methods.

Resolution power

The GC×GC comprehensive system revealed the presence of 76 peaks (Table 2.1 and Table 2.2), corresponding to 23 SFA, 26 MUFA, and 27 PUFA. Nine FA present in the standards, (C13:0$_{anteiso}$, C16:0$_{anteiso}$, C18:0$_{anteiso}$, C21:0. C15:1, C16:1*t*7, C19:1*t*12, C22:1*n*9 and the C22:1*n*11) were never found in any the 3 tissues analyzed, and were omitted from the analysis. Among the 76 FA detected, the which raw means and standard deviations are given in order of elution per band in Table 2.1, 51 FA were identified through the use of standards. Of the remaining 25 FA, 10 FA were identified on the basis of their position in the two-dimensional chromatogram, 6 were identified as uncertain isomers, and 9 unidentified FA only the pertaining group was identified. Some FA were absent in one or two tissues. In particular, the number of undetected FA in liver, sub-cutaneous fat and LT muscle were 5, 14 and 9, respectively. There were only two cases of a clear co-elution of FA as under the operative conditions adopted the GC×GC system was unable to clearly separate the C18:1*t*7 from the C18:1*t*9, and the C18:1*c*4 from the C18:1*t*2.

For each method and each tissues 18 observations were expected, but many FA evidenced some observations with null values (undetectable values). The incidence of undetectable values on the total number of expected observation, which depend on the sensitivity of the method used, for liver, fat and muscle, ranged 0.04 to 0.08, 0.05 to 0.06, and 0.05 to 0.12, respectively, with incidences greater for the J method compared to the other two for liver and muscle samples but not for subcutaneous fat. In the case of liver the highest incidence of null values with the J method was mainly observed for short chain FA (C8:0 and C10:0), whereas in the case of muscle the highest incidence of undetectable values with the J method was mainly observed for the C24:0, for two C20:1*t* unknown isomers, and for 3 PUFA (C20:3*n*3, C20:4*n*3, C22:5*n*6).

Sources of variation and effect of method (bias)

The effect of diet was not significant for almost all the various FA, with exception of C18:0 (P = 0.037), C14:1*n*5 (P =0.031), C18:1*c*9 (P = 0.018), for one of the two C20:1*t* (P = 0.046), C20:1*n*7 (P = 0.027), C22:5*n*3 (P = 0.026) and, more notably, for the CLA*t*10,*c*12 (P <0.001) and the CLA*t*9,*t*11 (P = 0.004) CLA isomers, that were added to diets, together with palmitic and stearic acids, to two of the three groups of young bulls fed rumen protected CLA during fattening (Table 2.3 and 2.4). On the opposite, the effect of tissue was always highly significant, even if the data were expressed as proportion of the total amounts of FA and not as absolute amount in the tissue sample. The effect of the interaction diet×tissue was highly significant (P <0.001) only in the case of the CLA*t*10,*c*12 isomer (Figure 1). Interesting, from the methodological point of view, were the significant effects of both the method and the interaction method×tissue for the majority of the FA. Significant effects of tissue, method and method×tissue were also observed for the various groups of FA and for the desaturase indices (Table 2.5). With regard to the amount of fat extracted, there were, as expected, significant effect of tissue, but the F and the A methods did not significantly differed (P = 0.66) and on average 27, 594 and 22 mg/g fresh sample were extracted from liver, sub-cutaneous fat and muscle, respectively.

Heteroscedasticity of residual variances and repeatability of the methods in different tissues

The results of the Levene's test evidenced, except for diet, as the variances among levels within tissue, method or method×tissue were not homoscedastic for the large majority of the FA (Table 2.6 and 2.7). In other words, the RMSE values given in these tables for each FA, considered as an index of repeatability, differed significantly not only among methods but also among the 6 levels of the interaction M×T. Nevertheless, the RMSE values for the various single FA were in the large majority of the cases smaller than 1 mg/g total FA. In the case of liver the overall mean of these coefficients was 0.68 0.89 and 0.47 $mg \times g^{-1}$ total FA for A, F and for J, respectively. In the case of sub-cutaneous fat the mean RMSE averaged 0.48, 0.42 and 0.30 $mg \times g^{-1}$ total FA, and in the case of muscle the RMSE averaged 0.46, 0.41 and 0.69 $mg \times g^{-1}$ total fat, respectively.

Nevertheless, when the various FA were grouped in SFA, MUFA and PUFA the effects of method and of method×tissue interaction became not significant (Table 2.8). For SFA the magnitude of the RMSE was in the order of 7, 5 and 6 $mg \times g^{-1}$ total FA for liver, fat and muscle, respectively, in the order of 0.01 ± 0.004 of the mean SFA proportion. In the case

of MUFA the RMSE values averaged 9, 5, and 10 mg×g^{-1} total FA for liver, sub-cutaneous fat and muscle, respectively, corresponding to about 0.06, 0.01, and 0.03 of the corresponding mean MUFA proportions. In the case of PUFA, the RMSE values were in the order of 12, 1, and 8 mg×g^{-1} total FA, for liver, fat and muscle, respectively, corresponding to relative errors of 0.04, 0.03 and 0.10 of the corresponding mean PUFA proportions. Among the various tissues, subcutaneous fat showed the better repeatability for MUFA and PUFA. Liver and muscle showed similar repeatability for MUFA, but for PUFA the repeatability observed on liver was better than that observed on muscle, likely because the liver contained a greater proportion of PUFA (295 mg×g^{-1} total FA) than muscle (86 mg×g^{-1} total FA), whereas the amount of fat extracted from these two tissues was similar (27 and 22 mg/g fresh tissues, respectively).

For the CLA groups homoscedastic variances among levels of method, and method×tissue were found, too, with the exception for Σ CLA *t*/*t* were a significant method×tissue interaction was found (P = 0.008). In this case the three methods showed a similar repeatability for liver, whereas in subcutaneous fat the J method showed the best repeatability and in muscle the best repeatability was observed for both F and A.

Heteroscedasticity among the 6 levels of the method×tissue interaction was also observed for the Ω6 and Ω3 FA groups, but not for their ratio, for the branched and the odd chain FA, likewise for the groups of FA with less or more than 16C in their chain. No influence of method or method×tissue was found for ΣC18:1 FA, whereas for ΣC18:2 there was a significant effect of method, not of the method×tissue interaction.

Method and method×tissue interaction evidenced homoscedastic variances for the major part of the desaturase indexes, including the total one, and for the thrombogenic index, whereas the method×tissue heteroscedastic variances were detected for the atherogenic index.

Relationships between methods for different tissues

The regression approach was used to compare the mean contents of all the FA within each tissue. Within tissue, when the mean values obtained for the 76 FA with the A or the J method were regressed against those obtained with the F method, considered as the reference, very strong relationships were found (Figure 2). The intercepts of these regression were never significant. The slopes of the regressions relating ASE with Folch et al. (1957) methods were always significantly different from the unity, whereas those relating the Jenkins (2010) with Folch et al. (1957) methods differed significantly from the unity only in the case of

subcutaneous fat. However, in all cases the slopes were very close to the unity and the significant difference with respect to the unity was due to the very low residual variation, as indicated by the RMSE values given in the figure. The logarithmic representation of the distribution of the data in the figure, that expand the small values and contracts the greater ones, evidenced that the linearity between methods was maintained even for very little FA concentrations.

There were evident deviations from the linearity only for few FA. In the liver deviation from linearity with respect to the F method were observed for the C8:0 measured with the J method, as the proportions of this FA were 0.70 and 0.15 $mg \times g^{-1}$ total FA, respectively for the two methods. It should be also remembered that the C10:0 was undetected with the J method whereas with both F and A the presence of this FA was about 0.57 $mg \times g^{-1}$ total FA (Table 2.3). With respect to F and A, the J method indicated a lower proportion of C19:1*c* (Table 2.3), and a greater amount of an unknown C18:2 isomer (Table 2.4). In the muscle there were deviation from linearity of J versus F method, for the proportions of about 6 FA. These were the C20:4*n*3, two unknown C20:1*t* isomers, the C22:5*n*6, the C24:0, and the C20:3*n*3, and the proportions of these FA were always smaller than 0.18 $mg \times g^{-1}$ total FA (Table 2.3 and 2.4).

The relationships between the RMSE values provided by A or J compared to those of F method are given in Figure 3. In this case the correlations between methods were still high but far from the identity. The distribution of the points above or below the y=x function evidenced that both the repeatability of the A and the J methods was correlated and proportional to that achieved from the F method, with few very exceptions. In general the repeatability of the J method was consistently smaller (more favorable) than that achieved by the other methods in the case of liver, slightly smaller in the case of fat, and slightly greater in that of muscle.

In the liver, the notable deviations from the y=x function, and the consequent low correlation with the F method ($R^2 = 0.54$), regarded in particularly the J method: C8:0 (we previously evidenced that this FA was likely underestimated with this method), C14:1*n*5, C14:0, C18:2 unknown isomer, and for the A method the C20:2. Even excluding these points the correlation of J with F had an $R^2 < 0.55$ because for a number of other important FA, among which the C16:0, C181*c*9, C18:2, C20:4*n*6, evidenced RMSE smaller than those obtained with F and A (Table 2.6 and 2.7). In subcutaneous fat for both A or J with F showed an $R^2 > 0.80$ and in muscle both A and J had correlations with F with $R^2 > 0.74$.

2.5 DISCUSSION

The statistical approach followed in this experiment was structured to provide a full representation of different sources of variation on the analytical results. Different sources of variation were analyzed: diet, method, tissue and interactions (diet×tissue and tissue×method). Despite the significant effect of diet, the methodological approaches used in this study, given a greater interest to the effects of tissue and method. In total GC×GC identify 76 peaks in one run. Fatty acids identified were the product of reactions that occur in rumen. These reactions, that transform dietary lipids, were composed by a first step of hydrolysis through microbial lipases, followed by a second step of bio-hydrogenation of unsaturated fatty acids through rumen bacteria. When the process of bio-hydorgenation was not complete, become available for deposition in microbial biomass and in animal tissues many intermediates, such as, odd- and branched-chain fatty acids (Or-Rashid et al., 2007). Odd- and branched- chain fatty acids were important for the influence on fluidity of cellular membranes and they were used also for calculated the concentration of volatile fatty acids (VFA), mainly acetate, propionate and butyrate (Castro Montoya et al, 2011; Kaneda, 1991). This FA distribution is tissue specific ($P<0.001$). SFA were higher in subcutaneous fat, MUFA in subcutaneous fat and muscle and the higher amount of PUFA was observed in liver. In these three tissue, C14:0, C16:0 and 18:0 were the main SFA. Palmitoleic (C16:1) and Oleic (C18:1*c*9) the main MUFA. Linoleic (C18:2*n*6) the most abundant PUFA. Nevertheless, some fatty acids were found only in liver and in muscle and others only in subcutaneous fat (C14:1*t*, C17:1*t*7 and C17:1). In liver many Ω6 and Ω3PUFA were found (C20:3*n*6, C20:3*n*3, C20:4*n*6, C20:4*n*3, C20:5*n*3, C22:4*n*6, C22:5*n*6, C22:5*n*3, C22:6*n*3). The concentration of PUFA in liver was 10-fold higher than the concentration in muscles from the same animals (Enser, 1998-b). Enser et al (1998), confirm the higher amount of Linoleic acid (12.3% of fatty acid) follow by C20:4*n*6 (7.6%), C20:3*n*6 (3.8%), C22:4*n*6 (2.6%) and C22:5*n*3 (3.0%). Differences, in ruminants, were study and are related to the activity of rumen, liable to produce Stearic Acid (C18:0) by hydrogenation of dietary Oleic acid (C81:1*c*9) (Turk and Smith, 2009). Nevertheless, many tissues were involved in fatty acids metabolism and the most important were subcutaneous fat, skeletal muscle and liver. The content of FA can be regulated by enzyme activities, in particular by many desaturases (Δ^5,Δ^6 and Δ^9) and elongase, that were involved in the conversion of C18PUFA to their long chain derivatives (Δ^5,Δ^6 desaturase) and in the insertion of a double bond at the ninth carbon atom of the fatty acid chain and in this way is responsible to the conversion of saturated (SFA) to monounsaturated (MUFA) (Δ^9-desaturase) (Malau-Aduli et

al.,1997; Mele et al., 2007; Wang et al., 2013). Tissues were the store of lipids and an high content of triacylglycerol (TAG), as in subcutaneous fat, causes a low content of very long chain Ω3PUFA. On the contrary, liver is rich in phospholipids and it had an high content of very long chain Ω3PUFA (Sinclair, 2007). Moreover, adipose tissue was considered a storage of fatty acids that may be released into the blood circulation for delivery to other tissue. Muscle was the principal substrate for oxidation, directly proportional to their total volume of mitochondria, which was higher in small animals than in ruminant. Liver produced long chain fatty acid (LCFA) mainly from plasma NEFA that were removed in 7-25% from blood flow. Therefore, hydrolysis of circulating triglycerides by a lipase may also be a minor source of hepatic LCFA (Hocquette and Bauchart, 1999; Frayn et al., 2006; Zabala et al., 2006).

FA distribution was method specific ($P<0.01$ and $P<0.001$). GC×GC has been recognized as a tool offering higher peak capacities than others chromatography techniques. Samples were separated on two different columns, with different polarity, ensuring an improve in resolution power with no loss of time (Adahchour et al., 2006; Adahchour et al., 2008; Manzano et al., 2011). The advantage of this improve of power resolution was that the odd numbered FAME, which normally were not present o present in small amount, can now be recognize easily (Adahchour et al., 2006a).

Compared with other biological samples, beef meat fat is a complex matrix. Particularly, the diversity of 18-carbon chain acids in meat fat is large in contrast to other biological materials. For example only seven C18-FA components were found in marine oil even with the highly efficient GC×GC (Western et al., 2002) and in milk at least 17 different C18-fatty acids have been identified with the use of GC×GC by Hyötyläinen et al. (2004). Brugiapaglia et al. (2014), using a traditional GC, identified 31 FA in the *Longissimus Thoracis* muscle of different breeds, among which 10 were C18-FA. In the tissues of current experiment, because of the use of various reference standards and the ordered structure of the GC×GC chromatograms, 76 different FA have been quantified, among these 26 were C18-FA. The GC×GC is also well suited for the analysis of samples where compounds are present in very different concentrations, for this reason, were identified fatty acids between 372 mg/g total FA for C18:0 in liver analyzed by ASE and 0.01 mg/g total FA for C20:1*t* and C20:4*n*3 in muscle analyzed by Jenkins. Considering the presence of FA in very different concentrations, the resolution achieved under the operative conditions applied was considered good. Even if, irrespective by the method of extraction and by the tissue, the incidence of null values with respect to the number of expected measures for the various FA averaged 0.06.

The presence of undetermined values can be due to different reasons. Among these, an important role is played by the concentration of FAME in the final solvent solution injected into the system. A low FAME concentration of this solution reduce the detection of FA present in trace, and on the opposite, a high FAME concentration increases the co-elution of FA present in large proportion but with similar retention times (C18:1*t*9 and C18:1*t*11). In the current experiment, after preliminary attempts to identify the best operative conditions of the GC×GC equipment, a concentration of about 22 mg fat/ml solvent was found to be the best compromise to achieve a good separation and quantification of FA over a very extended range of concentration. In the case of the two methods based on fat extraction, Folch and ASE, the preparation of the FAME-solvent solution was accurate as it was based on the measured amount of fat extracted from the sample. Differently, in the case of J method it was necessary to compute, assuming a given fat content, the amount of fresh tissue to be treated to achieve a solvent FAME solution FAME with approximately 22 g fat/ml. This uncertainness explains in part the slightly greater incidence of null values obtained with J for liver and muscle compared to that achieved with the other two methods. However, no explanation was found about the fact that the J method, compared to the other two, presented a greater incidence of null values and lower concentrations for some saturated short chain fatty acid (C8:0, C10:0 and C12:0) in the liver, not in other tissues, and a greater incidence of null values and lower concentrations of some long chain FA (C24:0, two C20:1*t* uncertain isomers, C20:3n3, C20:4n3, C22:5n6) only in muscle.

2.6 CONCLUSIONS

In conclusion, the results of this experiment confirm that fatty acid can be influence by different sources of variation. Despite the diet, the most important effects considered, are tissue and method. The experiment confirm that fatty acid are tissue specific and each tissue has a specific fatty acid profile related to lipids composition and the different type of lipid present. SFA were higher in subcutaneous fat, MUFA in subcutaneous fat and muscle and PUFA in liver. As confirm by other study PUFA in liver were 10-fold higher than the concentration in muscles from the same animals and the main Ω6 and Ω3 found were: C20:3*n*6, C20:3*n*3, C20:4*n*6, C20:4*n*3, C20:5*n*3, C22:4*n*6, C22:5*n*6 and C22:5*n*3. CLA isomers has the same concentration on the muscle and this concentration is lower than subcutaneous fat. Some fatty acids, as example C22:6*n*3(DHA) were identify only in liver and not in the other tissue. The result shown that 18 observation were expected but many FA

evidenced some observations with undetectable values: This depend on the sensibility of method and it was for liver, fat and muscle, ranged 0.04 to 0.08, 0.05 to 0.06, and 0.05 to 0.12, respectively. GC×GC chromatography techniques used in this experiment, caused to the use of two different columns with different polarity, offer the higher resolution power. In total GC×GC identify 76 peaks in one run because of the use of various reference standards and the ordered structure of the GC×GC chromatograms. The reference standard allowed the identification also of 5 CLA isomers, not only the two main isomers (CLA*c*9,*t*11 and CLA*t*10,*c*12) but also, CLA*c*11,*t*13, CLA*c*9,*c*11 and CLA*t*9,*t*11. FAME concentration influence the resolution power because of low FAME concentration reduce the detection of FA present in trace, and a high FAME concentration increases the co-elution of FA present in large proportion but with similar retention times (C18:1*t*9 and C18:1*t*11).

Table 2.1: Saturated (SFA) and monounsaturated fatty acids (MUFA) identified by standard (Std) or position (Pos) in liver, subcutaneous fat, and *longissimus thoracis* muscle and number of undetermined values for each tissue and each method of fat extraction [F = Folch et al. (1957); A = ASE; J = Jenkins et al. (2010)] (n = 18 within each tissue and method).

FA	Identification*	Mean mg×gFA^{-1}	SD	Number of undetermined values (on a total of 18 per group)								
				Liver			Fat			Muscle		
				F	A	J	F	A	J	F	A	J
C8:0	Std[1]	0.353	0.283			10						2
C10:0	Std[1]	0.565	0.282		1	18						
C12:0	Std[1], Std[2], Std[3]	0.593	0.288	1	2	8						
C13:0	Std[1], Std[2], Std[3]	8.491	3.770									
C14:0$_{iso}$	Pos	0.299	0.093	5	3	6		1				4
C14:0	Std[1]	23.08	13.86									
C15:0$_{iso}$	Std[3]	1.221	0.259									
C15:0$_{anteiso}$	Std[3]	1.719	0.539									
C15:0	Std[1], Std[2], Std[3]	3.135	0.794									
C16:0$_{iso}$	Std[3]	1.399	0.257									
C16:0	Std[1]	214.2	75.88									
C17:0$_{iso}$	Std[3]	3.117	0.529									
C17:0$_{anteiso}$	Pos	6.406	1.379									
C17:0	Std[1]	8.746	1.585									
C18:0$_{iso}$	Pos	1.230	0.228									
C18:0	Std[1]	248.0	90.13									
C19:0$_{iso}$	Pos	0.205	0.121	1	1	2	3	1	3	2	4	4
C19:0$_{anteiso}$	Pos	0.932	0.650			1		1	1		1	1
C19:0	Std[3]	1.364	0.778	5	4	6	3	3	9	3	4	5
C20:0	Std[1]	8.536	8.190									
C22:0	Std[1]	2.785	3.342				13	8	7			
C23:0	Std[1]	0.225	0.147	4		5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C24:0	Std[1]	0.050	0.062	1	1	3	n.d.	n.d.	n.d.	5	7	14
C14:1*t*9	Std[1]	0.130	0.177	n.d.	n.d	n.d.				n.d.	n.d.	n.d.
C14:1*n*5 (c9)	Std[1]	6.969	6.629			1						
C16:1*c*7	Std[1], Std[2], Std[3]	2.271	0.385									
C16:1*c*9	Std[1]	25.47	17.98									
C17:1*t*7	Std[1]	0.042	0.059	n.d.	n.d	n.d.	2	6	1	n.d.	n.d.	n.d.
C17:1c10	Std[1]	0.036	0.059	n.d.	n.d	n.d.	4	2	2	n.d.	n.d.	n.d.
C18:1*t*9 (+*t*7) [c]	Std[1]	25.03	6.822									
C18:1*c*9	Std[1]	235.9	102.8									
C18:1*c*7	Std[1]	14.71	3.206									
C18:1*n*6[b]	Pos	3.587	1.663			1	3	8	4			
C18:1[a]	Pos	2.226	1.817				7	6	3			
C18:1*c*4 (+*t*2) [c]	Pos	1.262	0.504				1	2				
C18:1*c*3[b]	Pos	0.680	0.419					1				
C18:1[a]	Pos	0.724	0.701		3	4	3	3	1	7	9	6
C19:1*t*9	Std[1]	0.164	0.143	n.d.	n.d	n.d.	1			1	1	5
C19:1*t* [a]	Pos	0.551	0.300									
C19:1*c*9[b]	Pos	0.527	0.356					1	1		2	1
C19:1*c* [b]	Pos	0.206	0.302	5	6	4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C20:1*t* [b]	Pos	0.456	0.592				6	5	2	6	5	17
C20:1*t* [b]	Pos	0.332	0.386				3	5	4	9	2	16
C20:1*n*9t	Std[1]	0.879	0.748							5	4	3
C20:1*n*11	Std[1]	1.645	0.642				n.d.	n.d.	n.d.			
C20:1*n*9c	Std[1]	0.391	0.370	2	2	2		3	4	2	5	5
C20:1*n*7[b]	Pos	0.195	0.274		1		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C20:1 [a]	Pos	0.095	0.125		4	4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C20:1 [a]	Pos	0.273	0.495			1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

[a] Unidentified isomers

[b] Uncertained isomers

[c] Coeluited isomers

* The term "Std" is used to evidence that identification of a given FA was based on standard, the number superscript indicates the type of standard (Std[1]: #674, Nu-chek prep inc., MN, USA; Std[2]: #463, Nu-chek prep inc., MN, USA; Std[3]: #47080-U Bacterial Acid Methyl Esters (BAMEs), Sigma-Aldrich, MO, USA; Std[4]:#UC-60M, Nu-chek Prep Inc., MN, USA; Std[5]:#UC-61M, Nu-Chek Prep, Inc. MN, USA; Std[6]: #1256, Matreya LLC, PA, USA; Std[7]: #1257, Matreya LLC, PA, USA; Std[8]: #1259, Matreya LLC, PA, USA; Std[9]: #47085-U PUFA-3 Menhaden Oil, Supelco, PA, USA. The term Pos means that idenification was based on the position of that FA in the bi-dimensional chromatogram in regions grouping specific kind of fatty acids.The single FA are presented in order of elution.

Table 2.2: Polyunsaturated fatty acids (PUFA) identified by standard (STD) or position (POS) in liver, subcutaneous fat, and *longissimus thoracis* muscle and number of undetermined values for each tissue and each method of fat extraction [F = Folch (1957); A = ASE; J = Jenkins (2010)].

FA	Identification*	Mean mg×gFA^{-1}	SD	Number of undetermined values (on a total of 18 per group)								
				Liver			Fat			*muscle*		
				F	A	J	F	A	J	F	A	J
C18:2*t*9,*t*12	Std[1]	0.557	0.330	4	4	5		1		1		
C18:2 [a]	Pos	2.164	0.991		1	1				1		5
C18:2 [a]	Pos	3.642	0.462									
C18:2*n*6 (c9,c12)	Std[1]	67.67	45.03									
C18:2 *c*11,*c*14[b]	Pos	0.192	0.150	1		1		2	3	1	6	1
C18:2 [a]	Pos	0.231	0.131								1	
C18:2 [a]	Pos	0.321	0.221	9	11	5					1	1
C18:3*n*6 (c6c9c12)	Std[2]	1.201	0.900									
C18:3*n*3 (c9,c12,c15)	Std[2]	2.724	1.201									
CLA*c*9,*t*11	Std[4]	2.965	1.012									
CLA*c*11,*t*13	Std[8]	0.216	0.098	7	6	6	1				1	
CLA*t*10,*c*12	Std[5]	0.316	0.264	7	8	5		1			2	4
CLA*c*9,*c*11	Std[6]	0.262	0.135					1		2	3	3
CLA*t*9,*t*11	Std[7]	0.640	0.192	1	1	2						1
C18:4n3	Std[9]	0.437	0.407	1	5	1	3	3	4	1	2	1
C18:5*n*3 [b]	Pos	0.459	0.427				1	1				1
C20:2*n*3 [b]	Pos	0.750	0.821				n.d.	n.d.	n.d.			
C20:2n6	Std[3]	1.507	1.528		2	1	1	3	1		4	3
C20:3*n*6	Std[1]	12.40	15.86									
C20:3*n*3	Std[1]	0.414	0.496				n.d.	n.d.	n.d.	2	1	10
C20:4*n*6	Std[1]	28.35	33.01						1			
C20:4*n*3	Std[9]	0.766	1.038				n.d.	n.d.	n.d.	7	7	17
C20:5*n*3 (EPA)	Std[1]	0.639	0.722				n.d.	n.d.	n.d.			
C22:4*n*6	Std[9]	7.165	8.769				5	4				1
C22:5*n*6	Std[1]	1.652	2.244				n.d.	n.d.	n.d.	2	3	13
C22:5*n*3	Std[3] Std[9]	2.904	3.812				n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C22:6*n*3 (DHA)	Std[3] Std[9]	0.771	1.140				n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
null/expected values				0.04	0.05	0.08	0.05	0.06	0.05	0.05	0.06	0.12

[a] Unidentified isomers
[b] Uncertained isomers
[c] Coeluited isomers
***** The term "Std" is used to evidence that identification of a given FA was based on standard, the number superscript indicates the type of standard (Std[1]: #674, Nu-chek prep inc., MN, USA; Std[2]: #463, Nu-chek prep inc., MN, USA; Std[3]: #47080-U Bacterial Acid Methyl Esters (BAMEs), Sigma-Aldrich, MO, USA; Std[4]:#UC-60M, Nu-chek Prep Inc., MN, USA; Std[5]:#UC-61M, Nu-Chek Prep, Inc. MN, USA; Std[6]: #1256, Matreya LLC, PA, USA; Std[7]: #1257, Matreya LLC, PA, USA; Std[8]: #1259, Matreya LLC, PA, USA; Std[9]: #47085-U PUFA-3 Menhaden Oil, Supelco, PA, USA. The term Pos means that identification was based on the position of that FA in the bi-dimensional chromatogram in regions grouping specific kind of FA. The single FA are presented in order of elution.

Table 2.3: Mean content of saturated and monounsaturated fatty acid (mg/g total FA) in 3 tissues (T: liver, subcutaneous fat and *longissimus thoracis* muscle) determined with 3 methods of fat extraction [M: F = Folch et al. (1957); A = ASE; J = Jenkins et al. (2010)], and effects (*P* values) of Diet (D), T, D×T, M and M×T [n=18; the various FA are listed in order of elution within band].

Tissue	Liver			Fat			Muscle			*P*				
Method	F	A	J	F	A	J	F	A	J	D[1]	T	D×T	M	M×T
C8:0	0.70	0.64	0.15	0.20	0.13	0.09	0.61	0.30	0.21	0.53	<0.001	0.68	<0.001	<0.001
C10:0	0.57	0.56	n.d.	0.61	0.55	0.51	0.98	0.67	0.65	0.91	<0.001	0.76	<0.001	<0.001
C12:0	0.19	0.22	0.19	0.82	0.79	0.79	0.78	0.64	0.66	0.27	<0.001	0.87	0.07	0.033
C13:0	10.2	9.8	9.7	6.1	5.6	3.8	9.0	6.90	15.4	0.26	<0.001	0.24	<0.001	<0.001
C14:0$_{iso}$	0.19	0.23	0.22	0.38	0.39	0.36	0.30	0.29	0.25	0.11	<0.001	0.22	0.013	0.028
C14:0	5.2	6.0	4.8	38.0	37.8	38.4	26.1	26.2	25.2	0.08	<0.001	0.78	0.028	0.001
C15:0$_{iso}$	1.11	1.15	1.17	1.49	1.50	1.50	1.03	1.02	1.03	0.08	<0.001	0.81	0.076	0.23
C15:0$_{anteiso}$	1.40	1.44	1.38	2.21	2.20	2.23	1.54	1.54	1.54	0.04	<0.001	0.74	0.87	0.44
C15:0	2.49	2.59	2.44	3.89	3.90	3.93	2.96	3.05	2.96	0.15	<0.001	0.27	0.012	0.10
C16:0$_{iso}$	1.23	1.30	1.27	1.65	1.66	1.67	1.26	1.25	1.31	0.61	<0.001	0.23	<0.001	0.001
C16:0	109	113	106	275	275	275	257	262	256	0.82	<0.001	0.45	<0.001	0.002
C17:0$_{iso}$	2.61	2.70	2.67	3.50	3.42	3.53	3.09	3.11	3.43	0.23	<0.001	0.41	0.006	0.028
C17:0$_{anteiso}$	5.03	5.25	5.11	7.92	8.05	8.09	5.94	6.05	6.21	0.42	<0.001	0.42	<0.001	0.017
C17:0	10.3	10.5	10.5	7.6	7.7	7.7	8.0	8.2	8.2	0.58	<0.001	0.16	<0.001	0.52
C18:0$_{iso}$	1.34	1.35	1.35	1.29	1.28	1.30	1.03	1.05	1.09	0.12	0.006	0.30	0.26	0.56
C18:0	367	372	370	168	168	164	206	201	217	0.037	<0.001	0.55	0.020	<0.001
C19:0$_{iso}$	0.22	0.31	0.30	0.12	0.12	0.12	0.27	0.14	0.23	0.16	<0.001	0.45	0.28	<0.001
C19:0$_{anteiso}$	1.67	1.76	1.85	0.52	0.51	0.67	0.37	0.40	0.60	0.30	<0.001	0.61	<0.001	0.57
C19:0	2.38	2.43	2.34	0.82	0.84	0.77	0.99	0.77	0.94	0.44	<0.001	0.52	0.24	0.002
C20:0	20.7	19.7	18.2	1.63	1.64	1.49	4.52	3.88	5.07	0.60	<0.001	0.09	0.16	0.009
C22:0	7.55	7.08	7.20	0.01	0.03	0.05	0.75	0.68	0.66	0.26	<0.001	0.26	0.06	0.08
C23:0	0.15	0.31	0.18	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.70	n.d.	n.d.	0.002	n.d.
C24:0	0.10	0.13	0.08	n.d.	n.d.	n.d.	0.06	0.05	0.03	0.33	<0.001	0.65	0.014	0.07
C14:1*t*	n.d.	n.d.	n.d.	0.40	0.27	0.17	n.d.	n.d.	n.d.	0.32	n.d.	n.d.	<0.001	n.d.
C14:1*n*5	0.71	0.87	0.44	14.73	14.51	15.01	5.28	5.54	5.25	0.031	<0.001	0.026	0.73	0.006
C16:1*c*7	2.58	2.53	2.88	2.13	2.19	2.18	1.96	2.01	1.99	0.60	<0.001	0.76	<0.001	<0.001
C16:1*c*9	5.4	5.9	5.0	47.2	45.8	46.6	24.1	26.0	23.3	0.15	<0.001	0.65	0.06	0.006
C17:1*t*7	n.d.	n.d.	n.d.	0.10	0.09	0.08	n.d.	n.d.	n.d.	0.30	n.d.	n.d.	0.78	n.d.
C17:1	n.d.	n.d.	n.d.	0.09	0.08	0.09	n.d.	n.d.	n.d.	0.46	n.d.	n.d.	0.69	n.d.
C18:1*t*9 (+*t*7) [c]	16.6	18.6	17.2	30.5	30.0	30.7	26.5	30.7	24.6	0.14	<0.001	0.016	<0.001	<0.001
C18:1*c*9	95	97	91	316	320	325	294	301	284	0.018	<0.001	0.46	<0.001	<0.001
C18:1*c*7	15.2	15.2	17.3	16.6	16.8	14.9	12.0	11.7	12.7	0.41	<0.001	0.32	0.039	<0.001
C18:1*n*6[b]	4.21	4.10	4.81	3.97	2.65	3.97	2.98	2.98	3.12	0.32	0.031	0.013	0.008	0.10
C18:1 [a]	1.07	1.21	1.13	2.31	2.61	2.48	3.08	3.04	3.10	0.08	<0.001	0.49	0.90	0.99
C18:1*c*4 (+*t*2) [c]	0.95	0.94	1.18	1.42	1.31	1.33	1.41	1.55	1.25	0.08	0.005	0.06	0.99	0.10
C18:1*c*3[b]	0.64	1.11	0.95	0.84	0.46	0.65	0.52	0.55	0.39	0.56	<0.001	0.14	0.78	<0.001
C18:1 [a]	1.09	1.35	1.08	0.28	0.73	0.33	0.35	0.36	1.00	0.31	<0.001	0.35	0.07	0.003
C19:1*t*9	n.d.	n.d.	n.d.	0.24	0.26	0.17	0.21	0.26	0.20	0.32	<0.001	0.52	0.29	0.07
C19:1*t* [a]	0.35	0.44	0.46	0.85	0.62	0.62	0.67	0.51	0.43	0.77	0.001	0.70	0.048	0.025
C19:1*c*9[b]	0.52	0.45	0.25	0.60	0.83	0.83	0.39	0.49	0.42	0.50	<0.001	0.47	0.23	0.013
C19:1*c* [b]	0.31	0.29	0.46	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.18	n.d.	n.d.	0.21	n.d.
C20:1*t* [b]	0.95	0.93	1.15	0.14	0.13	0.15	0.11	0.11	<0.01	0.55	<0.001	0.77	0.83	0.43
C20:1*t* [b]	0.72	0.74	0.78	0.14	0.10	0.12	0.06	0.13	0.01	0.046	<0.001	0.06	0.91	0.49
C20:1*t*9	1.51	1.37	1.74	n.d.	n.d.	n.d.	0.24	0.40	0.49	0.14	<0.001	0.20	0.08	0.38
C20:1*n*11	1.43	1.39	1.39	1.90	2.13	2.05	1.47	1.58	1.49	0.23	0.003	0.04	0.09	0.22
C20:1*n*9	0.15	0.14	0.20	0.65	0.51	0.30	0.60	0.50	0.52	0.32	0.002	0.31	0.11	0.10
C20:1*n*7[b]	0.50	0.41	0.51	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.027	n.d.	n.d.	0.31	n.d.
C20:1 [a]	0.21	0.18	0.25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.25	n.d.	n.d.	0.45	n.d.
C20:1 [a]	0.81	0.62	1.04	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.77	n.d.	n.d.	0.08	n.d.

[a] Unidentified isomers
[b] Uncertain isomers
[c] Co-eluted isomers

Table 2.4: Mean content of polyunsaturated fatty acid (mg/g total FA) in 3 tissues (T: liver, subcutaneous fat and *longissimus thoracis* muscle) determined with 3 methods of fat extraction [M: F = Folch et al. (1957); A = ASE; J = Jenkins et al. (2010)], and effects (*P* values) of Diet (D), T, D×T, M and M×T [n=18; the various FA are listed in order of elution].

	Liver			Fat			Muscle			*P* - value				
	F	A	J	F	A	J	F	A	J	D	T	D×T	M	M×T
C18:2 *t*9,*t*12	0.68	0.87	1.02	0.34	0.25	0.28	0.50	0.79	0.50	0.47	<0.001	0.88	0.010	<0.001
C18:2 [a]	1.14	0.95	1.54	3.20	2.91	3.18	2.24	2.07	2.17	0.54	<0.001	0.09	0.012	0.29
C18:2 [a]	3.21	4.47	3.63	3.73	3.74	3.64	3.34	3.61	3.39	0.17	0.037	0.55	<0.001	<0.001
C18:2*n*6	127.9	121.9	131.9	21.2	21.4	21.8	56.9	51.6	54.4	0.15	<0.001	0.25	<0.001	<0.001
C18:2 *c*11,*c*14[b]	0.20	0.23	0.25	0.13	0.11	0.10	0.22	0.17	0.29	0.71	0.002	0.52	0.30	0.23
C18:2 [a]	0.24	0.20	0.31	0.18	0.17	0.11	0.34	0.23	0.30	0.21	0.001	0.33	0.029	0.002
C18:2 [a]	0.08	0.09	0.24	0.36	0.33	0.42	0.59	0.44	0.33	0.94	<0.001	0.60	0.16	<0.001
C18:3*n*6	2.18	2.35	2.36	0.32	0.31	0.20	1.04	0.75	1.30	0.13	<0.001	0.10	<0.001	<0.001
C18:3*n*3	4.12	4.01	4.50	1.61	1.55	1.40	2.54	2.02	2.78	0.45	<0.001	0.16	<0.001	<0.001
CLA*c*9,*t*11	2.19	1.82	2.27	4.08	4.09	4.12	3.03	2.27	2.83	0.74	<0.001	0.96	<0.001	<0.001
CLA*c*11,*t*13	0.17	0.14	0.20	0.23	0.23	0.24	0.28	0.17	0.21	0.06	0.048	0.019	0.022	0.034
CLA*t*10,*c*12	0.23	0.20	0.20	0.33	0.32	0.30	0.41	0.25	0.38	<0.001	0.003	<0.001	0.006	0.006
CLA*c*9,*c*11	n.d.	n.d.	n.d.	0.29	0.27	0.29	0.23	0.17	0.32	0.57	0.21	0.06	0.003	0.19
CLA*t*9,*t*11	0.55	0.57	0.56	0.78	0.70	0.66	0.74	0.68	0.50	0.004	<0.001	0.008	<0.001	<0.001
C18:4	0.31	0.51	0.46	0.18	0.20	0.12	0.60	0.41	1.02	0.90	<0.001	0.41	0.010	<0.001
C18:5*n*3 [b]	0.29	0.44	0.45	0.22	0.24	0.14	0.87	0.52	0.97	0.61	<0.001	0.66	0.22	0.003
C20:2*n*3 [b]	0.54	0.56	0.56	n.d.	n.d.	n.d.	0.74	0.68	0.50	0.62	<0.001	0.89	<0.001	0.003
C20:2	3.13	3.41	3.76	0.32	0.34	0.36	0.71	0.64	0.64	0.99	<0.001	0.99	0.14	0.05
C20:3*n*6	34.59	33.34	34.69	0.36	0.38	0.37	2.76	2.35	2.77	0.11	<0.001	0.04	0.027	0.15
C20:3*n*3	0.76	1.51	0.54	n.d.	n.d.	n.d.	0.18	0.33	0.08	0.76	<0.001	0.34	<0.001	<0.001
C20:4*n*6	75.81	70.47	75.27	0.39	0.42	0.38	10.83	9.17	10.85	0.36	<0.001	0.29	<0.001	<0.001
C20:4*n*3	2.10	1.96	2.11	n.d.	n.d.	n.d.	0.02	0.02	0.01	0.20	<0.001	0.15	0.004	<0.001
C20:5*n*3 (EPA)	1.66	1.54	1.66	n.d.	n.d.	n.d.	0.33	0.25	0.31	0.08	<0.001	0.08	<0.001	<0.001
C22:4*n*6	19.26	18.06	18.97	0.05	0.10	0.10	1.46	1.28	1.36	0.08	<0.001	0.08	<0.001	<0.001
C22:5*n*6	4.86	4.43	4.81	n.d.	n.d.	n.d.	0.15	0.14	0.03	0.65	<0.001	0.74	<0.001	<0.001
C22:5*n*3	8.38	7.75	8.41	n.d.	n.d.	n.d.	0.63	0.55	0.41	0.026	<0.001	0.008	0.001	<0.001
C22:6*n*3(DHA)	2.38	2.17	2.38	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.33	n.d.	n.d.	0.10	n.d.

[a] Unidentified isomers
[b] Uncertain isomers
[c] Co-eluted isomers

Table 2.5: Mean content of solvent fat extracted (mg/g fresh sample) and of groups of fatty acid (mg/g total FA) in 3 tissues (T: liver, subcutaneous fat and *longissimus thoracis* muscle) determined with 3 methods of fat extraction [M: F = Folch et al. (1957); A = ASE; J = Jenkins et al. (2010)], and effects (*P* values) of Diet (D), T, D×T, M and M×T.

	Liver			Fat			Muscle			*P value*				
	F	A	J	F	A	J	F	A	J	D	T	D×T	M	M×T
Fat extracted	25	29	n.d.	602	586	n.d.	25	19	n.d.	0.45	<.0001	0.66	0.66	0.82
SFA	551	560	546	521	521	515	532	529	548	0.17	0.002	0.15	0.46	0.001
MUFA	151	156	150	441	441	447	378	390	365	0.029	<0.001	0.65	<0.001	<0.001
PUFA	298	284	304	38	38	38	91	81	87	0.09	<0.001	0.34	<0.001	<0.001
Σ CLA	3.08	2.72	3.14	5.70	5.59	5.60	4.67	3.49	4.11	0.11	<0.001	0.62	<0.001	<0.001
Σ CLA *c*/*t*	2.46	2.05	2.56	4.63	4.63	4.65	3.72	2.67	3.37	0.17	<0.001	0.79	<0.001	<0.001
Σ CLA *c*/*c*	-	-	-	0.29	0.27	0.29	0.23	0.17	0.32	0.57	0.210	0.06	0.003	0.19
Σ CLA *t*/*t*	0.55	0.57	0.56	0.78	0.70	0.66	0.74	0.68	0.50	0.004	0.001	0.008	<0.001	<0.001
Ω6	268	254	272	23	23	23	74	66	71	0.11	<0.001	0.43	<0.001	<0.001
Ω3	22	22	22	3	2	2	6	4	6	0.016	<0.001	0.030	<0.001	<0.001
Ω6 /Ω 3	12.3	11.8	12.1	11.5	11.8	13.8	13.5	15.1	12.7	0.90	0.003	0.78	0.17	<0.001
Branched	14.7	15.4	15.1	19.1	19.1	19.4	14.8	14.8	15.6	0.21	<0.001	0.41	<0.001	0.001
Odd Chain	38.1	39.1	37.8	35.9	35.5	33.7	34.6	32.4	41.5	0.24	0.05	0.53	<0.001	<0.001
<C16	23	23	20	69	68	67	49	46	53	0.036	<0.001	0.49	0.034	<0.001
C16	118	123	115	326	325	325	284	291	283	0.37	<0.001	0.90	<0.001	<0.001
>C16	859	854	865	605	607	608	667	662	664	0.16	<0.001	0.97	0.003	<0.001
ΣC18:1	129	133	127	366	370	373	336	347	326	0.040	<0.001	0.61	<0.001	<0.001
ΣC18:2	133	128	138	29	29	29	64	58	60	0.14	<0.001	0.22	<0.001	<0.001
Δ^9 desaturases[1]:														
C14	1.02	1.20	0.78	2.74	2.72	2.75	1.67	1.73	1.70	0.022	<0.001	0.20	0.019	0.002
C16	0.45	0.49	0.43	1.46	1.43	1.45	0.86	0.90	0.83	0.13	<0.001	0.80	0.11	0.16
C18	2.05	2.07	1.97	6.54	6.56	6.66	5.88	6.00	5.70	0.018	<0.001	0.61	<0.001	<0.001
CLA*c*9,*t*11	1.16	0.90	1.18	1.18	1.20	1.18	1.03	0.69	1.04	0.86	<0.001	0.30	<0.001	<0.001
CLA*t*10,*c*12	0.10	0.06	0.08	0.10	0.10	0.09	0.15	0.07	0.12	<0.001	0.043	0.048	0.001	0.08
Total Index	1.65	1.71	1.60	4.65	4.65	4.71	4.03	4.16	3.90	0.045	<0.001	0.80	<0.001	<0.001
AI[2]	2.90	3.12	2.76	8.97	8.90	8.89	7.79	7.86	7.95	0.43	<0.001	0.17	0.011	<0.001
TI[3]	4.50	4.52	4.49	2.07	2.07	2.01	2.38	2.30	2.51	0.036	<0.001	0.98	0.37	0.002

[1] Δ^9-desaturase indices
[2] Atherogenic Index
[3] Thrombogenic Index

Table 2.6: Coefficient of repeatability of the measurements (RMSE) of saturated and monounsaturated fatty acids (mg/g total FA) content in 3 tissues (T: liver, subcutaneous fat and *longissimus thoracis* muscle) obtained by 3 different methods of fat extraction [M: F = Folch et al.(1957), A = ASE, J = Jenkins et al. (2010)] and heteroscedasticity of the variances (*P* - values) among levels of Diet (D), T, D×T, M and M×T evaluated by the Levene's test.

	Liver			Fat			Muscle			Heteroscedasticity of variances (*P* value)				
	F	A	J	F	A	J	F	A	J	D	T	D×T	M	M×T
C8:0	0.218	0.390	0.003	0.046	0.029	0.016	0.246	0.117	0.032	0.53	<0.001	0.68	<0.001	<0.001
C10:0	0.135	0.189	n.d.	0.036	0.016	0.011	0.244	0.067	0.031	0.02	<0.001	0.08	<0.001	<0.001
C12:0	0.054	0.055	0.028	0.021	0.014	0.016	0.305	0.020	0.016	0.33	0.051	0.42	0.022	0.010
C13:0	1.211	0.790	0.908	0.713	0.936	0.300	1.009	0.761	2.253	0.63	0.003	0.05	0.05	<0.001
C14:0$_{iso}$	0.038	0.051	0.045	0.021	0.024	0.040	0.053	0.009	0.055	0.69	0.037	0.45	0.003	0.031
C14:0	1.039	1.806	0.116	1.272	0.336	0.325	0.998	0.301	0.844	0.09	0.11	0.16	0.38	0.004
C15:0$_{iso}$	0.023	0.034	0.029	0.037	0.042	0.023	0.033	0.018	0.161	0.80	0.27	0.89	0.09	0.004
C15:0$_{anteiso}$	0.040	0.172	0.131	0.052	0.057	0.043	0.064	0.024	0.127	0.50	0.16	0.54	0.49	0.66
C15:0	0.065	0.119	0.265	0.098	0.068	0.043	0.093	0.056	0.123	0.75	0.16	0.38	0.73	0.71
C16:0$_{iso}$	0.042	0.046	0.028	0.038	0.039	0.028	0.029	0.018	0.065	0.35	0.45	0.25	0.57	0.33
C16:0	5.047	6.161	0.995	2.589	2.056	1.545	3.297	1.573	1.295	0.36	0.002	0.19	0.14	0.055
C17:0$_{iso}$	0.050	0.064	0.053	0.048	0.447	0.054	0.039	0.041	0.606	0.78	0.29	0.30	0.20	0.007
C17:0$_{anteiso}$	0.096	0.150	0.058	0.158	0.149	0.099	0.113	0.125	0.392	0.27	0.69	0.59	0.41	0.027
C17:0	0.125	0.123	0.074	0.159	0.093	0.113	0.136	0.311	0.318	0.41	0.09	0.50	0.52	0.11
C18:0$_{iso}$	0.022	0.108	0.040	0.035	0.150	0.021	0.021	0.023	0.123	0.63	0.96	0.19	0.47	0.045
C18:0	4.938	7.706	4.806	3.870	3.185	2.312	1.129	2.814	3.809	0.14	0.018	0.51	0.87	0.22
C19:0$_{iso}$	0.130	0.085	0.045	0.048	0.069	0.070	0.136	0.144	0.106	0.58	0.027	0.76	0.90	0.96
C19:0$_{anteiso}$	0.224	0.111	0.080	0.114	0.088	0.180	0.352	0.033	0.334	0.75	0.21	0.43	0.001	0.002
C19:0	0.058	0.114	0.650	0.210	0.101	0.022	0.139	0.035	0.111	0.41	0.29	0.72	0.30	0.36
C20:0	1.883	3.502	2.754	0.047	0.060	0.275	0.405	0.455	0.800	0.56	0.003	0.67	0.10	0.08
C22:0	0.334	0.430	0.475	0.027	0.034	0.013	0.152	0.099	0.334	0.76	0.004	0.75	0.049	0.16
C23:0	0.114	0.125	0.105	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.23	n.d.	n.d.	<0.001	n.d.
C24:0	0.065	0.085	0.048	n.d.	n.d.	n.d.	0.024	0.012	0.087	0.59	0.003	0.67	0.11	0.05
C14:1*t*	n.d.	n.d.	n.d.	0.108	0.097	0.137	n.d.	n.d.	n.d.	0.05	n.d.	n.d.	0.77	n.d.
C14:1*n*5	0.627	0.437	0.056	1.196	0.303	0.206	0.243	0.283	0.272	0.47	0.043	0.25	0.14	0.07
C16:1*c*7	0.076	0.231	0.071	0.280	0.057	0.150	0.041	0.026	0.054	0.22	0.015	0.24	0.034	<0.001
C16:1*c*9	1.741	1.481	0.754	1.508	0.517	0.859	3.099	0.875	3.907	0.51	0.19	0.81	0.65	0.033
C17:1*t*7	n.d.	n.d.	n.d.	0.035	0.072	0.053	n.d.	n.d.	n.d.	0.12	n.d.	n.d.	0.07	n.d.
C17:1	n.d.	n.d.	n.d.	0.034	0.034	0.026	n.d.	n.d.	n.d.	0.99	n.d.	n.d.	0.07	n.d.
C18:1*t*9(+*t*7) [c]	0.587	2.014	2.034	1.884	0.990	0.499	0.792	0.703	2.297	0.72	0.047	0.16	0.12	0.002
C18:1*c*9	5.80	8.06	1.62	4.09	3.05	2.10	4.49	5.80	10.05	0.94	0.035	0.26	0.35	0.19
C18:1*c*7	0.226	1.146	0.390	0.900	0.807	0.924	0.544	0.396	1.024	0.44	0.040	0.62	0.58	0.55
C18:1*n*6[b]	0.129	0.217	0.191	1.835	3.033	1.747	0.082	0.093	0.063	0.15	<0.001	0.06	0.006	<0.001
C18:1 [a]	0.231	0.186	0.188	2.936	2.668	2.427	0.474	0.088	0.214	0.18	<0.001	0.14	0.66	0.84
C18:1*c*4(+*t*2) [c]	0.364	0.548	0.532	0.295	0.393	0.332	0.386	0.029	0.462	0.07	<0.001	0.96	0.05	0.005
C18:1*c*3[b]	0.190	0.211	0.251	0.549	0.366	0.382	0.309	0.248	0.271	0.26	0.002	0.82	0.03	0.015
C18:1 [a]	0.491	0.366	0.152	0.119	0.801	0.164	0.502	0.422	0.985	0.45	0.004	0.21	0.25	<0.001
C19:1*t*9	n.d.	n.d.	n.d.	0.186	0.172	0.183	0.077	0.070	0.057	0.73	0.032	0.41	0.21	0.10
C19:1*t* [a]	0.079	0.210	0.365	0.265	0.442	0.415	0.392	0.340	0.228	0.21	0.010	0.28	0.22	0.07
C19:1*c*9[b]	0.354	0.469	0.146	0.028	0.446	0.475	0.234	0.427	0.455	0.44	0.062	0.10	0.014	0.002
C19:1*c* [b]	0.339	0.262	0.400	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.61	n.d.	n.d.	0.07	n.d.
C20:1*t* [b]	0.939	0.442	0.776	0.032	0.055	0.083	0.031	0.060	0.023	0.87	<0.001	0.97	0.40	0.22
C20:1*t* [b]	0.519	0.343	0.495	0.085	0.043	0.059	0.091	0.039	0.058	0.20	<0.001	0.17	0.36	0.53
C20:1*t*9	0.828	0.411	0.752	n.d.	n.d.	n.d.	0.216	0.362	0.337	0.70	0.005	0.022	0.62	0.87
C20:1*n*11	0.057	0.239	0.378	0.651	0.068	0.326	0.381	0.252	0.377	0.29	0.44	0.024	0.34	0.019
C20:1*n*9	0.089	0.061	0.214	0.807	0.447	0.058	0.399	0.402	0.295	0.11	0.001	0.004	0.56	0.43
C20:1*n*7[b]	0.239	0.334	0.201	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.74	n.d.	n.d.	0.031	n.d.
C20:1 [a]	0.240	0.151	0.198	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.59	n.d.	n.d.	0.45	n.d.
C20:1 [a]	0.540	0.517	0.751	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.49	n.d.	n.d.	0.035	n.d.

[a] Unidentified isomers
[b] Uncertained isomers
[c] Coeluited isomers

Table 2.7: Coefficient of repeatability of the measurements (RMSE, mg/g total FA) of polyunsaturated fatty acids content in 3 tissues (T: liver, subcutaneous fat and *longissimus thoracis* muscle) obtained by 3 different methods [M: F = Folch et al. (1957); A = ASE; J = Jenkins et al. (2010)] and heteroscedasticity of the variances (*P* - values) among Diet (D), T, D×T, M and M×T evaluated by the Levene's test.

	Liver			Fat			Muscle			*P* value				
	F	A	J	F	A	J	F	A	J	D	T	D×T	M	M×T
C18:2 *t*9,*t*12	0.233	0.426	0.154	0.079	0.093	0.045	0.053	0.115	0.164	0.49	<0.001	0.41	0.08	0.207
C18:2 [a]	1.041	0.786	0.154	0.088	1.120	0.047	0.074	0.048	1.127	0.94	0.015	0.24	0.13	0.001
C18:2 [a]	0.122	0.142	0.349	0.149	0.155	0.105	0.222	0.221	0.465	0.80	0.006	0.75	0.92	0.820
C18:2*n*6	4.040	4.862	1.835	0.340	0.486	0.286	3.245	4.102	4.207	0.65	<0.001	0.009	0.07	0.155
C18:2 *c*11,*c*14[b]	0.193	0.111	0.244	0.059	0.081	0.065	0.073	0.073	0.406	0.67	0.15	0.31	0.07	0.235
C18:2 [a]	0.120	0.049	0.189	0.114	0.106	0.071	0.120	0.158	0.184	0.63	0.027	0.47	0.20	0.196
C18:2 [a]	0.124	0.129	0.146	0.312	0.204	0.069	0.316	0.177	0.195	0.52	0.028	0.10	0.007	0.005
C18:3*n*6	0.150	0.200	0.134	0.101	0.127	0.129	0.349	0.278	0.437	0.71	0.002	0.69	0.38	0.141
C18:3*n*3	0.130	0.245	0.210	0.108	0.117	0.066	0.275	0.152	0.413	0.18	<0.001	0.004	0.37	0.001
CLA*c*9,*t*11	0.211	0.156	0.107	0.160	0.145	0.074	0.258	0.197	0.261	0.06	0.004	0.37	0.79	0.088
CLA*c*11,*t*13	0.145	0.065	0.164	0.061	0.058	0.033	0.071	0.038	0.059	0.34	0.16	0.14	0.135	0.067
CLA*t*10,*c*12	0.203	0.133	0.205	0.092	0.084	0.025	0.198	0.016	0.082	0.19	0.016	0.27	0.198	0.981
CLA*c*9,*c*11	n.d.	n.d.	n.d.	0.054	0.103	0.076	0.106	0.144	0.110	0.26	0.021	0.37	0.412	0.108
CLA*t*9,*t*11	0.095	0.081	0.084	0.098	0.047	0.022	0.098	0.094	0.157	0.71	0.023	0.06	0.405	0.008
C18:4	0.322	0.202	0.353	0.164	0.118	0.084	0.379	0.193	0.726	0.40	<0.001	0.10	0.291	0.012
C18:5*n*3 [b]	0.246	0.293	0.276	0.088	0.216	0.074	0.740	0.343	0.768	0.28	<0.001	0.17	0.059	0.003
C20:2*n*3 [b]	0.112	0.159	0.075	n.d.	n.d.	n.d.	0.041	0.118	0.282	0.47	<0.001	0.027	0.213	<0.001
C20:2	1.696	0.170	1.168	0.112	0.072	0.011	0.039	0.142	0.259	0.13	0.009	0.05	0.263	0.040
C20:3*n*6	1.375	4.687	1.191	0.046	0.034	0.011	0.357	0.389	0.412	0.48	0.004	0.39	0.079	0.043
C20:3*n*3	0.140	0.247	0.163	n.d.	n.d.	n.d.	0.089	0.069	0.143	0.49	0.001	0.54	0.005	0.431
C20:4*n*6	4.111	5.935	1.338	0.098	0.089	0.021	1.316	1.669	1.580	0.53	<0.001	0.19	0.007	0.002
C20:4*n*3	0.135	0.099	0.057	n.d.	n.d.	n.d.	0.030	0.062	0.021	0.93	0.001	0.56	0.076	0.183
C20:5*n*3 (EPA)	0.066	0.186	0.067	n.d.	n.d.	n.d.	0.056	0.068	0.105	0.24	<0.001	0.35	0.019	0.001
C22:4*n*6	1.113	1.644	0.576	0.013	0.060	0.024	0.231	0.202	0.481	0.54	<0.001	0.21	0.022	0.001
C22:5*n*6	0.293	0.335	0.147	n.d.	n.d.	n.d.	0.049	0.089	0.032	0.90	<0.001	0.41	0.041	0.005
C22:5*n*3	0.499	1.149	0.288	n.d.	n.d.	n.d.	0.106	0.149	0.221	0.30	<0.001	0.11	0.006	<0.001
C22:6*n*3(DHA)	0.513	0.333	0.535	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.34	n.d.	n.d.	0.820	n.d.

[a] Unidentified isomers
[b] Uncertained isomers
[c] Coeluited isomers

Table 2.8: Coefficient of repeatability of the measurements (RMSE, mg/g total FA) of fat extracted, groups of FA, and the main indices content in 3 tissues (T: liver, subcutaneous fat and *longissimus thoracis* muscle) obtained by 3 different methods [M: F = Folch et al. (1957); A = ASE; J = Jenkins et al. (2010)] and heteroscedasticity of the variances (*P* - values) among Diet (D), T, D×T, M and M×T evaluated by the Levene's test.

	Liver			Fat			Muscle			*P* value				
	F	A	J	F	A	J	F	A	J	D	T	D×T	M	M×T
SFA	4.53	6.63	8.79	8.06	5.71	2.76	6.03	5.20	8.15	0.57	0.08	0.54	0.32	0.07
MUFA	10.65	12.26	4.87	7.25	4.79	3.27	4.78	9.82	15.45	0.65	0.033	0.28	0.98	0.62
PUFA	13.12	15.21	7.21	1.34	1.42	0.62	7.03	8.37	9.95	0.75	<0.001	0.05	0.05	0.06
Σ CLA	0.278	0.365	0.190	0.249	0.121	0.099	0.495	0.234	0.375	0.14	<0.001	0.37	0.41	0.37
Σ CLA *c*/*t*	0.167	0.247	0.109	0.168	0.112	0.076	0.238	0.131	0.223	0.27	0.004	0.20	0.84	0.15
Σ CLA *c*/*c*	n.d	n.d	n.d.	0.054	0.103	0.076	0.106	0.144	0.110	0.26	0.021	0.37	0.41	0.11
Σ CLA *t*/*t*	0.095	0.081	0.084	0.098	0.047	0.022	0.098	0.094	0.157	0.71	0.023	0.06	0.41	0.008
Ω 6	12.15	14.56	6.10	0.48	0.83	0.49	6.44	8.21	8.53	0.75	<0.001	0.06	0.03	0.047
Ω 3	1.01	1.74	1.04	0.28	0.39	0.20	0.96	0.67	1.32	0.75	0.001	0.16	0.15	0.039
Ω6 /Ω 3	2.99	4.13	5.67	13.61	20.39	16.40	21.14	27.93	30.28	0.26	<0.001	0.13	0.38	0.22
Branched	0.348	0.326	0.474	0.400	0.580	0.301	0.461	0.200	1.211	0.93	0.45	0.31	0.50	0.028
Odd Chain	2.434	1.993	3.056	1.144	1.995	1.022	1.249	1.179	3.186	0.15	0.001	0.009	0.017	<0.001
<C16	2.249	2.682	0.845	2.098	1.046	0.694	2.085	0.658	1.577	0.19	0.024	0.002	0.16	<0.001
C16	6.607	7.708	1.555	3.491	2.420	2.122	1.947	1.862	4.321	0.18	0.005	0.55	0.44	0.06
>C16	8.501	9.889	1.598	4.588	2.538	2.041	3.459	2.092	4.697	0.20	0.008	0.31	0.24	0.014
ΣC18:1	4.959	7.760	4.798	3.857	3.162	2.331	1.145	2.810	3.813	0.81	0.09	0.13	0.80	0.07
ΣC18:2	5.668	4.678	2.918	0.879	1.416	0.445	4.507	5.893	6.317	0.72	<0.001	0.015	0.11	0.25
Δ^9 desaturases[1]:														
C14	0.277	0.437	0.159	0.203	0.022	0.027	0.070	0.050	0.090	0.17	<0.001	0.23	0.32	0.018
C16	0.113	0.080	0.077	0.035	0.009	0.020	0.109	0.026	0.136	0.30	0.048	0.44	0.75	0.044
C18	0.169	0.235	0.061	0.107	0.086	0.055	0.045	0.088	0.167	0.62	0.016	0.42	0.34	0.62
CLA*c*9,*t*11	0.110	0.154	0.201	0.069	0.049	0.030	0.110	0.047	0.164	0.41	<0.001	0.26	0.002	0.12
CLA*t*10,*c*12	0.095	0.095	0.098	0.024	0.029	0.007	0.072	0.005	0.043	0.09	<0.001	0.013	0.31	0.38
Total Index	0.132	0.147	0.041	0.091	0.063	0.030	0.047	0.099	0.153	0.67	0.033	0.31	0.56	0.60
AI[2]	0.298	0.407	0.066	0.321	0.130	0.074	0.297	0.130	0.185	0.23	0.13	0.07	0.039	<0.001
TI[3]	0.143	0.207	0.168	0.066	0.054	0.026	0.059	0.039	0.117	0.58	0.008	0.64	0.22	0.23

[1] Δ^9-desaturase indices
[2] Atherogenic Index
[3] Thrombogenic Index

Figure 1: Effect of increasing dietary rumen protected conjugated linoleic acid (CLA) supplementation on CLA*c*10,*t*12 content (mg/g total FA) of liver, subcutaneous fat and *longissimus thoracis* muscle (vertical bars indicate RMSE, interaction of CLA dose × tissue: $P < 0.001$, n = 18).

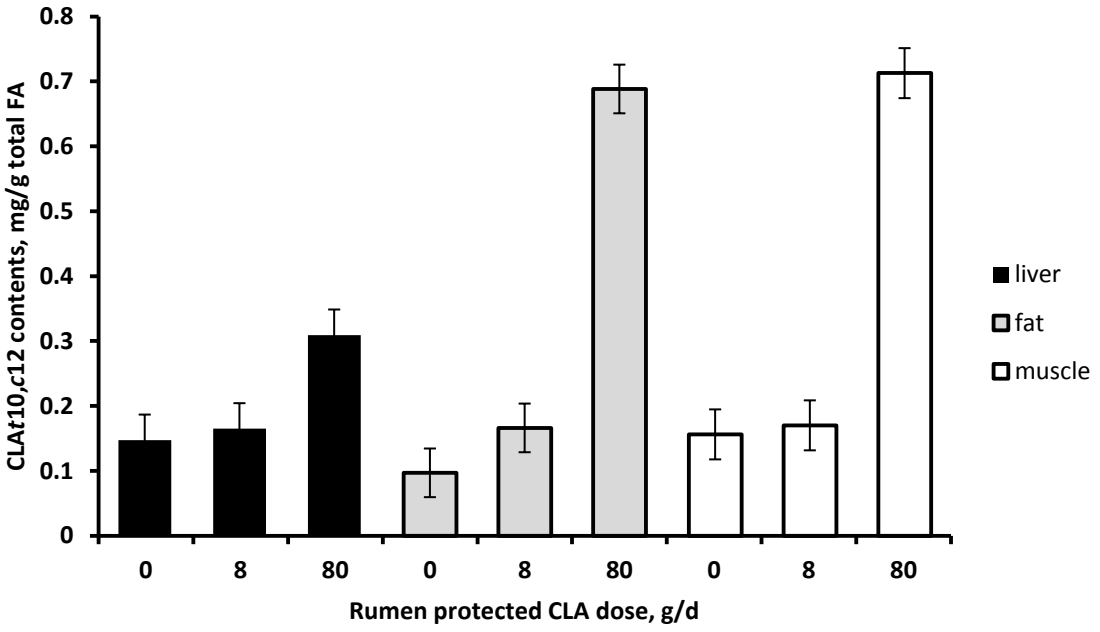
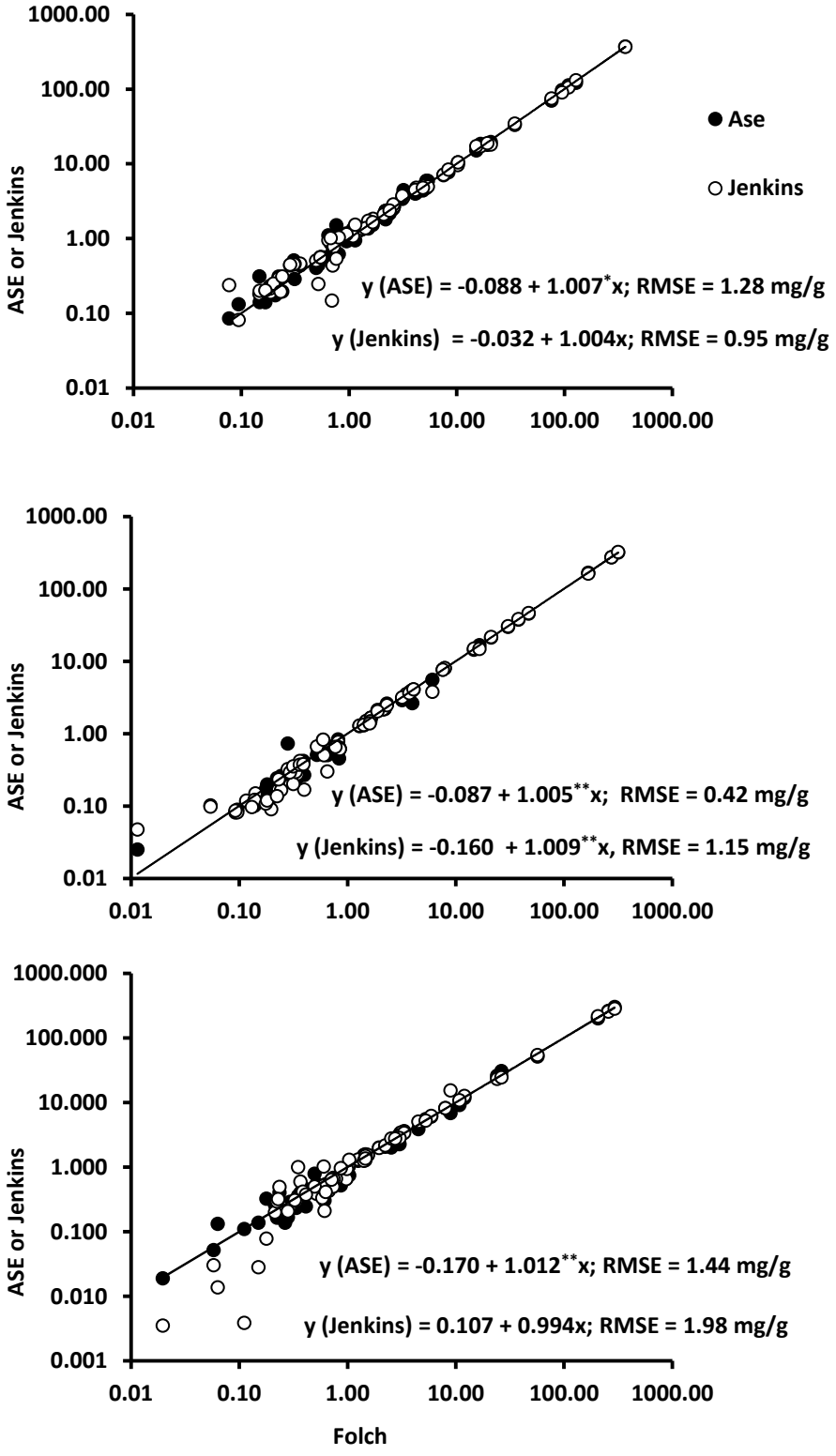


Figure 2: Single FA contents (mg/g total FA) in liver (a), subcutaneous fat (b) and *longissimus thoracis* muscle (c) determined with ASE or Jenkins et al (2010) plotted against those determined with Folch et al. (1957), and linear relationships between methods [Each point is one of the FA determined as a mean of 18 measures; data distribution is evidenced in logarithmic base 10 scale; Significance of the *F*-test was computed for a slope different from 1 and intercept different from 0 ($P < 0.05$).



CHAPTER 3

Effect of feeding system and rumen-protected conjugated linoleic acid (*rp*CLA) supplementation on a detailed fatty acid profile of three muscles, two fatty tissues and liver of lambs assessed by two-dimensional gas chromatography

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Ready to be submitted to Meat Science

3.1 ABSTRACT

The aim of this study was to investigate the effect of feeding system, gender and the tissue on the fatty acid (FA) profiles of lambs. Thirty-six weaned lambs, belonging to three autochthonous breeds of Veneto Region, were divided in three groups, balanced for breed, gender, age and weight, that were fed according to different systems: at pasture (P); penned in an open barn and fed with hay and concentrate (Indoor-control); and penned in the open barn and fed with hay and concentrate plus a rumen protected conjugated linoleic acid mixture (Indoor-CLA). Lambs were slaughtered at puberty and samples of 3 muscles (leg, rib eye, and other chop muscles), 2 fatty depots (sub-cutaneous cover and kidney fats) and liver were collected and analyzed for FA profile by two-dimensional gas chromatography. Data were analyzed considering the effect of the feeding system, breed, gender, and tissue. The random effect of animal was used to test feeding system, gender, and their interaction whereas the effects of tissue and corresponding interactions were tested on the residual.

Fifty-two FA were identified using standard references, among which 8 PUFA were found only in liver or kidney or both, other 70 were classified in 6 groups of FA according to their carbon chains length and unsaturation degree from their position in the two-dimensional chromatograms, and 12 FA were excluded because present in trace only in some samples. Irrespective by the tissue, the replacement of pasture with both the Indoor diets decreased the proportions of PUFA (-12%), ω3 FA (-45%), and increased the proportions of ω6 (+17%), branched (+36%) and odd chain FA (+20%) and the ω6/ω3 FA ratio was increased from 2.1 to 4.1 (P = 0.01). Compared to Indoor-control, Indoor-CLA increased the relative contents of CLA isomers (CLA*c*9,*t*11, P = 0.01, and CLA*t*10,*c*12, P = 0.002) without alteration of the relative contents of others FA, towards values similar to those observed for P. Despite the presence feeding system × tissue interaction for almost all FA, the effects on FA profile induced by the feeding systems with or without *rp*CLA showed similar trends for all tissues. Liver presented a FA profile very different from that of muscles and fatty tissues and, among fatty tissues, kidney fat was very peculiar. Considering the ω6/ω3 FA ratio suggested for human nutrition and those greater than 10.0 frequently observed for many ruminant meat products, the ω6/ω3 FA ratio of lamb fed indoor diets can be considered acceptable in the perspective of extending the period of seasonal availability of lambs in the market. The effectiveness of using rumen protected molecules to deliver specific bioactive FA in the lamb tissues was demonstrated.

Key words: Lambs, Fatty acids, Two-dimensional gas chromatography, Conjugated linoleic acid, liver, muscle.

3.2 INTRODUCTION

Fatty acid (FA) profile of meat from ruminants is often addicted as a cause of health problems for humans because of the high content of saturated FA (SFA) and also for the high ω6/ω3 FA ratio and the content of *trans* FA (Scollan et al., 2006; Shingfield et al., 2013), but it can be also an important source of beneficial FA, and for some of these high levels of biological activity have been reported, for example conjugated linoleic acid (CLA) (Wood and Fearon, 2009; Dilzer and Park, 2012). Nevertheless the effect of FA and micronutrient profiles on human health remains a controversial topic (Dannenberger et al., 2013). Ruminant meat has a high content of SFA and a low content of polyunsaturated fatty acids (PUFA). The main reason of this higher amount is the rumen hydrogenation of monounsaturated FA (MUFA) and PUFA (Gatellier et al., 2005). Following the guidelines for a healthy nutrition, meat quality can be improved by reducing SFA, and increasing MUFA and PUFA, reducing ω6/ω3 FA ratio, and increasing CLA content of red meat (Kouba and Mourot, 2011). Fatty acid composition can be affected by factors such as diet, species, breed, gender, age and weight, fatness, and fat deposit site (Scollan et al., 2006; Faria et al., 2012). With some feeding systems an increase of the ω3 PUFA content, in particular of the C18:3 α-linoleic, and a reduction of the ω6/ω3 FA ratio can be achieved (Realini et al., 2004; Webb and O'Neil, 2008). The CLA content can be increased by different dietary strategies, for example pasture feeding resulted in a significant increase of CLA*c*9,*t*11 in muscle lipids of German Holstein and German Simmental bulls compared with concentrate fed bulls (Nuernberg et al., 2005). However, pasture is subjected to seasonal availability, and has negative effects on the growth performance of animals, so that alternatives need be studied. For example, some feeds containing oil, such as soybean, linseed and sunflower seeds were found to influence the *n*-3 PUFA level in tissues (Kim et al., 2007), and a supply of a commercial rumen protected conjugated linoleic acid (*rp*CLA) was found to increase CLA content in the meat of growing cattle (Schiavon et al., 2011). The availability of new powerful analytical methodology and equipment, such as the two-dimensional gas-chromatography (GC × GC) allows to achieve a detailed and accurate assessment of FA profile (Manzano et al., 2011).

A better knowledge about the nutritional properties of meat can be important to improve the value of local endangered breeds reared at pasture. Sheep breeding has an important role

in rural, marginal and mountain areas, where the links with territory and traditions are pronounced and the use of native breed is predominant (Casabianca and Matassino, 2006). In Veneto region (North-East Italy) four native breeds are at risk of extinction (Pastore and Fabbris, 1999; Pastore, 2002; Bittante, 2011): Foza, Brogna, Alpagota, and Lamon with different characteristics (Bittante and Pastore, 1988; Ramanzin et al., 1991; Bittante et al., 1996). These breeds are used for the production of traditional dairy and meat products, for example "Alpagota lamb" is labelled by the "Slow Food Presidium" if lambs are reared according to specific norms (Slow Food Foundation, 2008), but it is also used for the production of a typical fermented sausage called Pitina (Bovolenta et al., 2007). The lambing of these local breeds is normally concentrated during late winter and both ewes and lambs are kept at pasture from spring to autumn. However, there is a tendency to distribute parturitions also in other seasons to offer in the market lamb carcasses for longer periods of time. This implies the use of an indoor feeding system based on hay and concentrate which would cause alterations of carcass and meat characteristics which need to be evaluated in relation to their possible effects on human health (Wood et al., 2003).

The aim of this study was to evaluate the effects of different feeding systems (lambs at pasture vs lambs fed indoor diets composed of hay and concentrate with or without *rp*CLA supplementation) and of gender on a detailed fatty acid profile and content of CLA isomers of different tissues (liver, fat depots and muscles) by means of a two-dimensional gas chromatography.

3.3 MATERIAL AND METHODS

Animals, feeding system and in vivo measurements

The present project followed the Guideline for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Consortium, 1988). This experiment was conducted at the "Lucio Toniolo" Experimental Farm of the University of Padova in Legnaro (Padova, Italy) and results about growth performance and carcass traits are given in a previous study of Pellattiero et al. (2011). Briefly, the lambs used for this research belongs to two flocks undergoing to an *in situ* conservation program. In total 36 weaned lambs belonging to 3 autochthonous Alpine breeds from the Veneto Region were used: 12 each, 6 males and 6 females, of Foza, Brogna, and Alpagota breeds. According to different feeding systems, lambs were divided in 3 groups (2 males and 2 females for each breed in each group): a) grazing a

permanent pasture (P), b) penned in an open barn and fed with hay and concentrate (Indoor-Control), and c) penned in the open barn and fed with hay, concentrate and daily supplemented with 8.0 g/head (Indoor-CLA) of a commercial *rp*CLA product (SILA, Noale, Venice, Italy). The first group (pasture) was introduced in a fenced meadow of the Experimental Farm, the second group (Indoor-control) was penned in an open barn and feed with hay from the same meadow given *ad libitum* plus, on average, 267 g/d of a commercial concentrate mixture. The last group (Indoor-CLA) was penned in the same open barn and fed the same diet of group (Indoor-Control) but the concentrate was daily supplemented with 8 g/head of *rp*CLA product containing 0.71 g/d of CLA*c*9,*t*11 and 0.69 g/d of CLA*t*10,*c*12. The characteristics and composition of the *rp*CLA product used is given by Schiavon et al. (2010). At the beginning of the trial lambs were weighed and evaluated for their body condition score (BCS) according to a scale from 1 (emaciated) to 5 (obese). The mean initial BW was 20.1 kg for ewe lambs and 21.8 kg for ram lambs, the initial BCS was 3.1 for ewe lambs and 3.0 for ram lambs. The trial lasted 113 d and the final BW was 28.1 and 31.7 kg for ewe and ram lambs, respectively. The average age, BW and BCS at slaughter and the killing out of the three groups are shown in Table 3.1.

Tissues sampling

Immediately after slaughter, liver and kidney fat samples were collected from all the animals and stored at -20°C. Carcasses were divided in two halves, that were weighed and cold stored at 4 °C. The day after slaughter, the semi-carcasses of each lamb were weighed again and the right semi-carcass was dissected into five cuts (hind-leg, fore-leg and shoulder, ribs-loin**,** withers, brisket). Each cut was weighed and the ribs-loin and the hind-leg were collected and stored under vacuum for 6 d at 4°C. Thereafter, these two cuts were dissected and weighed. Rib-loin was divided in ribs and loin. Ribs were dissected, and weighed, in rib eye muscle, other chop muscles, bones and subcutaneous fat. From hind-legs, only the inner part of the leg (*Quadriceps femoris*) was separated and weighed. Samples of subcutaneous fat, rib eye muscle, other chop muscles and leg muscle were collected and stored at -20°C prior the analysis.

Lipid extraction, FA esterification and GC × GC analysis

All the tissues collected were ground and mixed for 10 s at 4500 × *g* (Grindomix GM200, Retsch, Haan, Düsseldorf, Germany). Fresh samples were weighted and homogenized with Hydromatrix (Phenomenex, Castel Maggiore, Bologna, Italy) and transferred into 10 mL stainless steel extraction cells for ASE (Dionex ASE 350, Thermo Fisher Scientific Inc., MA, USA) with petroleum ether as solvent (#32044, Sigma-Aldrich, MO, USA). The conditions of extraction, reported in the ASE Application note 334 for meat samples (ASE, Dionex, Sunnyvale, USA), were those also applied by Schafer (1998) and Toschi et al. (2003). After the extraction the solution was heated at 50 °C under N_2 stream for about 20 min to complete solvent evaporation, placed in an oven at 60 °C for 15 min, cooled in a drier and the resulting extracted fat (EE) was weighted. About 40 mg of extracted fat were transferred to test tubes to be methylate according to Christie (2001) using sodium methoxide 1 *M* in methanol at room temperature. Briefly, 2 mL of n-exane, containing 2 mg/mL of methyl 12-tridecenoate as internal standard (#U-35 M, Nu-chek prep inc., MN, USA), 100 µl of sodium methoxide (1 *M*) in methanol were added to the EE obtained from lipid extraction. The solution containing the EE and solvents were mixed for 10 min using a laboratory multi mixer (717+, ASAL s.r.l., FI, Italy) at room temperature. After this step 150 µL of oxalic acid in ethyl ether was added to stop the reaction and obtained the fatty acids methyl esters (FAME) solution. The solution was mixed for 30 s and centrifuged at 8000 × *g* for 10 min (Sigma Laborzentrifugen 3K15, Osterode am Harz, Germany). The upper phase was collected (1 mL) and transferred to a vial for the GC × GC analysis. The resulting FAME solution was stored in GC vials at −20 °C prior the analysis.

Gas chromatographic analysis

The samples obtained were analyzed for their FA profile by GC × GC (Agilent Technologies 7890A, CA, USA) with two columns in series, equipped with a modulator (G3486A CFT, Agilent, CA, USA), an automatic sampler (7693, Agilent, CA, USA) and a flame ionization detector (FID) connected with a chromatography data system software (Agilent Chem Station, CA, USA). This instrument was used because the use of a double column allows a much better separation and identification of FA on a two-dimensional basis, compared to the traditional one column GC (Cesaro et al., 2013). Between the two columns the modulator unit collects in a fixed volume channel the analyte bands of the first column

and these are successively launched into the short second column in narrow bands. The operative conditions of the GC apparatus were:

- First column of 20 m × 100 µm (internal diameter) × 0.1 µm of film thickness (J&W 127-0122 Agilent Technologies, CA, USA), H_2 carrier flow of 0.3 mL/min.
- Second column of 5 m × 250 µm (internal diameter) × 0.15 µm of film thickness (Agilent 19091N-030, Agilent Technologies, USA), H_2 carrier flow of 24 mL/min.
- Planned oven temperature variation: increase from 40 °C (held for 2 min) and then increased to 260 °C (held for 5 min) at 5 °C/min.
- Valves: modulation delay, 0.115 min; modulation period, 2.0 s; sample time, 1.75 s.
- Detector (FID): heater, 250°C, H_2 carrier flow 20mL/min, air flow 450mL/min.
- Splitless Inlet: temperature 250°C, pressure 37.758 MPa, Septum Purge 3mL/min, Split Ratio 15mL/min.
- The resulting three-dimensional chromatograms were analyzed with the comprehensive GC × GC software (Zoex Corp., TX, USA) to evaluate the cone volume of each FA.

Identification and quantification of FA

The identification was made first by comparing the cone position in the chromatogram with the cone position of FA contained in reference standards. The reference standards used were mixtures of pure FA (#674, Nu-chek prep inc., MN, USA; Bacterial Acid Methyl Esters (BAMEs), Sigma-Aldrich, MO, USA) plus CLA*c*9,*t*11 (#UC-60M, Nu-chek Prep Inc., MN, USA) and CLA*t*10,*c*12 (#UC-61M, Nu-Chek Prep, Inc. MN, USA). The second FA identification method was based on the elution order and position of each FA in the two-dimensional chromatogram of the comprehensive GC × GC software (GC Imagine Software, Zoex Corporation, TX, USA). In the region of the C18:1 isomers different peaks were detected and two of them C18:1*t*9 (elaidic acid) and C18:1*t*11 (vaccenic acid) were partially co-eluted. Quantification of each FA was made considering the cone volume of each FA peak with respect to the volume of total FA and it was expressed in terms of g FA/g of total FA ×100 on the basis of the concentration in weight of the methyl 12-tridecenoate contained in the solution used as internal standard. In the statistical analysis and in the tables some peaks were excluded (C4:0, C6:0, C7:0, C13:0$_{iso}$, C13:0$_{anteiso,}$ C19:0$_{anteiso,}$ C23:0, C24:0, C13:1unknown isomer, ΣC14:1unknown isomers, ΣC19:1unknown isomers, ΣC20:1unknown isomers, C23:1, C24:1) because the relative peaks were lacking in many samples. Some long

chain PUFA (LC-PUFA) were reported separately because they were detected in significant amount only in liver and kidney fat.

Moreover, the various FA were summed in categories according to various criteria as follow.

- Saturated FA (SFA) was the sum of: C4:0, C6:0, C7:0, C8:0, C9:0 C10:0, C11:0 C12:0, C13:0, C13:0$_{iso}$, C13:0$_{anteiso}$, C14:0, C14:0$_{iso}$, C15:0, C15:0$_{iso}$, C15:0$_{anteiso}$, C16:0, C16:0$_{iso}$, C16:0$_{anteiso}$, C17:0, C17:0$_{iso}$, C17:0$_{anteiso}$, C18:0, C18:0$_{iso}$, C19:0, C19:0$_{iso}$, C19:0$_{anteiso}$, C20:0, C21:0, C22:0, C23:0 and C24:0;
- Monounsaturated fatty acids (MUFA) were the sum of: C13:1unknown isomer, C14:1*c9*, ΣC14:1unknown isomers, ΣC15:1others, C16:1*n*5, C16:1*t*7, C16:1*c*7, C16:1*c*9, ΣC16:1others, C17:1*c*10, ΣC17:1others, C18:1*t*7, C18:1*t*9(+*t*11), C18:1*c*9, C18:1*c7*, C18:1*n*3, ΣC18:1others, C19:1*n*9t, C19:1*n9c*, ΣC19:1unknown isomers, C20:1*n*7, C20:1*n*9, C20:1*n*11, C20:1*n*9*t*, ΣC20:1unknown isomers, C23:1, C24:1;
- Polyunsaturated FA (PUFA) were the sum of: ΣC16:2unknown isomers, C18:2*n*6, ΣC18:2unknown isomers, ΣC19:2unknown isomers, C20:2*n*6, C20:2*n*3, ΣC20:2unknown isomers, ΣC16:3unknown isomers, C18:3*n*3, C18:3*n*6, ΣC18:3unknown isomers, C20:3*n*3, C20:3*n*6, ΣC20:3unknown isomers, C18:4*n*3, C20:4*n*3, C20:4*n*6, ΣC20:4unknown isomers, C20:5*n*3 (EPA), C20:5*n*6, ΣC20:5unknown isomers, C22:2*n*6, C22:4*n*6, C18:5*n*3, C22:5*n*6, ΣC22:5unknown isomers, C22:6*n*3 (DHA), CLA*c*9, *t*11 CLA*t*10, *c*12, and ΣCLA unknown isomers;
- Unknown FA are the sum of 10 peaks not identified by standard or by position of the peaks in relationship to elution time.
- Branched FA were calculated according to Raes et al. (2004) and were the sum of: C13:0$_{iso}$, C13:0$_{anteiso}$,C14:0$_{iso}$, C15:0$_{iso}$, C15:0$_{anteiso}$,C16:0$_{iso}$, C16:0$_{anteiso}$, C17:0$_{iso}$, C17:0$_{anteiso}$, C18:0$_{iso}$, C19:0$_{iso}$ and C19:0$_{anteiso}$;
- Odd chain FA were calculated according to Or-Rashid et al. (2007);
- The sum of the identified CLA isomers was indicated as ΣCLA and was calculated by the sum of isomers identify by standard and position: CLA*c*9, *t11* CLA*t*10, *c12* and ΣCLA unknown isomers. Other CLA isomers, such as the CLA*t*7, *c9* and CLA*c*11, *t13*;
- The sum of Omega-3 PUFA (ω3) or Omega-6 PUFA (ω6) were calculated according to Givens et al. (2000) and Connor (2000), as example ω3 PUFA was computed as the sum of C18:3*n*3, C18:4*n*3 C18:5*n*3, C20:2*n*3, C20:3*n*3, C20:4*n*3, C20:5*n*3(EPA), C22:5*n*3, C22:6*n*3 (DHA);

- The Δ9-desaturase indices were calculated, according to Kelsey et al. (2003), for four pairs of FA that represent products and substrates for Δ9-desaturase. These fatty acid pairs were 14:1*c*9/14:0, 16:1*c*9/16:0, 18:1*c*9 /18:0, and C18:2*c*9,*t*11/C18:1*t*11. We defined the desaturase index as follows: [product of Δ9-desaturase]/[product of Δ9-desaturase + substrate ofΔ9-desaturase];
- The Atherogenic Index (AI) and Thrombogenic Index (TI) were calculated according to Ulbricht and Southgate (1991) as: AI = (C12:0 + 4 × C14:0 + C16:0)/(ω3 PUFA + ω6 PUFA + MUFA); and TI = (C14:0 + C16:0 + C18:0)/(0.5 × MUFA + 0.5 × ω6 PUFA + 3 × ω3 PUFA/ω6 PUFA).

Statistical analysis

Statistical analysis of experimental data was performed using MIXED procedure of SAS (SAS, 2005). Data were analyzed using a linear mixed model considering the effect of the feeding system, breed, gender, age and tissue as fixed effects. The random effect of animal was used to test feeding system, breed, gender and age, whereas the effects of tissue and of its interaction with feeding system were tested on the residual. The model includes also the orthogonal contrasts listed in the corresponding tables. As the effect of breed and of age of lambs were seldom significant and as a preliminary analysis excluded interactions with the other fixed effects considered, their least square means were not reported in the tables and discussed in the text.

3.4 RESULTS

The GC×GC comprehensive system revealed the presence of 52 peaks identified using standard references, 21 SFA, 15 MUFA, and 16 PUFA. Fifty-four others peaks were found but as their FA identification was not based on standard references they were grouped in 6 categories (ΣC15:1 others, ΣC16:1 others, ΣC17:1 others, ΣC18:1 others, ΣC16:2 others, and ΣC18:2 others) on the basis of their position in the ordered GC×GC chromatograms. Twelve peaks were excluded because present in trace only in some samples.

Effects of feeding system, gender and the interaction between these two effects

The least square means of the proportions of single SFA (Table 3.2), MUFA and PUFA (Table 33.) are based on the data of all tissue analyzed, with the omission of the unsaturated FA found only in liver, kidney or both which are separately given in Table 4.

Feeding system affected the proportion of the large majority of individual FA. Lambs reared at pasture exhibited a FA profile different from those reared in the barn pens fed hay and concentrates, as evidenced by the *P*-values achieved for the pasture *vs* B contrast. The only important FA which were not affected by pasture were C14:0 among SFA, and C16:1*c*9, and C18:1*c*9 among MUFA. Even if pasture differed from the other treatments for the large majority of individual FA proportion, when the FA were summed in groups it resulted that the feeding system had little influence the proportions of SFA and of MUFA (Table 3.5). The PUFA proportion was slightly greater in lambs reared at pasture than the average value found in lambs fed the indoor diets mainly because of the increase of C18:3*n*3, C20:5*n*3 (EPA), and C22:6*n*3 (DHA) proportions in grass fed lambs. Pasture caused also an increase of the sums of CLA, of ω3 FA, and a decrease of ω6 FA proportions so that the ω6/ω3 ratio was lowered compared to the other feeding treatments. In addition, pasture increased long chain FA, decreased the proportions of medium and short chain FA as well as the incidence of both branched and odds FA. The effect of pasture on the desaturation indices was null or small, but the atherogenic index was reduced compared to the other treatments (Table 5). The effect of *rp*CLA supplementation with the indoor diet affected only individual CLA isomers (Table 3), their sum and the C18:2*c*9*t*11desaturation index (Table 3.5).

Gender affected only marginally the FA profile of lamb tissues (the proportion of C15:0, C15:0$_{anteiso}$, and C16:1c7 was always greater in males compared to females) and interact with feeding system only for C15:0.

Differences among tissues FA profile and interaction with feeding system

Notable differences of FA profile were observed among tissues or groups of tissues (Table 3.6, 3.7). The FA profile of liver differed from that of the other tissues for the large majority of the single SFA, MUFA PUFA. In addition, 5 long chain FA were found only in liver samples, among which the C22:6n3 (DHA) and other 3 long chain FA were detected only in liver and kidney fat (Table 3.8).

Compared to the other tissues, liver contained smaller proportions of SFA and MUFA and a much greater proportion of PUFA (Table 3.9). In addition, the proportion of unknown FA

was greater in liver compared to the average of the other tissues. Liver tended to contain a slightly greater proportion of ΣCLA (P = 0.06), but also contained a greater proportion of ω3 and ω6 FA, being the ω3 and the ω6 contents 6 to 7-fold and 3 to 4-fold greater than the average of the other tissues, respectively. The ω6/ω3 ratio in liver was about 50% smaller than the average value found for the other tissues. Liver also evidenced a smaller proportion of short- and medium-chain FA, and a greater proportion of long- and odd-chain FA compared to the other tissues. The overall Δ^9-desaturation index was lower in liver than in other tissues (Table 3.9), mainly because the incidence of smaller values found for the C18 index. On the opposite, the CLA*c*9,*t*11 desaturation index was greater in liver compared to the other tissues. Both the thrombogenic and atherogenic indices values computed for liver were about 50% smaller (more favorable) than the values computed for the other tissues.

The FA profile of the two fatty tissues was different from that of muscles, with exceptions for some FA (Table 3.6). In the case of saturated FA the differences between fat and muscle tissues in the FA proportions regarded the large majority of individual FA, in particular of the minor FA (those representing less than 1% of total FA). In the case of unsaturated FA, differences in the FA proportions between fats and muscles were found also for all the major FA (Table 3.7).

In terms of FA categories (Table 3.9), fatty depots had greater SFA and smaller MUFA and PUFA proportions, greater proportions of unknown FA and ω3 FA, smaller proportion of ω6 FA, lower ω6/ω3 ratio, similar short- medium- and long-chain FA proportions, and greater proportions of branched and odds chain FA compared to muscles. The total desaturation index of fatty tissues was lower and the thrombogenic and atherogenic indices were greater compared with that of muscles.

The FA profile of kidney fat frequently differed from that of cover fat, particularly for the greater proportion of SFA (Table 3.9), almost exclusively due to a greater incidence of C18:0 (Table 3.6), and a smaller proportion of MUFA, mainly due to a smaller proportion of C18:1*c*9 (Table 3.7). In addition, in kidney fat 7 very long-chain unsaturated FA that were not detected in the subcutaneous cover fat were found (Table 3.8). Kidney fat compared to subcutaneous cover fat was characterized by the presence of unknown FA in trace, a greater proportion of ΣCLA, a smaller proportion of ω6 FA, and a lower ω6/ω3 ratio. Kidney, had greater proportion of short- and long-chain FA, a smaller proportion of medium-chain FA, and a greater incidence of odd-chain FA, compared to cover fat. In kidney fat the values of the desaturase indices were smaller, particularly for C16 and C18, and the thrombogenic and atherogenic indices were greater compared to cover fat (Table 3.9).

Also within the 3 muscles several differences were found in all classes of FA, but in this case, even if statistically significant, the magnitude of the differences among muscles was smaller compared to that found in the previous comparisons (Tables 3.6, 3.7 and 3.9). The general trend was that the other chop muscles tended sometimes to have a FA profile intermediate between that of the leg and rib-eye muscles and that of the fatty depots.

Lastly, the large majority of individual FA and of their groups and indices showed interactions regarding the effects of feeding system in different tissues. The number of traits that evidenced a significant interaction (41 among FA, groups and indices) and the complexity of the interaction (18 least square means with 10 df for each one) make impossible to detail all these interactions, but some examples of interest for human health are presented and discussed. Pasture and *rp*CLA supply increased the proportion of the CLA*t*10,*c*12 isomer in all tissues compared to the system based on hay and concentrate, and the magnitude of response to *rp*CLA supply was similar to that achieved with pasture, except for kidney fat where a notable increase of this isomer was found when *rp*CLA was offered to the lambs (Figure 1). Among tissues, liver evidenced the highest proportions of both ω3 and ω6 FA, and in all tissues pasture increased the proportion of ω3, decreased the proportion of ω6 and decreased the ω6/ω3 ratio compared to the other feeding systems (Figure 2). The ω6/ω3 ratio was lowest with pasture in all tissues. Irrespective of the feeding treatment this ratio ranged 1.0 to 2.5 and 2.5 to 6.0 in liver and other tissues, respectively. With pasture the proportions of branched and odd FA were lower compared to those found for the other feeding systems in almost all tissues, but pasture decreased the proportion of these FA more in liver and kidney than in other tissues (Figure 3).

3.5 DISCUSSION

The GC×GC technique offers high separation efficiency and enhanced sensitivity compared to single column GC (Adahchour et al., 2008). A further characteristic of the GC×GC technique is the ordered structure of the chromatograms (Vlaeminck et al., 2007; Adahchour et al., 2008), which makes the identification of compounds more reliable than in traditional GC, particularly when columns with different polarity are used (Manzano et al., 2011). The GC×GC is also well suited for the analysis of samples where compounds are present in very different concentrations. Compared with other biological samples, beef meat fat is a complex matrix. To our knowledge this is one of the first time that such technique has been applied to analyse the FA profile of lamb's meat. The potential of this technique is highlighted by the

106 different FA detected. This would open new perspectives to improve the knowledge of lipid metabolism in ruminants.

Feeding system

Dietary manipulation is considered to be one of the most effective way to improve the FA composition of sheep meat (Nuernberg et al., 1998; Shingfield et al., 2013; Sinclair, 2007). Pasture exerts a positive effect on the FA profile of meat of ruminants because it increases the proportion of MUFA and PUFA and decreases the proportion of SFA with respect to indoor fed animals (Nuernberg et al., 2002; Realini et al., 2004). Also in the case of growing lambs pasture increased the proportion of PUFA, in particular of ω3 PUFA, and decreased the proportion of ω6 PUFA (Aurousseau et al., 2004; Serra et al., 2009; Gallardo et al., 2011) compared to diets based on hay and concentrates, so that the ω6/ω3 ratio was lowered (Kouba and Mourot, 2011). The main reason of this influence of pasture on meat FA profile is that grass is a natural source rich of C18:3*n*3, a precursor of other ω3 FA, whereas concentrates are rich in C18:2*n*6, a precursor of others ω6 FA (Sinclair, 2007).

These effects of pasture were confirmed in the current experiment where it was found that grazing lambs had an average ω3/ω6 FA ratio of 2.1 and that lambs fed hay and concentrate reached an average ω3/ω6 FA ratio of 4.1. For healthy human diets it has been suggested that the optimal ω3/ω6 ratio should range between 2.3 to 8.1, according to different authors (Kim et al. 2006) with a preferred ratio ≤ 5.0 (Nurberg et al., 2005). Kim et al. (2006) observed that typical western type foods that are consumed have an average ω3/ω6 FA ratio of equal or greater than 10:1, as also confirmed in the meat of different beef breeds produced in Italy by Brugiapaglia et al. (2014). This suggests that, for conditions similar to those of current trial, lambs grew on hay and concentrates would be characterized by a ω3/ω6 FA ratio still within the range considered to be adequate for human health.

Conjugated linoleic acids are considered to be beneficial FA for human health, especially the C18:2*c*9,*t*11 and the C18:2*t*10,*c*12 isomers, even if the topic remains controversial (Park, 2009; Penedo *et al*., 2013). The C18:2*c*9,*t*11 is the main CLA isomer in ruminant tissues and it is formed as partial hydrogenation product during ruminal fermentation (Williams, 2000; Perfield et al., 2007; Bhattacharya, Banu, Rahman, Causey & Fernandes, 2006). The major part of it is synthesized in tissues from *trans* vaccenic acid (C18:1*t*11) in a desaturation reaction catalyzed by the stearoyl-CoA-desaturase enzyme (SCD), encoded by a specific gene (Griinari *et al*., 2000; Pariza et al., 2001; Cecchinato *et al.*, 2011). The C18:1*t*11 is formed in

the rumen from C18:1*n*9, C18:2*n*6 and C18:3*n*3 (Kim *et al.*, 2007), but diets that promoted the greatest activity of SCD (i.e. starchy concentrate diets) were also associated with a lower duodenal flow of the C18:1*t*11 precursor, with forage-based diets resulting in the highest flow of this FA from the rumen (Sinclair, 2007). As a consequence tissues of ruminants kept on pasture commonly present greater content of C18:1*t*11 and C18:2*c*9*t*11 compared to those of animals fed concentrate diets (Sinclair, 2007). Drying and storage of forages decrease C18:3*n*3, precursor of CLA, and increase C16:0 (Sinclair, 2007). In addition an immature fresh grass has a higher rate of rumen lipolysis and hydrogenation that would reduce C18:1*t*11 formation in comparison with grass from mature pasture (Realini et al., 2004; Dhiman et al., 2005; Scerra et al., 2007).

These results were confirmed in the current experiment, as the proportions of C18:1*t*11, even if this FA was partially co-eluted with C18:1*t*9, and of C18:2*c*9,*t*11 were greater on tissues of lambs kept on pasture compared to those of lambs fed hay and concentrate. In addition, pasture decreased the proportion of both branched and odd FA in the various tissues, compared with the other treatments. According to Serra et al. (2009) this would reflect a lower rumen activity and a likely greater rumen escape of intermediary products of bio-hydrogenation, as also suggested by the greater proportions of PUFA and CLA found in the tissues of lambs kept at pasture compared to the other treatments of current experiment. The increased proportion of branched FA, caused by the use of the concentrated-based indoor diets compared to pasture, might also be due to a greater availability of propionic acid for lipogenesis, occurring, in particular, in adipose tissues (Demirel *et al.*, 2004).

From experiments conducted with monogastrics, it was suggested that C18:2*c*9,*t*11 would exert a prevalent action on protein metabolism increasing lean growth and feed efficiency (gain:feed intake), whereas the C18:2*t*10,*c*12 is effective on lipid metabolism, particularly in lactating cows and sheep where it induces a dose-dependent milk fat depression (Pariza et al., 2001; Pulina et al., 2006). The effects of these isomers on lipid and protein metabolism are likely inter-related (Pariza et al., 2001). For example the C18:2*t*10,*c*12 reduces the SCD activity and its gene expression so that an increased presence of this isomer would also reduce the endogenous synthesis of the C18:2*c*9,*t*11 by desaturation of C18:2*t*11 (Dhiman *et al.*, 2005). To this regard it is interesting to observe that in double-muscle Piemontese young bulls, a *rp*CLA supplementation did not affected fat deposition (Schiavon and Bittante, 2012), but increased feed efficiency (gain:feed) and N efficiency (N retained/N intake), particularly with low-protein diets (Dal Maso et al., 2009; Schiavon et al., 2012). Differently, in dairy cows *rp*CLA supply increased feed efficiency (milk yield/DMI) with conventional protein

diets, not with low protein diets (Schiavon et al., 2014), but in any case a notable reduction of milk fat content was found, in agreement with others (Baumgard et al., 2000; Bauman et al., 2008;Glasser et al., 2010). This was observed also in lactating ewes of the local sheep breeds of Veneto, with an alteration of milk technological properties (Bittante et al., 2014).

The C18:2*t*10,*c*12 isomer is produced in the rumen from the bio-hydrogenation of linoleic acid, especially when starchy and low fiber diets are used in cows, sheep and lambs (Bauman et al., 1999; Pulina et al., 2006) or when oils rich in linoleic acid are supplemented (Mir et al., 2000; Antongiovanni et al., 2004; Dhiman et al., 2005). However, the proportion of this CLA isomer in ruminant products is commonly low (Park, 2009), and a supply of *rp*CLA is considered the most effective way to increase the presence of both these two CLA isomers. In the current study, the indoor diet with *rp*CLA increased the average tissue contents of CLA*c*9,*t*11 and of CLA*t*10,*c*12 by about 30% and 100%, respectively, compared to the same diet without *rp*CLA supply. The different trends observed for the two isomers partially depend by the average tissue level observed for the indoor control diet (without *rp*CLA addition), which was notably lower for CLA*t*10,*c*12 compared to CLA*c*9,*t*11 (0.05 vs 0.43 % of total FA, respectively), so that the same daily dietary supply of the two isomers with the commercial *rp*CLA mixture had a relative greater effect for C18:2*t*10*c*12, compared to C18:2*c*9,*t*11. In a trial on Mule × Charolais ewe lambs fed a concentrate diet supplemented with a mixture of *rp*CLA at high dosage, Wynn et al. (2006) observed a notable increase of the tissues content of both isomers and an effect also on the proportions of other FA in different tissues. On Ripollesa young lambs Terrè et al. (2011) tested different doses of not rumen protected CLA, smaller than that tested by Wynn et al. (2006) but greater than that used in the present study. They observed a dose dependent increase of the proportion of CLA*c*9,*t*11 and of CLA*t*10,*c*12 in the *Longissimus thoracis* muscle that ranged +15 to +50% and -7 to +254%, respectively. In agreement with current study, they did not observed significant effects on other FA proportions. This would suggest that in growing lamb the effects of *rp*CLA supply has an effect on the tissue FA profile smaller than that which is commonly observed for the milk produced by the mammary gland (Pellattiero et al., 2014).

Gender

Gender is an important factor for FA composition, also because its relation with carcass fatness (Vacca et al., 2008). In this study no significant effect of gender was observed likely

because the fatness of males and females did not differed as they were slaughtered at the onset of puberty (Pellattiero et al., 2011).

Tissues and interaction tissue × feeding system

The tissues analyzed in current experiment had very different FA profile, in general agreement with literature (Enser et al., 1998; Mir et al., 2000; Demirel et al., 2004). The liver in ruminants has less effect on *de novo* synthesis and disposal of lipids than adipose tissue or muscles (Herdmann et al., 2010). Nevertheless, there is a low rate of *de novo* biosynthesis where acetate is the main carbon source, and the pool of long chain FA in the liver is mainly derived from plasma NEFA (Hocquette and Bouchart, 1999). In the current experiment the mean FA profile of liver was particularly rich in PUFA (21%) when in other tissues they represented not more than 6.3%, in agreement with literature (Demirel et al., 2004). This notable proportion of PUFA in the liver was mainly due to a greater incidence of C18:2, C18:3 and of FA with chains longer than 18 carbon atoms, including some FA present in trace or not detectable in other tissues. The most important, in quantitative terms, were C20:4n6 (4.2%), C20:5n3 (1.2%), and C22:6n3 (1.4%). Greater incidences in the liver compared with other tissues were also detected for very long chain SFA, C20:0 (1.3%), C22:0 (0.9%) and C23:0 (0.1%). In addition, the liver showed the higher content of both ω3 (6.5%) and ω6 (10.8%) FA and the most favorable ω3/ω6 FA ratio (1.99) compared to all other tissues. This confirm that lamb's liver is potentially a good source of beneficial FA for human diet (Enser et al., 1998), although it represents a minor part of the edible carcass (Demirel et al., 2004).

The adipose tissue is one of the major site of *de novo* FA synthesis, with palmitic acid (C16:0) being the main product, which can be further elongated to stearic acid (C18:0), and desaturated to oleic acid by SCD (Sinclair, 2007). Ward et al. (1998) demonstrated that in sheep SCD is produced from a single gene whose expression is under hormonal control, independent of total fat synthesis, and whose activity varies with fat depot, being higher in subcutaneous fat that in internal depots. In the current experiment cover fat and kidney fat differed substantially, being the first richer in C16:0 (22.6 *vs* 20.4%) and C18:1c9 (28 *vs* 19%, respectively) but poorer in C18:0 (26 *vs* 34%).

In the current experiment differences among muscles in the FA profile were less pronounced than those observed between fat depots, and in general the 3 muscles showed greater proportions of C18:1*c*9 (32%), C18:2n6 (3.1%), similar proportion of C16:0 (23.1%) and smaller proportion of C18:0 (21.5%) compared to subcutaneous fat. Differences among

muscles are related to differences in the presence of fibre with different oxidative, glycolytic or oxido-glycolitic properties nature of the muscles (Garcia et al., 2008). These fibres are present in various proportion in muscles depending on the animal species, the location of the muscle in the body and other nutritional and physiological characteristics such as physical movement. Red oxidative fibers have a higher proportion of phospholipids than the white and, in consequence, a higher percentage of PUFA (Hocquette and Buchart, 1999).

In the present study, interaction between feeding system and tissues was significant for many FA. Liver presents a much greater proportion of both ω3 and ω6 PUFA than any other tissue sampled, and the effect of pasture, compared to both indoor diets, is greater in liver than in any other tissues (Figure 1). About CLA isomers, only the CLA*t*10,*c*12 isomer showed a significant FS × T interaction, mainly caused by the different trends observed for the two fatty tissues (Figure 2). In fact, in subcutaneous cover fat the proportion of this isomer was not affected by the feeding system, but in kidney fat there was a large difference between the two indoor diet as a result of the *rp*CLA supplementation, and the responses in lean tissues were intermediate. Also on beef cattle the response to *rp*CLA was much more pronounced for CLA*t*10,*c*12 isomer than for CLA*c*9,*t*11, and for muscles compared to subcutaneous fat (Schiavon et al. 2011). Another example of interaction regards the proportions of branched and odd-chain FA. The major differences regard the comparison between the lambs reared at pasture or indoor (Figure 3). This difference is much greater for liver and kidney fat compared to the tissues sampled from the carcass (subcutaneous cover fat and muscles).

3.5 CONCLUSIONS

The results of this experiment confirms that the tissues FA profile of lambs kept on hay and concentrate was altered compared to that based on pasture, with a decrease of the proportions of PUFA (-12%) and ω3 FA (-45%), and an increase of ω6 FA (+17%), branched (+36%) and odd chain FA (+20%). Nevertheless in the lambs fed hay and concentrate the tissues ω6/ω3 FA ratio averaged 4.1, an in the 3 muscles this ratio ranged from 4.5 to 6.0. In the perspective of extending the period of availability of lamb in the marked using indoor diets, these values can be still considered acceptable, if compared to the values suggested for human nutrition (< 5.0) and to those greater than 10.0 commonly observed for others typical western meat products. The *rp*CLA supply in diets based on hay and concentrate permits to increase the tissues proportions of CLA, especially the C18:2*t*10,*c*12, towards values similar or greater than those achievable on pasture. The results of this experiment also evidence the

effectiveness of using rumen protected molecules to deliver specific bioactive molecules in the lamb tissues. The use of *rp*CLA does not appear to exert notable influence on the tissues proportions of the various categories of FA, except the CLA component itself. This experiment also evidences that the effects on FA profile induced by the feeding systems with or without *rp*CLA had similar trends in all the tissues analyzed.

3.6 ACKNOWLEDGEMENTS

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Table 3.1: Descriptive statistics of lambs used for the study.

	Feeding system[1]		
	Pasture	Indoor-Control	Indoor-CLA
Rearing and feeding conditions	Pasture	Barn (pens)	Barn (pens)
Lambs, n.	12	12	12
Males, n.	6	6	6
Females, n.	6	6	6
Feedstuffs offered:			
grass	*ad libitum*	-	-
hay	-	*ad libitum*	*ad libitum*
concentrate, g/d	-	267	267
*rp*CLA supplement[2] g/d	-	-	9
Slaughter:			
age, d	221 ± 26	220 ± 24	216 ± 28
body weight, kg	33.9 ± 3.6	27.5 ± 6.5	28.6 ± 9.2
body condition score[3]	3.4 ± 0.3	3.2 ± 0.3	3.1 ± 0.3
killing out, %	45.4 ± 2.4	41.1 ± 1.9	40.3 ± 2.5

[1] B-CLA: Barn feeding based on hay and concentrates without supplementation of *rp*CLA; B+CLA: Barn feeding with supplementation of *rp*CLA;
[2] rpCLA: Rumen protected CLA (SILA, Noale, Venice, Italy).
[3] Body condition score on a 1 to 5 points scale, where 1 = Emaciated and 5 = Obese

Table 3.2: Effects of feeding system (P: pasture; B-CLA: barn feeding based on hay and concentrates; B+CLA: barn feeding with supplementation of *rp*CLA), gender and of their interaction (FS×G) on the proportion of saturated FA (g/g total FA × 100) of six different tissues of lambs autochthonous of Veneto region.

	Feeding system (FS)			FS contrasts (*P*)		Gender (G)			FS×G	RMSE
	Pasture	Indoor		Pasture *vs* Indoor	CLA *vs* control	Ram	Ewes	*P*	*P*	
		Control	CLA							
C8:0	0.03	0.03	0.03	0.59	0.59	0.03	0.03	0.69	0.86	0.01
C10:0	0.17	0.20	0.19	0.06	0.28	0.18	0.19	0.58	0.97	0.04
C12:0	0.16	0.19	0.19	0.18	0.88	0.18	0.17	0.56	0.57	0.06
C13:0	0.33	0.30	0.33	0.61	0.31	0.32	0.31	0.80	0.65	0.18
C14:0$_{iso}$	0.10	0.13	0.14	<0.001	0.46	0.13	0.11	0.07	0.34	0.03
C14:0	2.70	3.04	2.95	0.17	0.74	2.93	2.87	0.79	0.73	0.58
C15:0$_{iso}$	0.30	0.37	0.37	<0.001	0.71	0.35	0.34	0.18	0.99	0.06
C15:0$_{anteiso}$	0.33	0.43	0.44	<0.001	0.56	0.43	0.38	0.03	0.36	0.08
C15:0	0.83	0.91	0.93	0.006	0.64	0.93	0.85	0.01	0.01	0.13
C16:0$_{iso}$	0.25	0.37	0.37	<0.001	0.91	0.34	0.33	0.43	0.72	0.06
C16:0	20.7	22.1	21.6	0.02	0.40	21.3	21.6	0.49	0.72	1.55
C17:0$_{iso}$	0.52	0.66	0.64	<0.001	0.40	0.62	0.59	0.12	0.97	0.08
C17:0$_{anteiso}$	0.68	0.95	0.93	<0.001	0.65	0.87	0.84	0.48	0.83	0.12
C17:0	1.71	1.96	1.94	0.005	0.85	1.88	1.86	0.74	0.70	0.25
C18:0$_{iso}$	0.18	0.26	0.25	<0.001	0.41	0.23	0.23	0.53	0.40	0.04
C18:0	26.3	24.1	24.9	<0.001	0.40	25.2	25.0	0.75	0.66	3.03
C19:0$_{iso}$	0.03	0.03	0.03	0.30	0.55	0.03	0.03	0.78	0.83	0.02
C19:0	0.22	0.29	0.27	<0.001	0.51	0.27	0.25	0.33	0.48	0.07
C20:0	0.34	0.41	0.41	0.02	0.86	0.41	0.36	0.07	0.21	0.15
C22:0	0.21	0.17	0.18	0.46	0.81	0.21	0.16	0.18	0.46	0.18
C23:0	0.02	0.02	0.02	0.37	0.91	0.02	0.02	0.98	0.68	0.02

[1] P: Pasture; B:Barn; CLA -: B-CLA; CLA +: B+CLA.

Table 3.3: Effects of feeding system (Pasture: grass grazing; Indoor-Control: barn feeding based on hay and concentrates; Indoor-CLA: barn feeding with supplementation of *rp*CLA), gender and of their interaction (FS×G) on the proportion of monounsaturated and polyunsaturated fatty acids and unidentified isomers sums (g/g total FA × 100) of six different tissues of lambs autochthonous of Veneto region.

	Feeding system (FS)			FS contrasts (*P*)		Gender (G)			FS×G	RMSE
	Pasture	Indoor		Pasture *vs* Indoor	CLA *vs* control	Ram	Ewes	*P*	*P*	
		Control	CLA							
C14:1*c*9	0.07	0.08	0.08	0.66	0.98	0.08	0.07	0.80	0.95	0.04
ΣC15:1 others	0.06	0.07	0.08	0.01	0.45	0.07	0.07	0.85	0.91	0.04
C16:1n7	0.04	0.02	0.03	0.03	0.60	0.03	0.03	0.91	0.68	0.03
C16:1t7	0.16	0.09	0.10	<0.001	0.44	0.11	0.12	0.49	0.27	0.06
C16:1*c*7	0.53	0.53	0.52	0.95	0.73	0.55	0.50	0.01	0.93	0.12
C16:1*c*9	1.05	1.18	1.12	0.11	0.38	1.12	1.11	0.75	0.76	0.26
ΣC16:1 others	0.21	0.24	0.25	0.40	0.84	0.25	0.22	0.56	0.76	0.31
C17:1*c*10	0.53	0.61	0.62	0.02	0.87	0.58	0.59	0.64	0.26	0.13
ΣC17:1 others	0.31	0.38	0.34	0.33	0.48	0.37	0.32	0.23	0.14	0.21
C18:1*t*9(+ *t*11)*	4.23	3.48	3.72	<0.001	0.27	3.81	3.81	0.97	0.12	0.61
C18:1*c*9	26.2	27.3	26.2	0.46	0.17	26.2	26.9	0.25	0.50	2.48
ΣC18:1 others	1.19	0.82	0.83	0.004	0.93	0.95	0.95	0.99	0.88	0.59
C19:1*n*9t	0.01	0.03	0.03	0.01	0.28	0.02	0.03	0.83	0.75	0.02
C19:1n9c	0.11	0.12	0.11	0.84	0.48	0.12	0.11	0.48	0.37	0.07
C20:1*n*9t	0.02	0.02	0.02	0.81	0.49	0.02	0.02	0.63	0.57	0.02
C20:1*n*9	0.03	0.06	0.05	0.002	0.65	0.05	0.05	0.86	0.90	0.03
C20:1*n*7	0.03	0.05	0.05	0.01	0.24	0.04	0.04	0.96	0.46	0.04
ΣC16:2 others	0.06	0.03	0.03	<0.001	0.25	0.04	0.04	0.77	0.52	0.02
C18:2*n*6	2.94	3.22	3.49	0.007	0.12	3.15	3.29	0.32	0.94	0.56
ΣC18:2 others	1.27	0.92	0.91	<0.001	0.92	1.05	1.02	0.70	0.80	0.37
CLA*c*9*t*11	0.56	0.43	0.52	0.003	0.010	0.51	0.49	0.59	0.61	0.13
CLA*t*10*c*12	0.07	0.05	0.10	0.64	0.002	0.09	0.07	0.11	0.21	0.06
C18:3*n*6	0.06	0.12	0.11	<0.001	0.39	0.10	0.10	0.82	0.30	0.05
C18:3*n*3	1.44	0.72	0.71	<0.001	0.95	0.98	0.93	0.50	0.87	0.30
C18:4*n*3	0.06	0.06	0.05	0.90	0.58	0.07	0.05	0.21	0.90	0.06
C20:2*n*6	0.03	0.03	0.04	0.81	0.89	0.03	0.04	0.23	0.65	0.01
C20:3*n*6	0.09	0.12	0.13	0.002	0.80	0.11	0.11	0.88	0.08	0.05
C20:4*n*6	1.12	1.58	1.71	0.005	0.52	1.52	1.42	0.58	0.40	0.05
C20:5*n*3 (EPA)	0.59	0.30	0.37	0.01	0.63	0.51	0.33	0.10	0.42	0.06

[1] P: Pasture; B:Barn; CLA -: B-CLA; CLA +: B+CLA;
*Co-eluted isomers.

Table 3.4: Effects of feeding system (P: pasture; B-CLA: barn feeding based on hay and concentrates; B+CLA: barn feeding with supplementation of *rp*CLA), gender and of their interaction (FS×G) on the proportion of long chain monounsaturated and polyunsaturated FA (g/g total FA × 100) present only in liver and kidney fat of lambs autochthonous of Veneto region.

		Feeding system (FS)			FS contrasts (*P*)		Gender (G)			FS×G	RMSE
		Pasture	Indoor		Pasture *vs* Indoor	CLA *vs* control	Ram	Ewes	*P*	*P*	
			Control	CLA							
C18:1*c*7	L,K	0.52	0.78	0.78	<0.001	0.83	0.69	0.69	0.99	0.88	0.11
C20:1n11	L,K	0.03	0.05	0.05	0.01	0.24	0.05	0.05	0.87	0.45	0.03
C20:2*n*3	L,K	0.15	0.40	0.35	<0.001	0.32	0.31	0.29	0.70	0.57	0.15
C20:3*n*3	L	0.07	0.09	0.08	0.23	0.82	0.09	0.07	0.14	0.49	0.04
C20:4*n*3	L	0.07	0.03	0.03	<0.001	0.31	0.05	0.04	0.26	0.22	0.02
C22:4*n*6	L	0.14	0.51	0.55	<0.001	0.57	0.43	0.38	0.42	0.64	0.17
C22:6*n*3 (DHA)	L	2.03	1.01	1.23	0.004	0.47	1.55	1.31	0.41	0.67	0.63
C22:5*n*6	L	0.09	0.32	0.34	<0.001	0.75	0.28	0.23	0.26	0.66	0.12

[1] L: liver, K = kidney fat;
[2] P: Pasture; B: Barn; CLA -: B-CLA; CLA +: B+CLA.

Table 3.5: Effects of feeding system (P: pasture; B-CLA: barn feeding based on hay and concentrates; B+CLA: barn feeding with supplementation of *rp*CLA), gender and of their interaction (FS×G) on the proportion of groups of fatty acids (g/g total FA × 100) and on their indices in six different tissues of lambs autochthonous of Veneto region.

	Feeding system (FS)			FS contrasts (*P*)		Gender (G)			FS×G	RMSE
	Pasture	Indoor		Pasture *vs* Indoor	CLA *vs* control	Ram	Ewes	*P*	*P*	
		Control	CLA							
SFA	56.1	57.0	57.3	0.19	0.75	57.0	56.6	0.58	0.85	2.82
MUFA	34.8	35.1	34.2	0.83	0.25	34.4	35.0	0.31	0.56	2.51
PUFA	8.91	7.78	8.33	0.03	0.22	8.44	8.23	0.57	0.79	1.98
FA unknow	0.17	0.08	0.08	<0.001	0.96	0.12	0.11	0.16	0.16	0.06
ΣCLA	0.80	0.56	0.71	0.002	0.007	0.72	0.66	0.24	0.62	0.20
ω 3	2.70	1.46	1.54	<0.001	0.70	2.00	1.80	0.24	0.63	1.10
ω 6	3.76	4.41	4.75	<0.001	0.17	4.26	4.36	0.64	0.82	0.95
ω 6/ω3	2.11	4.05	4.19	<0.001	0.63	3.33	3.56	0.36	0.85	1.13
<C16	5.07	5.77	5.84	0.008	0.82	5.72	5.40	0.22	0.90	0.88
C16	22.9	24.5	24.0	0.01	0.41	23.7	23.9	0.65	0.83	1.67
>C16	71.9	69.7	70.1	0.009	0.62	70.5	70.6	0.92	0.94	2.17
Branched	2.37	3.26	3.21	<0.001	0.69	3.04	2.85	0.14	0.95	0.43
Odd chain	5.88	7.11	7.04	<0.001	0.80	6.84	6.51	0.16	0.93	0.89
Desaturases:[2]										
C14	0.04	0.02	0.03	0.18	0.75	0.03	0.03	0.41	0.18	0.07
C16	0.05	0.05	0.05	0.21	0.52	0.05	0.05	0.32	0.54	0.01
C18	0.49	0.53	0.51	0.07	0.32	0.50	0.52	0.39	0.51	0.05
CLA*c*9,*t*11	0.12	0.11	0.13	0.60	0.04	0.12	0.12	0.51	0.67	0.03
Total Index	0.39	0.39	0.38	0.73	0.38	0.39	0.39	0.43	0.47	0.03
IT[3]	1.81	1.83	1.86	0.57	0.67	1.85	1.82	0.63	0.97	0.22
IAT[4]	0.77	0.85	0.84	0.03	0.92	0.83	0.81	0.69	0.78	0.09

[1] P: Pasture; B:Barn; CLA -: B-CLA; CLA +: B+CLA;
[2] Δ^9-desaturase indices: calculated according to Kelsey et al. (2003);
[3] Thrombogenic Index: calculated according to Ulbricht and Southgate's (1991);
[4] Atherogenic Index: calculated according to Ulbricht and Southgate's (1991).

Table 3.6: Proportion of saturated fatty acids of lamb (g/g total FA × 100) in six different tissues of lambs autochthonous of Veneto region.

	Tissue[1] (T)						T contrasts (*P* values)					FS×T	RMSE
	Liver	Fat depots		Muscles			Liver *vs* (fats+muscles)	Fats *vs* Muscles	KF *vs* CF	Leg *vs* Chop	RE *vs* OM	*P*	
		KF	CF	Leg	Chop								
					RE	OM							
C8:0	0.03	0.03	0.03	0.03	0.03	0.03	0.36	0.14	0.27	0.32	0.13	0.10	0.01
C10:0	-	0.18	0.19	0.21	0.19	0.18	-	-	0.71	0.09	0.07	0.34	0.04
C12:0	0.04	0.15	0.17	0.31	0.20	0.21	<0.001	<0.001	0.25	0.20	0.80	0.53	0.06
C13:0	0.91	0.60	0.07	0.06	0.04	0.23	<0.001	0.30	<0.001	0.01	<0.001	0.17	0.18
C14:0$_{iso}$	0.08	0.15	0.15	0.12	0.10	0.12	<0.001	<0.001	0.77	<0.001	<0.001	0.09	0.03
C14:0	0.89	2.76	3.13	3.98	3.27	3.36	<0.001	0.42	0.01	0.88	0.49	0.83	0.58
C15:0$_{iso}$	0.31	0.43	0.44	0.27	0.28	0.35	<0.001	<0.001	0.42	<0.001	<0.001	<0.001	0.06
C15:0$_{anteiso}$	0.38	0.47	0.47	0.36	0.33	0.4	0.08	<0.001	0.95	<0.001	<0.001	<0.001	0.08
C15:0	0.78	0.91	1.02	0.89	0.77	0.97	<0.001	<0.001	0.001	<0.001	<0.001	0.04	0.13
C16:0$_{iso}$	0.28	0.38	0.40	0.32	0.30	0.33	<0.001	<0.001	0.23	<0.001	0.01	<0.001	0.06
C16:0	16.4	20.4	22.6	23.7	23.2	22.4	<0.001	0.72	<0.001	0.05	0.05	0.32	1.55
C17:0$_{iso}$	0.64	0.61	0.66	0.57	0.58	0.59	0.02	0.001	0.003	0.01	0.70	0.002	0.08
C17:0$_{anteiso}$	0.79	0.91	1.00	0.77	0.80	0.86	0.001	<0.001	0.001	<0.001	0.05	<0.001	0.12
C17:0	1.77	2.06	2.20	1.62	1.66	1.91	0.01	<0.001	0.03	<0.001	<0.001	<0.001	0.25
C18:0$_{iso}$	0.23	0.19	0.27	0.22	0.25	0.23	0.45	0.81	<0.001	0.51	0.25	<0.001	0.04
C18:0	26.5	33.9	25.7	19.6	20.4	24.5	0.003	<0.001	<0.001	<0.001	<0.001	0.002	3.03
C19:0$_{iso}$	0.04	0.06	0.01	0.02	0.01	0.03	0.001	0.03	<0.001	0.24	0.01	<0.001	0.02
C19:0	0.53	0.36	0.17	0.13	0.12	0.25	<0.001	0.33	<0.001	<0.001	<0.001	<0.001	0.07
C20:0	1.27	0.35	0.16	0.15	0.13	0.24	<0.001	<0.001	<0.001	0.02	0.003	0.03	0.15
C22:0	0.94	0.08	0.03	0.01	0.02	0.03	<0.001	<0.001	0.30	0.94	0.94	0.56	0.18
C23:0	0.06	0.02	0.01	0.01	0.03	0.01	<0.001	0.02	0.01	0.02	0.05	0.45	0.02

[1] KF = kidney fat, CF = subcutaneous cover fat, Leg = leg inner muscle (*Quadriceps femoris*); RE = rib eye muscle, OM = other chop muscles

Table 3.7: Content of monounsaturated, polyunsaturated fatty acids and unidentified isomers sum (gFA×100 g total FA) in six different tissues of lambs autochthonous of Veneto region.

	Tissue[1] (T)						T contrasts (*P* values)					FS×T	RMSE
	Liver	Fat depots		Muscles			Liver *vs* (fats+muscles)	Fats *vs* Muscles	KF *vs* CF	Leg *vs* Chop	RE *vs* OM	*P*	
		KF	CF	Leg	Chop								
					RE	OM							
C14:1*c*9	0.02	0.04	0.07	0.12	0.10	0.09	<0.001	<0.001	0.02	0.007	0.11	0.80	0.04
ΣC15:1 others	0.06	0.10	0.07	0.05	0.05	0.08	0.26	<0.001	0.004	0.002	<0.001	0.69	0.04
C16:1n7	0.03	0.03	0.03	0.02	0.04	0.03	0.64	0.98	0.64	0.20	0.28	0.25	0.03
C16:1t7	0.23	0.08	0.09	0.12	0.10	0.07	<0.001	<0.001	0.72	0.27	0.20	0.12	0.06
C16:1*c*7	0.59	0.56	0.53	0.47	0.51	0.48	<0.001	0.08	0.37	0.97	0.44	0.06	0.12
C16:1*c*9	0.82	0.65	1.18	1.46	1.37	1.22	<0.001	<0.001	<0.001	0.003	0.02	0.03	0.26
ΣC16:1 others	0.48	0.30	0.10	0.17	0.16	0.19	<0.001	0.23	0.009	0.78	0.73	0.78	0.31
C17:1*c*10	0.11	0.39	0.77	0.74	0.75	0.75	<0.001	0.79	<0.001	0.79	0.99	0.66	0.21
ΣC17:1 others	0.73	0.41	0.17	0.27	0.15	0.32	<0.001	0.10	0.004	0.15	0.02	<0.001	0.13
C18:1*t*9(+ *t*11)*	3.32	4.99	4.19	3.38	3.49	3.50	<0.001	<0.001	<0.001	0.007	0.99	0.003	0.61
C18:1*c*9	15.8	19.2	28.4	32.9	33.7	29.6	<0.001	<0.001	<0.001	<0.001	<0.001	0.007	2.48
ΣC18:1 others	1.51	1.59	0.63	0.68	0.51	0.77	<0.001	0.005	<0.001	0.12	0.07	<0.001	0.59
C19:1*n*9t	0.03	0.02	-	-	-	0.02	-	-	-	-	-	0.04	0.02
C19:1n9c	0.07	0.02	0.14	0.15	0.14	0.18	<0.001	<0.001	<0.001	0.29	0.04	0.11	0.07
C20:1*n*9t	0.06	0.02	0.01	0.01	0.02	0.01	<0.001	0.33	0.34	0.52	0.43	0.003	0.02
C20:1*n*9	0.11	0.05	0.02	0.03	0.03	0.04	<0.001	0.002	<0.001	0.37	0.07	<0.001	0.03
C20:1*n*7	0.03	0.06	0.02	0.05	0.04	0.06	0.04	0.28	<0.001	0.99	0.02	0.28	0.04
ΣC16:2 others	0.05	0.07	0.03	0.03	0.04	0.03	0.002	<0.001	<0.001	0.56	0.55	<0.001	0.02
C18:2*n*6	5.39	1.93	2.54	3.33	3.19	2.92	<0.001	<0.001	<0.001	<0.001	0.05	0.26	0.56
ΣC18:2 others	1.45	1.17	0.98	0.88	1.02	0.72	<0.001	0.25	0.02	0.03	<0.001	0.06	0.37
CLA*c*9*t*11	0.52	0.51	0.42	0.53	0.47	0.56	0.30	0.004	0.003	0.34	0.002	0.25	0.13
CLA*t*10*c*12	0.04	0.07	0.10	0.06	0.08	0.11	<0.001	0.13	0.11	0.14	0.16	0.02	0.06
C18:3*n*6	0.32	0.08	0.04	0.04	0.05	0.05	<0.001	<0.001	0.004	0.96	0.56	<0.001	0.05
C18:3*n*3	1.34	0.87	0.90	0.83	0.89	0.90	<0.001	0.01	0.74	0.89	0.86	<0.001	0.30
C18:4*n*3	0.16	0.09	0.02	0.02	0.02	0.04	<0.001	0.72	0.02	0.73	0.52	0.52	0.06
C20:2*n*6	0.09	0.01	-	0.02	-	0.02	<0.001	<0.001	-	-	-	0.001	0.01
C20:3*n*6	0.38	0.02	-	0.03	-	0.02	-	-	-	-	-	<0.001	0.05
C20:4*n*6	4.21	0.06	-	-	-	0.14	-	-	-	-	-	<0.001	0.05
C20:5*n*3(EPA)	1.17	0.07	-	-	-	0.01	-	-	-	-	-	0.003	0.06

[1] KF = kidney fat, CF = subcutaneous cover fat, Leg = leg inner muscle (*Quadriceps femoris*); RE = rib eye muscle, OM = other chop muscles

Table 3.8: Content of long chain monounsaturated and polyunsaturated fatty acids present only on liver and kidney fat (gFA×100 g total FA) of lambs autochthonous of Veneto region.

		Tissue (T)		Tissue	FS×T	RMSE
	Tissue[1]	Liver	KF	*P*	*P*	
C18:1*c*7	L, KF	0.83	0.56	<0.001	<0.001	0.11
C20:1n11	L, KF	0.08	0.02	<0.001	0.01	0.03
C20:2*n*3	L, KF	0.57	0.03	<0.001	<0.001	0.15
C20:3*n*3	L	0.08	-	-	-	0.04
C20:4*n*3	L	0.04	-	-	-	0.02
C22:4*n*6	L	0.41	-	-	-	0.17
C22:6*n*3(DHA)	L	1.43	-	-	-	0.63
C22:5*n*6	L	0.26	-	-	-	0.12

[1] L = liver, KF = kidney fat;

Table 3.9: Content of groups of fatty acids of lamb (gFA×100 g total FA), and on their indices in six different tissues of lambs autochthonous of Veneto region.

	Tissue[1] (T)						T contrasts (*P* values)					FS×T	RMSE
	Liver	Fat depots		Muscles			Liver *vs* (fats+muscles)	Fats *vs* Muscles	KF *vs* CF	Leg *vs* Chop	RE *vs* OM	*P*	
		KF	CF	Leg	Chop								
					RE	OM							
SFA	53.2	65.6	58.8	53.3	52.7	57.3	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	2.82
MUFA	25.1	28.9	36.1	40.3	41.0	36.9	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	2.51
PUFA	21.2	5.44	5.15	6.31	6.18	5.79	<0.001	<0.001	0.54	0.09	0.42	0.98	1.98
FA unknow	0.56	0.05	0.02	0.03	0.01	0.01	<0.001	<0.001	0.009	0.92	0.99	<0.001	0.06
ΣCLA	0.75	0.74	0.58	0.67	0.59	0.80	0.06	0.19	0.001	0.02	<0.001	0.07	0.20
ω 3	6.50	1.13	0.90	0.94	0.96	0.98	<0.001	<0.001	0.39	0.95	0.93	<0.001	1.10
ω 6	10.8	2.09	2.58	3.71	3.52	3.14	<0.001	<0.001	0.03	0.001	0.10	<0.001	0.95
ω 6/ω3	1.99	2.16	3.61	4.65	4.43	3.85	<0.001	<0.001	<0.001	0.004	0.04	0.28	1.13
<C16	3.57	6.23	5.73	6.35	5.40	6.08	<0.001	<0.001	0.02	0.008	0.002	0.32	0.88
C16	18.8	22.4	24.9	26.3	25.6	24.7	<0.001	0.48	<0.001	0.02	0.02	0.70	1.67
>C16	77.1	71.2	69.3	67.4	69	69.2	<0.001	0.26	<0.001	0.53	0.68	0.92	2.17
Branched	2.91	3.26	3.34	2.64	2.64	2.88	0.65	<0.001	0.46	<0.001	0.03	<0.001	0.43
Odd chain	7.76	7.68	6.87	5.59	5.57	6.59	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.89
Desaturases:[2]													
C14	0.03	0.02	0.02	0.03	0.03	0.05	0.80	0.09	0.88	0.63	0.16	0.44	0.07
C16	0.05	0.03	0.05	0.06	0.05	0.05	0.48	<0.001	<0.001	0.12	0.28	0.01	0.01
C18	0.37	0.36	0.53	0.63	0.62	0.55	<0.001	<0.001	<0.001	<0.001	<0.001	0.004	0.05
CLA*c*9,*t*11	0.14	0.09	0.09	0.14	0.12	0.14	<0.001	<0.001	0.73	0.63	0.005	0.37	0.03
Total Index	0.30	0.31	0.40	0.45	0.45	0.41	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.03
IT[3]	1.15	2.73	2.02	1.61	1.59	1.90	<0.001	<0.001	<0.001	<0.001	<0.001	0.20	0.22
IAT[4]	0.48	0.99	0.90	0.88	0.80	0.88	<0.001	<0.001	<0.001	<0.001	0.001	0.15	0.09

[1] KF = kidney fat, CF = subcutaneous cover fat, Leg = leg inner muscle (*Quadriceps femoris*); RE = rib eye muscle, OM = other chop muscles
[2] Δ^9-desaturase indices: calculated according to Kelsey et al. (2003) and Capoprese et al. (2010);
[3] Thrombogenic Index: calculated according to Ulbricht and Southgate's (1991);
[4] Atherogenic Index: calculated according to Ulbricht and Southgate's (1991).

Figure 1. Effect of feeding system (Indoor-Control refers to indoor feeding based on hay and concentrate, Indoor-CLA refers to the same diet supplemented with CLA) on content of ω3 and ω6 fatty acids and on their ratio in six lamb's tissues.

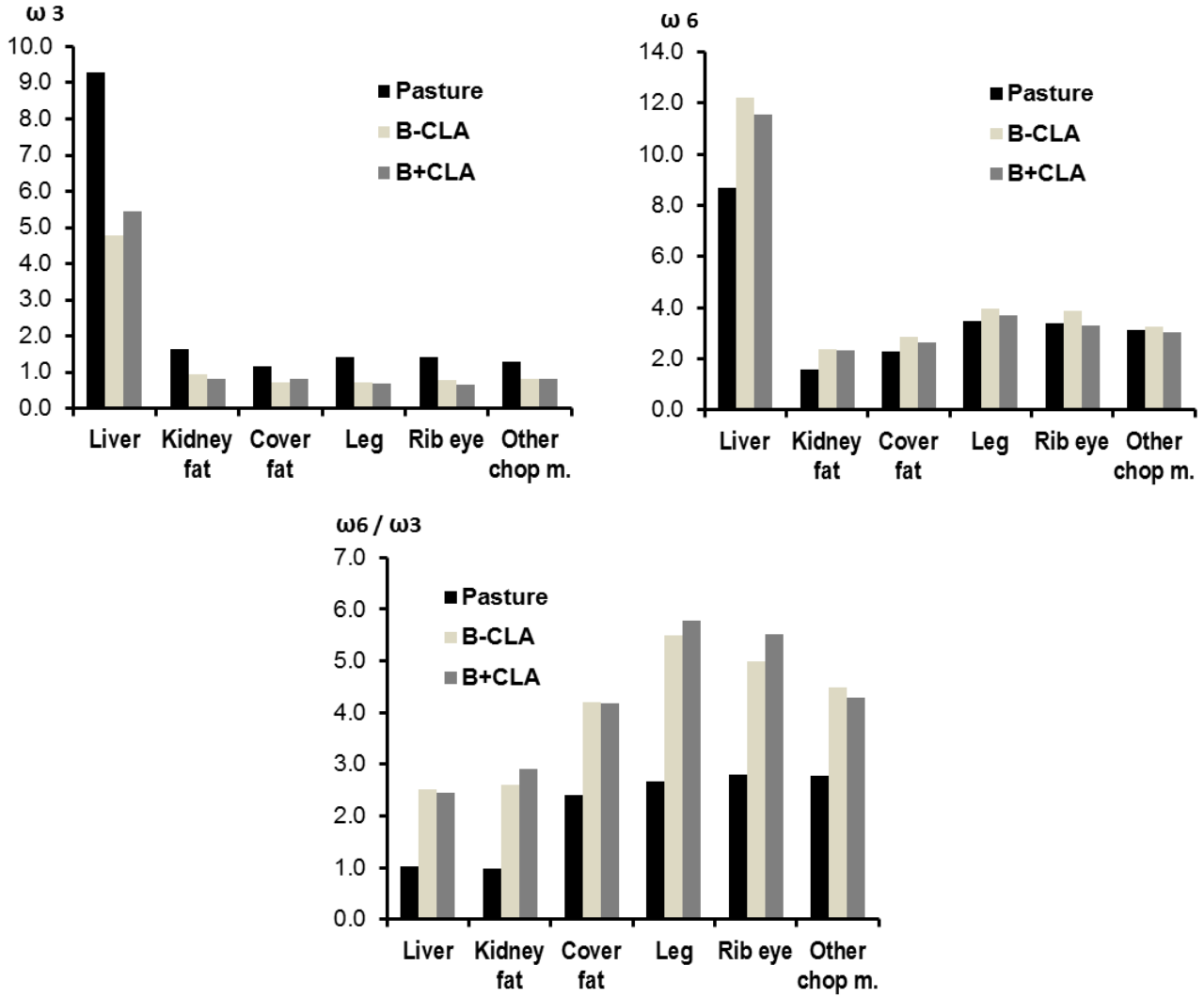


Figure 2. Effect of feeding system (Indoor-Control refers to indoor feeding based on hay and concentrate, Indoor-CLA refers to the same diet supplemented with rumen protected CLA) on content of CLA*t*10,*c*12 in six lamb's tissues.

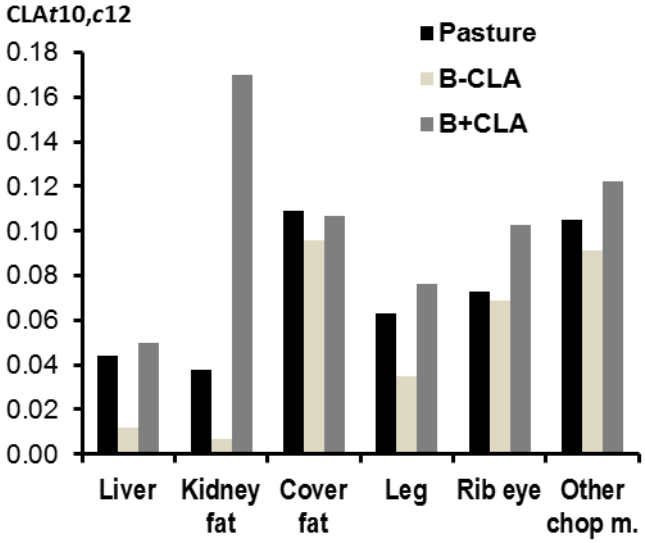
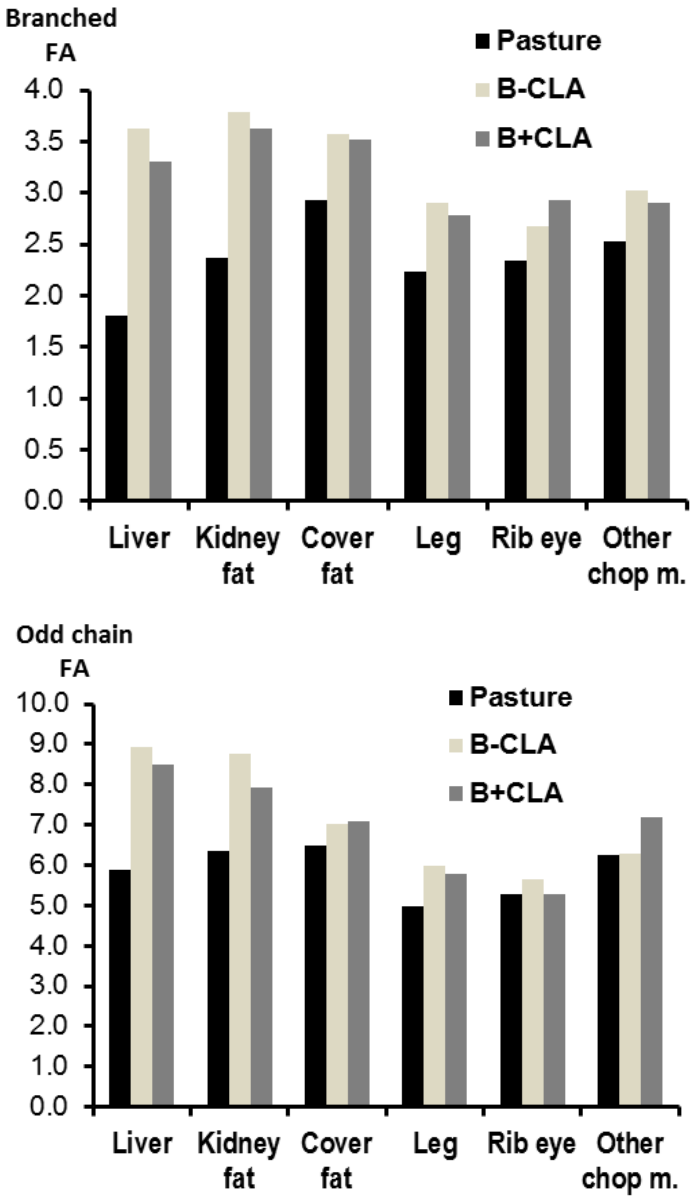


Figure 3. Effect of feeding system (Indoor-Control refers to indoor feeding based on hay and concentrate, Indoor-CLA refers to the same diet supplemented with CLA) on content of branched chain and odd chain fatty acids in six lamb's tissues.



CHAPTER 4

Effect of rumen protected conjugated linoleic acid (CLA) supplementation of breed, individual animal and lactation stage on a detailed fatty acid profile of sheep milk analyzed by two-dimensional gas chromatography

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4.1 ABSTRACT

The aim of this study was evaluate the effects of rumen protected conjugated linoleic acid (rpCLA) supplementation, breed, initial days in milk, sampling period, and number of lambs suckling, on fatty acid (FA) profile and CLA content of milk from ewes of 3 autochthonous breeds of the Veneto Alps (Brogna, Foza and Lamon). Twenty-four ewes with their 31 suckling lambs were used. They were divided in 6 pens (2 pens/ breed), according to DIM (38 ± 23 d) and live weight (61 ± 13 kg) of ewes. The ewes and their offspring of 3 pens (one pen/breed) were fed a total mixed ration offered *ad libitum* (Control), and the animals of the others pens were fed the control diet top dressed with 12 g/d per ewe, plus 4 g/d for each lamb older than 30 d, of a rpCLA mixture. The study lasted 63 d. Every 10 d, in the morning, the ewes were separated from their offspring for 2 h, and milk samples were individually collected and analyzed for fat and protein content. An aliquot of each milk sample was conserved at -80 °C. Individual milk samples were thawed and pooled in two composite samples for each ewe: the first regarded the 3 milk samples collected during the first month of trial (period A) and the second the 3 samples collected during the second month of trial (period B). These composite samples were analyzed for their FA profile by two-dimensional gas chromatography. Data of milk were analyzed by PROC MIXED of SAS considering the effect of the diet, breed, number of lambs suckling, DIM and sampling period. The random effect of animal was used to test diet, breed, and DIM, whereas the effects of period was tested on the residual. From the results was confirm that many effects can influence fatty acid profile of milk, such as period of sampling and lactation. Diet and in particular CLA supplementation was considered one of the principal effect, because of it can increase the two main CLA isomers. However, in milk was rich in CLA isomers, not only the two main components of the supplementation (CLA*c*9,*t*11 and CLA*t*10,*c*12) but also the others (CLA*c*11,*t*13, CLA*c*9,*c*11, CLA*c*10,*c*12 and CLA*t*9,*t*11).

Key words: conjugated linoleic acid (CLA); sheep milk; fatty acids; sheep breeds.

4.2 INTRODUCTION

Compared to bovine, ovine products are known for their greater content of some fatty acids (FA), considered to be beneficial for human health (Sinclair, 2007; Tsiplakou and Zervas, 2008; Barłowska, 2011), such as polyunsaturated fatty acids (PUFA), and among these linolenic acid, Ω3 PUFA and isomers of conjugated linoleic (CLA) (Dilzer and Park,

2012; Shingfield et al., 2013). Sheep milk is seldom consumed as fresh product, so that these effects would be exploited by the consumption of processed milk (Prandini et al., 2007), especially cheese (Nudda et al., 2005; Buccioni et al., 2010; Cattani et al., 2014), or meat of suckling lambs of sheep populations (Sinclair, 2007). However, the recovery from milk to ripened cheese have been recently found to be very high and greater than 80% for the majority of PUFA and CLA in the case of bovine milk (Cattani et al., 2014).

The FA profile of ovine milk partially depends by the genetic and physiological characteristics of the ovine species, but also because ewes are most commonly reared on pasture, according to traditional extensive techniques particularly in harsh environment (Pulina et al., 2006; Sanz Sanpelajo et al., 2007; De La Fuente et al., 2009). Fresh grass from pasture is rich in PUFA, particularly of linolenic acid, which is precursor of PUFA of the Ω3 series, vaccenic acid (C18:1*t*11), and CLA isomers which are found in the rumen and in animal tissues and milk (Bauman et al., 2008). Milk obtained from animals kept on grass-based diets is commonly richer in these FA compared to that obtained from animals kept on hay-, silage- or concentrate-based diets (Jutzeler van Wijlen and Colombani, 2010; Shingfield et al., 2013). The FA profile of milk and cheese also depends by the type of grass used (Addis et al., 2005). The intensification of the production systems, with increased use indoor of corn silage and concentrates, and the abandonment of seasonality in favor of continuous production system, are factors that would impair the quality of the lipid component of sheep milk. Nevertheless, the use of some rumen protected fat can be a tool to deliver beneficial FA in tissues and milk, and the use of rumen protected CLA (rpCLA) supplements, containing the two most promising CLA isomers, the C18:2*c*9,*t*11 and the C18:2*t*10, *c*12, has been found to be effective both for beef meat (Schiavon et al., 2011) and cow's milk (Pappritz et al., 2011; Schiavon et al., 2014). Less known is the possibility to influence the FA profile of sheep milk (Huswéth et al., 2010; Weerasinghe et al., 2012) and lamb tissues (Mir et al., 2000; Terré et al., 2011) through a supplementation of rpCLA on indoor diets based on corn silage and concentrate-based diets.

In recent years, improved analytical methods and equipment permit to obtain detailed FA profiles, including a number of CLA isomers even if present in very small amount. One of the most powerful technique is the GC×GC or two-dimensional gas-chromatography (Manzano et al., 2011; Pellattiero et al., 2014).

The aim of this study was evaluate, by GC×GC, the effects of rpCLA supplementation and of other factors (breed, days in milk, sampling period, number of lambs suckling, individual

animal) on a detailed FA profile of milk from ewes belonging to 3 breeds autochthonous of the Veneto Alps (Brogna, Foza and Lamon).

4.3 MATERIAL AND METHODS

Animals, feeding and milk sampling

This experiment was carried out at the "Lucio Toniolo" Experimental Farm of the University of Padova in Legnaro (Padova, Italy) on animals undergoing an *in situ* conservation program of the sheep breeds autochthonous of the Alpine areas of the Veneto region (Northeast Italy). Animals were treated according to the Guideline for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Consortium, 1988).

Twenty-four ewes with their 31 suckling lambs, of Brogna, Foza and Lamon breeds, were allotted in 6 pens (3 m × 6 m each) of an open barn (two pens/ breed), homogeneous for DIM (38 ± 23 d) and ewe's live weight (61 ± 13 kg). The animals of 3 pens (one pen/breed) were fed a control diet (Control), and those of the other pens received the same diet top dressed with 12 g/d per ewe, plus 4 g/d for each lamb older than 30 d, of a commercial rpCLA supplement (SILA, Noale, Venice) that contained, as detailed in Schiavon et al. (2010), 79.2 and 76.8 g/kg of C18:2*c*9,*t*11 and C18:2*t*10,*c*12 isomers, respectively.

The experimental condition and the composition of diets have been described in detail by Bittante et al. (2014). Briefly, the control diet was composed by 37.3, 26.0, 11.1, 11.0, 6.4, 6.6, 1.6 % of DM by corn grain, corn silage, dried sugar beet pulp, soybean meal, wheat bran, wheat straw and a vitamin mineral mixture, respectively. From chemical analysis performed on each feed ingredient and from ingredient composition of diet resulted that the diet contained 13.0, 29.3, 14.6, and 34.7% of DM of crude protein (CP), NDF, ADF, and starch, respectively, and that the metabolizable energy content was 11.4 MJ/kg DM (Bittante et al., 2014). Diets ingredients were mixed with addition of water to reach an average dietary DM content of 50.4% and fed as total mixed ration (TMR), offered *ad libitum*. The amount of each feed ingredient loaded into the mixer-wagon and the weight of the mixture uploaded in the manger of each pen were recorded daily. The orts remained in the mangers were weighed by pen weekly. The study lasted 63 d. At the beginning and at the end of the trial the ewes and their offspring were individually weighed and scored for body condition (BCS), and change in body weight (ADG) and BCS.

Every 10 d, in the morning, the ewes were separated from their offspring for two hours, and milk samples were collected from each ewe (6 times over the whole experiment),

refrigerated and analyzed for fat, protein and lactose content using a MilkoScan FT2 (Foss, Hillerød, Denmark) according to Bittante et al. (2014). For the purposes of current work an aliquot of each milk sample was conserved at -80°C. Prior of the analysis, milk samples were thawed and pooled in two composite samples for each ewe: the first pooled the 3 milk samples collected during the first month of the trial (period A) and the second pooled the 3 samples collected during the second month of the trial (period B).

Lipid extraction and Fatty Acid methylation

The lipid extraction procedure was performed according to Hara and Radin (1978) and Chouinard et al. (1999) using as a solvent hexane/isopropanol (3:2, v/v) solution at room temperature. Fresh milk samples were mixed, homogenized and 5 ml were poured in a Hollow glassware (Duran Group GmbH, Mainz, Germany). Samples were blended 3 times with hexane/isopropanol (3:2, v/v). The washings were made with 7.5 ml of hexane and 10 ml of isopropanol and the solution was allowed to rest for one hour to allow the phases separation. In each of the 3 washing steps after resting, the two phases were separated, and the lipid isopropanol one was transferred into another Hollow glassware, blended by adding a saline solution (Na_2SO_4 0.47 *M*, 5 ml/g) to permit the separation of the upper hexane-water phase from the lipid isopropanol phase. The final isopropanol solution was collected and transferred into a flat bottom flask, heated at 35 °C under N_2 stream for about 20 min to complete solvent evaporation and the resulting extracted fat material was weighted. About 44 mg of fat were transferred into culture tubes to be immediately methylated according to Christie (2001) using sodium methoxide 1 *M* in methanol at room temperature. Two mL of n-exane, containing 2 mg/mL of methyl 12-tridecenoate as internal standard (#U-35 M, Nu-chek prep inc., MN, USA), 100 µl of Sodium Methoxide (1M) in methanol were added to the extracted fat (EE). The solution containing the EE and solvent was mixed for 10 min using a multi mixer (717+, ASAL s.r.l., Florence, Italy) at room temperature. After this step 150 µl of oxalic acid in ethyl ether was added to stop the reaction in the solution containing the FA methyl esters (FAME). The solution was agitated for 30 s and centrifuged at 8000 × *g* for 10 min (Sigma Laborzentrifugen 3K15, Osterode am Harz, Germany). The upper phase was taken (1 ml) and transferred to a vial for the GC×GC analysis.

Gas chromatographic analysis

The samples obtained were analyzed for their fatty acid profile using a GC×GC instrument (Agilent Technologies 7890A, CA, USA) with two columns in series, equipped with a modulator (Agilent G3486A CFT, CA, USA), an automatic sampler (Agilent 7693, CA, USA) and a flame ionization detector (FID) connected with a chromatography data system software (Agilent Chem Station, CA, USA). This instrument was used because the use of a double column allows a much better separation and identification of FA on a two-dimensional basis, compared to the traditional one column GC (Cesaro et al., 2013). Between the two columns a modulator unit collects in a fixed volume channel the analyte bands of the first column and these are successively launched into the short second column in narrow bands. The operative conditions of the GC apparatus were:

- First column of 75 m × 180 µm (internal diameter) × 0.14 µm of film thickness (23348U, Supelco, Sigma Aldrich, PA, USA), H_2 carrier flow of 0.22 mL/min;
- Second column of 3.8 m × 250 µm (internal diameter) × 0.25 µm of film thickness (J&W 19091-L431, Agilent Technologies,CA, USA), H_2 carrier flow of 22 mL/min;
- Planned oven temperature variation: increase from 50 °C (held for 2 min), then increased to 150 °C (held for 15 min) at 50 °C/min and then increased to 240 °C (held for 84 min) at 2 °C/min
- Valves: modulation delay, 1.00 min; modulation period, 2.90 s; sample time, 2.77 s.
- Detector (FID): heater, 250°C, H_2 carrier flow 20mL/min, air flow 450mL/min.
- Splitless Inlet: temperature 270°C, pressure 20.698 MPa, Septum Purge 3mL/min, Split Ratio 20mL/min.
- The resulting bi-dimensional chromatograms were analyzed with the comprehensive GC×GC software (Zoex Corp., TX, USA) to evaluate the cone volume of each FA.

Identification and quantification of Fatty Acid Methyl Esters (FAME)

The FA identification was completed in two different ways, the first by comparison of the cone position in the chromatogram with the cone position of FA contained in the GC reference standards. The reference standards used were mixtures of pure fatty acids [(#674, Nu-chek prep inc., MN, USA), (#463, Nu-chek prep inc., MN, USA), (47080-U Bacterial Acid Methyl Esters - BAMEs, Sigma-Aldrich, MO, USA), (47085-U PUFA-3 Menhaden Oil, Supelco, PA, USA)] plus individual CLA isomers: CLA*c*9,*t*11 (#UC-60M, Nu-chek Prep Inc., MN, USA), CLA*t*10,*c*12 (#UC-61M, Nu-Chek Prep, Inc. MN, USA), CLA*c*9,*c*11 (#1256,

Matreya LLC, PA, USA), CLA*t*9,*t*11 (#1257, Matreya LLC, PA, USA) and CLA*c*11,*t*13 (#1259, Matreya LLC, PA, USA). The second FA identification procedure was completed by considering the elution order and the position of each peak in the two-dimensional chromatogram on the basis of comprehensive GC×GC software (GC Imagine Software, Zoex Corporation, TX, USA). In the region of the C18:1 isomers were detected different peaks and two of them C18:1*t*9 (Elaidic Acid) and C18:1*t*11 (Vaccenic Acid) were partially co-eluted. Quantification of each FA was made considering the cone volume of each FA peak with respect to the volume of total FA and it was expressed in terms of g FA/g of total FA ×100 on the basis of the concentration in weight of the methyl 12-tridecenoate contained in the solution used as internal standard. The various FA were summed in categories according to various criteria as follow.

- Saturated fatty acids (**SFA**) categories were the sum of: C4:0, C6:0, C7:0, C8:0, C9:0 C10:0, C11:0, C11:0$_{iso}$, C12:0, C12:0$_{iso}$, C13:0, C13:0$_{iso}$, C13:0$_{anteiso}$, C14:0, C14:0$_{iso}$, C14:0$_{anteiso}$, C15:0, C15:0$_{iso}$, C15:0$_{anteiso}$, C16:0, C16:0$_{iso}$, C16:0$_{anteiso}$, C17:0, C17:0$_{iso}$, C17:0$_{anteiso}$, C18:0, C18:0$_{iso}$, C18:0$_{anteiso}$, C19:0, C19:0$_{iso}$, C19:0$_{anteiso}$, C20:0, C21:0, C22:0, C23:0 and C24:0.
- Monounsaturated fatty acids (**MUFA**) were the sum of C10:1, C11:1, C12:1, C12:1*t*, C12:1*c*, ΣC13:1, C14:1*c*9, C14:1*t*9, ΣC14:1, C15:1, C15:1*t*, ΣC15:1, C16:1*t*7, C16:1*c*7, C16:1*c*9, ΣC16:1, C17:1*c*10, C17:1*t*, ΣC17:1, C18:1*t*9(+*t*11), C18:1*c*9, C18:1*c7*, C18:1*t*12, C18:1*c*12, ΣC18:1, C19:1, C19:1*c*, C19:1*c*9, C19:1*t*9, C19:1*n*12, ΣC19:1, C20:1*n*9, C20:1*n*7, ΣC20:1, ΣC21:1, ΣC22:1 and C24:1.
- Polyunsaturated fatty acids (**PUFA**) were the sum of ΣC16:2, ΣC17:2, C18:2*n*6, ΣC18:2, ΣC19:2, C20:2*n*6, C20:2*n*3, ΣC20:2, ΣC16:3, C18:3*n*3, C18:3*n*6, C20:3*n*3, C20:3*n*6, ΣC20:3, ΣC16:4, C18:4*n*1, C18:4*n*3, C18:4*n*4 C20:4*n*3, C20:4*n*6, ΣC20:4, C18:5*n*3, C20:5*n*3(EPA), C20:5*n*6, ΣC20:5, C22:4*n*6, C22:5*n*3, C22:5*n*6, C22:6*n*3(DHA), CLA*c*9,*t*11 CLA*t*10,*c*12, CLA*c*11,*t*13, CLA*t*9,*t*11, CLA*c*9,*c*11 and CLA*c*10,*c*12.
- Branched fatty acids were calculated according to Raes et al. (2004) as the sum of C13:0$_{iso}$, C13:0$_{anteiso}$,C14:0$_{iso}$, C14:0$_{anteiso}$, C15:0$_{iso}$, C15:0$_{anteiso}$,C16:0$_{iso}$, C16:0$_{anteiso}$, C17:0$_{iso}$, C17:0$_{anteiso}$, C18:0$_{iso}$, C18:0$_{anteiso}$, C19:0$_{iso}$ and C19:0$_{anteiso}$.
- Odd Chain fatty acids were calculated according to Or-Rashid et al. (2007) as sum of: C7:0, C9:0, C11:0$_{iso}$, C11:0, C13:0$_{iso}$, C13:0$_{anteiso}$, C13:0, C15:0$_{iso}$, C15:0$_{anteiso}$, C15:0, C17:0$_{iso}$, C17:0$_{anteiso}$,C17:0, C19:0$_{iso}$, C19:0$_{anteiso}$, C19:0, C21:0, C23:0, C15:1*t*isomer, C15:1*t*isomer, C15:1*n*5*t*, C13:1isomer,C15:1isomer, C15:1isomer, C15:1, C17:1*n*7*t*,

C17:1*n*9, C17:1*n*7, C19:1*n*12*t*, C19:1*n*9*t*, C19:1*n*12, C19:1*n*9*c*,C19:1*c*, C19:2isomer, C19:2isomer, C19:2*n*6, C19:4isomer, C19:5.

- The sum of the identified CLA isomers (**ΣCLA**) was calculated as the sum of: CLA*c*9,*t*11 CLA*t*10,*c*12, CLA*c*11,*t*13, CLA*c*9,*c*11, CLA*c*10,*c*12 *and* CLA*t*9,*t*11.
- The sum of Omega-3 PUFA (**Ω3**) or Omega-6 PUFA (**Ω6**) were calculated according to Givens et al. (2000) and Connor (2000), as example Ω3 PUFA was computed as the sum of C18:3*n*3, C18:4*n*3 C18:5*n*3, C20:2*n*3, C20:3*n*3, C20:4*n*3, C20:5*n*3 (EPA), C22:5*n*3, C22:6*n*3 (DHA).
- The Δ^9-desaturase indices were calculated, according to Kelsey et al. (2003), for four pairs of FA that represent products and substrates for Δ^9-desaturase. These FA pairs were 14:1*c*9/14:0, 16:1*c*9/16:0, 18:1*c*9 /18:0, and C18:2*c*9,*t*11/C18:1*t*11. We defined the total desaturase index as follows: [products of Δ^9-desaturase]/[products of Δ^9-desaturase + substrates ofΔ^9-desaturase];
- The Atherogenic Index (AI) and Thrombogenic Index (TI) were calculated according to Ulbricht and Southgate (1991) as: AI = (C12:0 + 4 × C14:0 + C16:0)/(Ω3PUFA + Ω6PUFA + MUFA); and TI = (C14:0 + C16:0 + C18:0)/(0.5 × MUFA + 0.5 × Ω6PUFA + 3 × Ω3 PUFA/Ω6PUFA).

Statistical Analysis

Fatty acid proportion (g FA×100 g total FA) of milk samples were analyzed using MIXED procedure of SAS (SAS, 2005). Data were analyzed with a linear mixed model considering the effect of the diet (Control vs rpCLA), breed (Brogna, Foza and Lamon), number of suckling lambs (single vs twins), linear covariate of DIM at the beginning of the trial, and period of sampling (period A vs B) as fixed effects. The random effect of animal (24 ewes, 18 df) was used to test diet, breed and number of suckling lambs and DIM, whereas the effects of period of sampling was tested on the residual. The following orthogonal contrast were calculated: Brogna vs (Foza+Lamon), i.e. the dairy vs the average of the two meaty breeds, and Foza vs Lamon breed. Data regarding ewes and lambs live weight, growth rate and BCS variation (one observation per animal) were analyzed using a linear model similar to that used for FA profile, but without the effect of period and the random effect of animal. Lastly, data regarding average DM intake (one observation per pen) were analyzed with a linear model including only the effects of diet and breed.

4.4 RESULTS

The GC×GC comprehensive system revealed the impressive presence in milk of 170 peaks corresponding to 33 SFA, 53 MUFA, 76 PUFA, and other 8 unidentified FA peaks. For reason of space, FA partially identified were summed in groups according to the length of their carbon chain and degree of unsaturation. In total, 29 SFA, 17 MUFA and 5 sums of other MUFA, 9 PUFA and 3 sums of other PUFA, and 6 CLA isomers are presented in the tables. As the effect of the number of suckling lambs never influenced the milk FA profile, with the only exception of C17:0$_{anteiso}$ (0.65 vs 0.73 % for milk of ewes suckling 1 or 2 lambs, respectively, P = 0.04), results about this effect were ignored. The rpCLA supplementation had no effect on liveweight, average daily gain, body condition score variation and DM intake of ewes and their suckling lambs (Table 4.1). Results about major milk constituents contents, as well as on cheese making properties, have been previously given in Bittante et al. (2014), but some results, for the purpose of current work, are summarized in Figure 1. The rpCLA supplementation reduced milk protein content, and numerically reduced milk fat content by 10% compared to the control diet. In this last case the difference was not significant because the residual variation observed for this trait was great.

Effects of breed and animal within breed on fatty acids profile of ewe's milk

Difference in milk SFA profile due to the breed was scarce (Table 4.2). Milk from Foza and Lamon (large size meaty breeds) compared to that of Brogna (small size, multipurpose breed) differed for only some *iso* forms and odd-numbered SFA: a lower content of C15:0$_{iso}$, a tendency for lower content of both C17:0$_{iso}$ and C17:0$_{anteiso}$, and a greater content of C19:0. Milk from Foza ewes contained a greater proportion of *iso* forms of C13:0, C14:0, C15:0 and C16:0 compared to that from Lamon ewes. The relative incidence of the animal within breed variance on the sum of residual plus animal variance, an index of animal repeatability, showed a large range of variation among FA (Table 4.2). For even-numbered FA this index was low or null for C4:0, C10:0, C20:0 and C22:0, and moderate for other FA of the same category ranging from 0.15 to 0.30 for C6:0 and C14:0, respectively. For odd-numbered not branched SFA this index was small or null for short-chain FA (till C13:0), great for C15:0 (0.44) and C17:0 (0.62), and low for C19:0 (0.12) and C21:0 (0.05). For short chain branched SFA animal repeatability was small (≤ 0.22), with the only exception of C13:0$_{anteiso}$ (0.65), but for long chain branched SFA animal repeatability was equal or greater than 0.23.

The effect of breed on the proportion of various MUFA and PUFA was scarce, too (Table 4.3). Milk from Brogna ewes differed from that of the other two breeds only for a smaller proportion of ΣC19:1 FA, not for C19:1*n*12, and of C18:2*c*9,*t*11. Compared to the milk obtained from Lamon ewes, that collected from Foza ewes had a smaller proportion of ΣC19:1 and a greater proportion of C12:1, C14:1*t*9, ΣC20:2, C18:3*n*6, CLA*c*11*t*13, and C18:4*n*3. Animal repeatability was medium-high for all MUFA, as for 15 FA or sums the value of this index was equal or greater than 0.30, with only some exceptions with null values for ΣC16:1 and C17:1*c*10, and moderate to low values for C10:1 (0.22), C18:1*c*9 (0.13), C18:1*c*7 (0.23), and for the two C19:1 groups. On opposite, animal repeatability for the various PUFA was always lower than 0.30, and often null, with exception for C18:2*n*6 (0.49), CLA*t*10,*c*12 (0.39), and C20:4*n*6 (0.37).

In terms of FA categories and indices (Table 4.4), the milk produced by Brogna tended to present smaller proportion of PUFA, ΣCLA, and branched FA compared to the other breeds, and Foza ewes produced a milk with smaller proportion of medium-chain FA (C16) compared to the Lamon ewes.

Animal repeatability was moderate to great (0.15 to 0.48) for all the FA categories and indices considered, except for the sums of unknown FA and for the ratio Ω 6/Ω 3 that showed null values.

Effects of CLA supplementation, of DIM and sampling period on fatty acids profile of ewe's milk

The rpCLA supply increased the proportion of C4:0, decreased that of all other SFA with chains ranging 6 to 13 carbons, excluding the branched FA, tended to increase that of C20:0 and notably increased that of C22:0 (Table 4.5). Initial stage of lactation had small effect of the SFA proportions, but a decrease of C6:0, C8:0 and C18:0 proportions, and a tendency for an increase of C14:0 and C15:0 proportion was observed with increasing initial stage of lactation. Differently, sampling period affected almost all the SFA proportions, as compared to the first period the second one increased the proportions of short chain SFA (till C14:0) and a decrease those of SFA with longer carbon chain.

The rpCLA supply also affected the proportion of several MUFA (Table 4.6), as it decreased or tended to decrease those regarding C10:1, C12:1, ΣC16:1, and ΣC20:1, and increased those of C16:1*t*7, C18:1*c*9 and the sum of C18:1*t*9 and C18:1*t*11 (in the GC×GC chromatograms these last two FA were co-eluted). In general, the rpCLA supply did not influenced the total proportion of PUFA, but increased the proportions of ΣC16:2, of the two

CLA isomers supplied with the rumen protected mixture (CLA*c*9,*t*11 and CLA*t*10,*c*12), and also of the *cis-cis* and the *trans-trans* forms of isomers with double bounds in position 10 and 12 (CLA*c*10,*c*12 and CLA*t*10,*t*12).

Like in the case of SFA, also in the case of MUFA and PUFA (Table 4.6) the stage of lactation at the start of the trial had small influences with some increase of the proportion of C12:1*t*, ΣC14:1, ΣC16:1, C16:1*c*7, CLA*c*9,*t*11, and CLA*c*9*c*11. Differently, the period of sampling affected the large majority of unsaturated FA. In this case, passing from the first to the second period of sampling it was observed a relevant decrease of C18:1*c*9 which was compensated by an increased proportion of major part of others unsaturated FA.

Considering the FA categories given in Table 4.7, it was observed that rpCLA supply decreased the proportion of SFA and short chain FA and increased those of MUFA and long chain FA and the sum of CLA isomers. The rpCLA addition also increased the Ω6/Ω3 FA ratio, the CLA*t*10,*c*12 desaturation index and the total desaturation index. The initial stage of lactation affected only the Ω6/Ω3 FA ratio and the C14 and C18 desaturation indices, but the sampling period influenced the large majority of FA categories and indices.

4.5 DISCUSSION

Differences in milk fatty acid profile among sheep breeds

Brogna, Foza, Lamon and Alpagota are native breeds reared in the Veneto region with different morphological and production abilities (Pastore and Fabbris, 1999; Pastore, 2002; Bittante, 2011). Brogna is represented by few thousands of red spotted medium-sized sheep reared in the hills of Verona province (Bittante et al., 1990). This breed is similar to the Alpagota breed, not considered in current work, which is represented by few thousands of a medium-sized black spotted sheep reared in small farms of Alpago mountain and known for lamb production labelled by the Slow Food Presidium as "*Agnello dell'Alpago*" or Alpagota lamb (Slow Food Foundation, 2014). The Brogna ewes are also commonly used for the production of milk to be processed in a cheese locally called "*Pegorin*". Foza and Lamon breeds are represented by large-sized black spotted sheep used for lamb production. In the past these breeds were widely reared in the pre-Alps mountains of Vicenza and Belluno provinces by transhumant shepherds, but they are currently endangered of extinction (Bittante and Pastore, 1988; Ramanzin et al., 1991; Bittante et al., 1996). In the companion study conducted with the same animals, Bittante et al. (2014) showed that milk composition for major nutrients was not affected by the breed of ewes, with the only exception of a slightly

lower content of non-fat solids of the milk from Lamon compared to that from Foza ewes (11.7% vs 12.1%, respectively, $P = 0.05$).

In the current paper, as the typical flavor of sheep milk and cheese is strongly influenced by the presence of some FA (Guinee and O'Brien, 2010), and considering that current literature on sheep milk commonly reports data about a limited number of major FA, major emphasis was given to achieve a detailed FA profile of milk, and to evaluate the influences of breed and other possible sources of variation. The effect of breed on the FA profile of sheep milk have been object of some studies with contradictory results, and none of them studied the autochthonous sheep breeds of North-East Italy. Signorelli et al. (2008), comparing the FA profile (15 fatty acids and 3 groups) of the milk of three Italian sheep breeds in very different conditions (pasture-based in the Southern Italy) compared to the present study, found several breed's differences. In particular, the milk from the specialized dairy Sarda ewes, respect to that produced by Altamurana and Gentile di Puglia local ewes, was characterized by a tendency for a smaller proportion of the even-numbered short-chain SFA and of the MUFA with 10, 14 and 16 carbon chain a greater proportion of C18:0. None of these fatty acids was affected by breed in the case of the breeds from North-East Italy of the present study. On the contrary, the fatty acids affected were the odd- and branched-chain fatty acids and some PUFA (Table 4.3 and 4.3), that were not analyzed (odd- and branched-chain fatty acids) or only as a group (C18:2 and C18:3) or individual isomer (CLA*c*9*t*11) by Signorelli et al. (2008).

Tsiplakou et al. (2008) compared the fatty acid profile (16 fatty acids,5 groups and 2 indices) of milk obtained from ewes belonging to four breeds of very different origin and characteristics: Awassi, Lacaune, Friesland and Chios. The ewes were reared indoor during winter and on pasture from spring. The level of milk production was not very different among breeds and the fatty acid profile was profoundly affected by season/feeding system but not by breed. The interaction between breed and season/feeding system was often significant reflecting a different entity of individual breed reaction to environmental factors, but not a opposite trend.

Mierlita et al. (2011) found greater differences in milk fatty acid profile (23 fatty acids, 7 groups and 4 indices) due to breed (Spanca and Turcana, Romania) respect to those found in the present study. The major differences regarded the even-numbered SFA between C6 and C16, the C18:1*c*9 and *t*11, the CLA*c*9,*t*11 and all groups and indices with the exception of Ω6/ Ω3 ratio.

Recently Soják et al. (2013) compared the fatty acid profile (15 fatty acids, 6 groups and 2 indices) of the milk of a specialized sheep dairy breed of French origin, Lacaune, with two Slovakian breeds, Tsigai and Improved Valachian, reared at pasture. Also these authors reported several differences between all the breeds. The specialized breed tended to produce milk with a greater proportion of C10:0, C12:0, and C14:0 fatty acids and a smaller proportion of C4:0, C16:0, C16:1*c*9, and C18:1c9. Like in the present study, also Soják et al. (2013) found no breed effect on Ω6 and Ω3 fatty acids, while the dairy sheep breed was characterized by a slightly lower desaturase activity respect to local breeds.

Regarding the CLA isomers content of sheep milk fat, while in the present study was observed a smaller amount of the most important isomers, the C18:2*c*9,*t*11, in the Brogna ewes respect to the large-sized Foza and Lamon sheep, Rozbicka-Wieczorek et al. (2013) found significant difference between two Polish sheep breeds only for the sums of *cis*-*cis* CLA isomers.

Variability of milk fatty acid profile among individual ewes within breed

It is interesting to note that in the present study it does not appear some relationships between the significance of the breed effects and the entity of variability among individuals within breed. If a significant individual variability is expected for even-numbered normal SFA, it is not expected that a similar individual variability was found for odd-numbered and branched-chain SFA in the interval C14-C19 (Table 4.2). Moreover, MUFA generally presented a medium-great individual variability while PUFA did not (Table 4.3).

In the literature, few studies analyzed variation of milk fatty acid profile of individual ewes within breed. De La Fuente et al. (2009), with a different approach than that used in the present study, found that individual ewe explained a greater fraction of total variation of milk fatty acid proportions respect to age and lactation stage of the animal and a much smaller fraction respect to flock and to day of testing. Moreover individual variation was greater for SFA, excluded C16:0, than UFA, excluded C18:2*c*9,*c*12. Soják et al. (2013) observed that the ranking of individual ewes for the CLA proportion in their milk, changed a lot moving from indoor feeding to pasture.

In a large survey on a back-cross Sarda×Lacaune population, Carta et al. (2008) calculated the individual repeatability of fatty acids proportion in milk and obtained estimates varying from about 15% of the C18:3 fatty acid to almost 60% of C4:0 and C17:0. Also the desaturase indices were characterized by large variations among individual ewes. The order of magnitude of the common fatty acids was not much different in that study and in the present one,

suggesting that a genetic variability could affect fatty acid profile and be the basis for a possible selection aimed at increase the fatty acids characterized for favorable effects on human health and decrease the negative ones. This was corroborated by the estimates from the same authors who found that the sire variance seldom was greater than 10% of total variance and specifically it happened for C4:0, C10:0, C12:0, C16:0, and C17:0, but was never null.

More recently, from another very large survey on Churra sheep in Spain, Sánchez et al. (2010) estimated heritability values of fatty acid proportion in sheep milk. The obtained values were low, generally below 10%, and around half the value found for milk yield and fat and protein content.

In bovine species several heritability estimates were carried out on the proportion of 8 to 17 milk fatty acids analyzed by gas chromatography with results going from values similar to those found on sheep (Mele et al., 2009) to values much greater (Stoop et al, 2008). Only recently Cecchinato et al. (2013) estimated the heritability coefficients of a detailed fatty acid profile (47 fatty acids) obtaining values greater than 20% for C10, C12, C16, and C18 SFA, for C14:1c9, and C16:1c9 MUFA. All the other fatty acids and also the groups and indices were characterized by smaller heritability estimates (<20%).

New prospective can interest also the improvement of the knowledge of phenotypic and genetic aspects of fatty acid profile of sheep milk because it can also be predicted using Fourier-transform Infrared Spectroscopy at population level (Soyeurt et al. 2007; De Marchi et al., 2011; Bittante and Cecchinato, 2013).

Lastly new knowledge could be achieved from molecular genetics. In fact, some authors found significant associations between the proportion of some sheep milk fatty acid and specific candidate genes (Moioli et al., 2012).

4.4 CONCLUSION

The results of this experiment confirms that different source of variation can influence FA profile of ewe's milk. Amoung the various effects, CLA supplementation was considered one of the most important. It can decrease the proportions of SFA (carbon chain 6 to 13) and MUFA and increase C20:0, C22:0, C16:1*t*7, C18:1*c*9, CLA*c*9,*t*11 and CLA*t*10,*c*12 that were isomers supplied with the rumen protected mixture. The CLA addition increase also the Ω6/Ω3 ratio, total desaturase index and CLA*t*10,*c*12 desaturase index.

Moreover, stage of lactation and period of sampling affect the composition fo few FA, such as, MUFA that increase in particular in the second period of sampling.

In conclusions from these results, is important underline the high number of peaks identify through GC×GC and considered milk fatty acids composition because of it is important for the effect that ewes milk can have on lamb's tissues.

Table 4.1: Effect of rpCLA supplementation on liveweight, growth rate, variation of body condition score (BCS) and dry matter intake (DMI) of ewes and suckling lambs belonging to three breeds autochthonous of the Veneto Alps.

	Diet (LSM):		*P-value*	RMSE
	Control	CLA		
Ewes' liveweight:				
initial, kg	64.2	61.1	0.57	11.8
final, kg	65.4	63.5	0.72	11.6
ADG, g/d	40	20	0.44	56
Lambs' liveweight:				
initial, kg	12.9	13.7	0.51	3.0
final, kg	20.9	21.5	0.72	4.0
ADG, g/d	134	130	0.76	33
BCS variation:				
ewes, score	0.38	0.25	0.25	0.25
lambs, score	0.21	0.11	0.10	0.15
DMI, g/d/ewe	2.67	2.63	0.74	0.11

Table 4.2: Effects of breed, and of animal within breed on the proportion of Saturated Fatty Acids (g FA×100 g total FA) of milk of three sheep breeds autochthonous of the Veneto Alps (the individual FA are listed in order of elution).

	Breed LSM:			*Contrasts (P-value)*[1]		Animal effect[3]	RMSE[4]
	Brogna	Foza	Lamon	B vs(F+L)	F vs L		
C4:0	3.44	3.45	3.77	0.38	0.15	0.00	0.49
C6:0	3.01	3.02	2.93	0.66	0.37	0.15	0.20
C7:0	0.15	0.18	0.14	0.82	0.23	0.00	0.07
C8:0	2.87	2.86	2.70	0.54	0.31	0.27	0.28
C9:0	0.17	0.19	0.17	0.54	0.37	0.00	0.05
C10:0	9.48	9.19	7.39	0.11	0.04	0.01	1.85
C11:0$_{iso}$	0.03	0.04	0.04	0.41	0.65	0.06	0.01
C11:0	0.25	0.29	0.25	0.61	0.33	0.00	0.08
C12:0	6.46	6.48	5.56	0.35	0.09	0.16	1.04
C13:0$_{iso}$	0.05	0.05	0.07	0.50	0.02	0.00	0.02
C13:0$_{anteiso}$	0.03	0.03	0.03	0.59	0.55	0.65	0.01
C13:0	0.25	0.27	0.25	0.49	0.39	0.00	0.06
C14:0$_{iso}$	0.16	0.16	0.22	0.22	0.03	0.22	0.05
C14:0	12.4	11.7	11.7	0.30	0.99	0.30	1.21
C15:0$_{iso}$	0.33	0.37	0.43	0.03	0.09	0.00	0.08
C15:0$_{anteiso}$	0.74	0.78	0.84	0.20	0.35	0.15	0.12
C15:0	1.44	1.51	1.56	0.31	0.63	0.44	0.15
C16:0$_{iso}$	0.32	0.33	0.40	0.14	0.06	0.30	0.06
C16:0	23.5	22.2	24.1	0.59	0.04	0.16	1.65
C17:0$_{iso}$	0.48	0.54	0.56	0.09	0.66	0.23	0.08
C17:0$_{anteiso}$	0.65	0.72	0.71	0.09	0.70	0.23	0.07
C17:0	0.81	0.87	0.87	0.31	0.99	0.62	0.08
C18:0$_{iso}$	0.11	0.11	0.14	0.36	0.33	0.50	0.03
C18:0	6.64	6.26	7.17	0.89	0.16	0.23	1.16
C19:0$_{iso}$	0.02	0.03	0.01	0.99	0.23	0.98	0.00
C19:0	0.08	0.04	0.06	0.04	0.20	0.12	0.03
C20:0	0.19	0.19	0.22	0.66	0.28	0.07	0.07
C21:0	0.05	0.05	0.06	0.46	0.63	0.05	0.03
C22:0	0.10	0.10	0.11	0.64	0.53	0.05	0.05

[1] B=Brogna, F=Foza, L=Lamon;
[2] Slope of the linear regression on initial DIM (g FA×100 g total FA × d^{-1});
[3] Expressed as the ratio between the variance of animals within breed and the total variance (animal plus residual);
[4] Residual RMSE=Root mean square error.

Table 4.3: Effects of breed and of animal within breed on the proportion of Monounsaturated and Polyunsaturated Fatty Acids (g FA×100 g total FA) of milk of 3 sheep breeds autochthonous of the Veneto Alps (the individual FA are listed in order of elution).

	Breed			*Contrasts (P-value)*[1]		Animal effect[3]	RMSE[4]
	Brogna	Foza	Lamon	B vs (F+L)	F vs L		
C10:1	0.35	0.37	0.32	0.87	0.14	0.22	0.06
C12:1*t*	0.11	0.12	0.09	0.64	0.11	0.35	0.02
C12:1	0.19	0.21	0.15	0.77	0.04	0.30	0.05
ΣC14:1	0.22	0.24	0.20	0.96	0.11	0.49	0.04
C14:1*t*9	0.02	0.03	0.01	0.68	0.05	0.69	0.00
C14:1*c*9	0.33	0.32	0.29	0.61	0.61	0.76	0.05
ΣC15:1	0.13	0.14	0.13	0.77	0.61	0.40	0.03
C15:1*t* isomer*	0.06	0.07	0.07	0.73	0.95	0.36	0.02
C15:1*t* isomer*	0.03	0.03	0.02	0.63	0.31	0.32	0.01
C15:1*t*5	0.02	0.02	0.02	0.48	0.80	0.96	0.00
ΣC16:1	0.40	0.47	0.38	0.69	0.24	0.00	0.16
C16:1*t*7	0.08	0.12	0.11	0.36	0.68	0.58	0.04
C16:1*c*9	0.42	0.47	0.45	0.18	0.44	0.67	0.03
C16:1*c*7	1.20	1.16	1.22	0.96	0.73	0.57	0.19
C17:1*c*10	0.29	0.32	0.16	0.55	0.12	0.00	0.22
ΣC18:1	0.47	0.56	0.53	0.22	0.68	0.36	0.11
C18:1*t*9(+*t*11)	1.39	2.07	1.69	0.25	0.42	0.31	0.78
C18:1*c*9	15.2	15.3	16.4	0.40	0.19	0.13	1.62
C18:1*c*7	0.56	0.61	0.58	0.49	0.61	0.23	0.10
ΣC19:1 others	0.01	0.02	0.03	0.04	0.04	0.09	0.00
C19:1*n*12	0.02	0.02	0.02	0.53	0.75	0.16	0.01
ΣC20:1	0.06	0.07	0.09	0.41	0.51	0.63	0.03
ΣC16:2	0.08	0.10	0.10	0.21	0.92	0.20	0.03
ΣC18:2	0.66	0.78	0.73	0.13	0.55	0.26	0.13
C18:2isomer*	0.24	0.23	0.24	0.89	0.53	0.00	0.06
C18:2isomer*	0.26	0.29	0.27	0.41	0.37	0.06	0.04
C18:2*n*6	2.17	2.64	2.32	0.16	0.19	0.49	0.32
ΣC20:2	0.02	0.03	0.01	0.52	0.06	0.00	0.01
C18:3*n*6	0.13	0.15	0.12	0.75	0.03	0.03	0.03
C18:3*n*3	0.30	0.35	0.34	0.11	0.69	0.24	0.06
CLA*c*9*t*11	0.36	0.48	0.43	0.01	0.20	0.09	0.08
CLA*t*10*c*12	0.04	0.04	0.05	0.78	0.64	0.39	0.03
CLA*c*11*t*13	0.02	0.05	0.03	0.90	0.06	0.26	0.02
CLA*c*9*c*11	0.02	0.03	0.02	0.51	0.14	0.00	0.02
CLA*c*10*c*12	0.01	0.02	0.02	0.10	0.70	0.00	0.01
CLA*t*9*t*11	0.04	0.04	0.04	0.66	0.49	0.05	0.02
C18:4*n*3	0.01	0.02	0.01	0.77	0.06	0.00	0.01
C18:5*n*3	0.01	0.01	0.02	0.26	0.19	0.00	0.00
C20:3*n*6	0.02	0.03	0.03	0.34	1.00	0.00	0.02
C20:4*n*6	0.20	0.24	0.21	0.22	0.15	0.37	0.03

* Isomer identifies by position in the chromatograms. [1] B=Brogna, F=Foza, L=Lamon;
[2] Slope of the linear regression (g FA×100 g total FA × d^{-1});
[3] Expressed as the ratio between the variance of the animals within breed and the total variance (animal plus residual);
[4] Residual RMSE=Root mean square error.

Table 4.4: Effects of breed and of animal on milk fat content (%) and on the proportion of groups of Fatty Acids (g FA×100 g total FA) and on some indices of milk of three sheep breeds autochthonous of the Veneto Alps.

	Breed			*Contrasts (P-value)*[1]		Animal effect[3]	RMSE[4]
	Brogna	Foza	Lamon	B vs(F+L)	F vs L		
Milk fat content	5.23	3.91	5.47	0.42	0.18	0.73	0.85
Milk protein content	5.77	6.19	5.24	0.75	0.56	0.85	0.46
Milk lactose content	5.11	5.29	5.18	0.94	0.46	0.04	0.51
SFA	74.2	72.0	72.4	0.12	0.78	0.48	1.88
MUFA	21.3	21.3	23.0	0.14	0.89	0.40	1.63
PUFA	4.35	5.26	4.73	0.08	0.18	0.40	0.59
$FA_{unknown}$	0.07	0.06	0.05	0.21	0.66	0.00	0.03
ΣCLA	0.49	0.63	0.56	0.07	0.21	0.25	0.11
Ω6	2.53	3.08	2.68	0.14	0.13	0.47	0.35
Ω3	0.33	0.38	0.36	0.17	0.48	0.15	0.06
Ω 6/ Ω 3	7.75	8.10	7.49	0.91	0.22	0.00	1.11
Branched	2.91	3.13	3.42	0.04	0.14	0.19	0.36
Odd chain	6.10	6.61	6.51	0.16	0.79	0.46	0.50
<C16	42.6	42.1	39.3	0.23	0.13	0.17	3.41
C16	26.1	25.1	26.9	0.93	0.05	0.20	1.64
>C16	31.3	32.9	33.9	0.24	0.61	0.44	2.85
Desaturase indices:							
C14	0.02	0.03	0.03	0.25	0.35	0.15	0.01
C16	0.04	0.05	0.05	0.23	0.69	0.12	0.01
C18	0.70	0.71	0.70	0.63	0.31	0.37	0.02
CLA*c*9,*t*11	0.22	0.21	0.21	0.76	0.98	0.27	0.04
Total	0.29	0.32	0.31	0.14	0.66	0.28	0.03
IT	2.07	1.81	2.02	0.12	0.06	0.39	0.16
IAT	3.33	2.92	2.97	0.12	0.85	0.33	0.45

[1] B=Brogna, F=Foza, L=Lamon;
[2] Slope of the linear regression (g FA×100 g total FA × d^{-1});
[3] Expressed as the ratio between the variance of the animals within breed and the total variance (animal plus residual);
[4] Residual RMSE=Root mean square error.

Table 4.5: Effects of rumen protected CLA supplementation, of the days in milk at the beginning of the trial (DIM), and of sampling period on the proportion of Saturated Fatty Acids (g FA×100 g total FA) of milk of three sheep breeds autochthonous of the Veneto Alps (the individual FA are listed in order of elution).

	CLA supplementation			DIM		Sampling period		
	Control	CLA	*P-value*	Slope	*P-value*	A	B	*P-value*
C4:0	3.28	3.82	<0.01	-0.002	0.64	3.30	3.80	<0.01
C6:0	3.18	2.79	<0.01	-0.007	<0.01	2.90	3.08	0.01
C7:0	0.18	0.13	0.08	0.0003	0.66	0.13	0.18	0.04
C8:0	3.13	2.49	<0.001	-0.008	0.04	2.67	2.95	<0.01
C9:0	0.20	0.15	0.01	0.0003	0.52	0.16	0.20	0.02
C10:0	9.56	7.82	0.01	-0.002	0.89	7.65	9.73	<0.01
C11:0$_{iso}$	0.03	0.04	0.29	0.0003	0.09	0.03	0.04	0.01
C11:0	0.31	0.22	0.01	0.001	0.25	0.23	0.30	0.01
C12:0	6.88	5.46	<0.01	0.012	0.31	5.47	6.87	<0.001
C13:0$_{iso}$	0.06	0.06	0.84	-0.0002	0.45	0.06	0.06	0.73
C13:0$_{anteiso}$	0.03	0.02	0.14	0.0002	0.39	0.03	0.03	0.02
C13:0	0.28	0.24	0.07	0.001	0.11	0.24	0.28	0.03
C14:0$_{iso}$	0.18	0.18	1.00	-0.0001	0.82	0.21	0.15	<0.01
C14:0	12.0	11.9	0.79	0.028	0.09	11.5	12.4	0.02
C15:0$_{iso}$	0.37	0.38	0.76	-0.0004	0.61	0.42	0.33	<0.01
C15:0$_{anteiso}$	0.78	0.79	0.78	-0.0003	0.83	0.86	0.71	<0.01
C15:0	1.51	1.50	0.91	0.004	0.09	1.57	1.43	0.01
C16:0$_{iso}$	0.34	0.35	0.78	-0.0002	0.84	0.38	0.31	<0.01
C16:0	23.1	23.5	0.56	0.017	0.36	23.9	22.6	0.02
C17:0$_{iso}$	0.50	0.56	0.11	-0.0003	0.76	0.60	0.46	<0.001
C17:0$_{anteiso}$	0.67	0.71	0.14	0.236	0.24	0.76	0.62	<0.001
C17:0	0.83	0.86	0.58	-0.003	0.10	0.93	0.76	<0.001
C18:0$_{iso}$	0.12	0.12	0.90	-0.001	0.14	0.13	0.11	0.02
C18:0	6.30	7.08	0.13	-0.044	0.01	7.63	5.75	<0.001
C19:0$_{iso}$	0.02	0.01	0.37	0.0005	0.10	0.02	0.02	0.04
C19:0	0.06	0.06	0.72	-0.0004	0.21	0.06	0.06	0.44
C20:0	0.18	0.22	0.09	0.0002	0.81	0.19	0.21	0.44
C21:0	0.05	0.05	0.80	0.0001	0.59	0.06	0.04	0.06
C22:0	0.08	0.13	0.01	0.0003	0.62	0.12	0.09	0.07

Table 4.6: Effects of rumen protected CLA supplementation, of the days in milk at the beginning of the trial (DIM), and of sampling period on the proportion of Monounsaturated and Polyunsaturated Fatty Acids (g FA×100 g total FA) of milk of three sheep breeds autochthonous of the Veneto Alps (the individual FA are listed in order of elution).

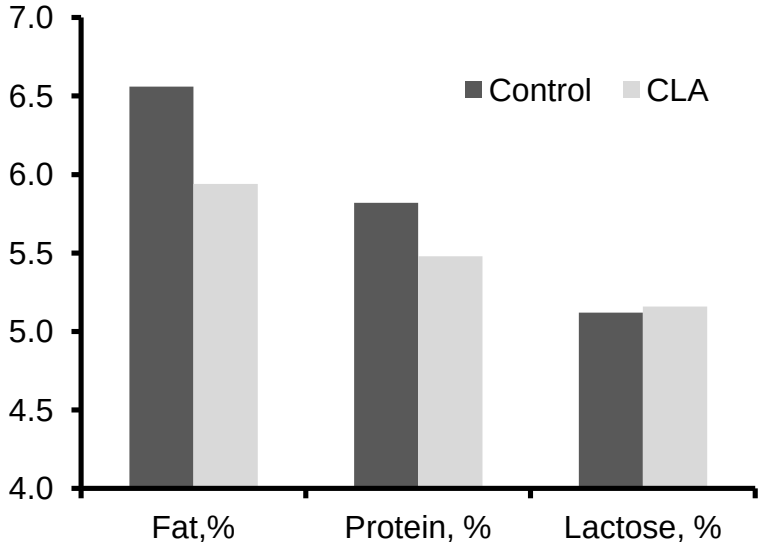
	CLA supplementation			DIM		Sampling period		
	Control	CLA	*P-value*	Slope	*P-value*	A	B	*P-value*
C10:1	0.40	0.30	<0.001	0.001	0.05	0.29	0.41	<0.001
C12:1*t*	0.12	0.10	0.13	0.001	<0.01	0.08	0.13	<0.001
C12:1	0.22	0.15	0.01	0.001	0.06	0.13	0.23	<0.001
ΣC14:1	0.23	0.21	0.36	0.002	0.05	0.19	0.25	<0.01
C14:1*t*9	0.02	0.02	0.28	-0.00003	0.84	0.02	0.02	0.56
C14:1*c*9	0.30	0.33	0.48	0.004	0.01	0.26	0.37	<0.001
ΣC15:1	0.12	0.14	0.27	0.0003	0.57	0.15	0.11	<0.01
C15:1*t* isomer*	0.06	0.07	0.79	0.0003	0.25	0.07	0.06	0.01
C15:1*t* isomer*	0.03	0.03	0.59	0.0001	0.72	0.03	0.03	0.08
C15:1*t*5	0.02	0.02	0.50	-0.00004	0.84	0.02	0.03	0.13
ΣC16:1	0.46	0.37	0.09	0.003	0.05	0.31	0.52	<0.001
C16:1*t*7	0.08	0.13	0.07	0.0002	0.77	0.11	0.10	0.38
C16:1*c*9	0.44	0.45	0.71	0.0002	0.79	0.46	0.44	0.09
C16:1*c*7	1.16	1.23	0.61	0.01	0.03	1.13	1.26	0.05
C17:1*c*10	0.26	0.26	0.94	-0.003	0.21	0.27	0.24	0.68
ΣC18:1	0.48	0.56	0.15	-0.0002	0.91	0.49	0.54	0.12
C18:1*t*9(+*t*11)	1.33	2.10	0.05	0.01	0.60	1.86	1.57	0.24
C18:1*c*9	14.9	16.4	0.02	-0.02	0.19	16.6	14.7	<0.01
C18:1*c*7	0.58	0.58	0.95	0.0002	0.84	0.58	0.59	0.64
ΣC19:1	0.02	0.02	0.06	0.02	0.31	0.01	0.02	0.14
C19:1n12	0.02	0.02	0.79	0.000	0.44	0.02	0.02	0.19
ΣC20:1	0.09	0.05	0.08	0.001	0.34	0.08	0.06	0.36
ΣC16:2	0.08	0.11	0.02	-0.0003	0.38	0.08	0.11	0.01
ΣC18:2	0.71	0.74	0.62	-0.0001	0.94	0.71	0.74	0.39
C18:2isomer*	0.25	0.22	0.21	-0.0001	0.90	0.21	0.26	0.01
C18:2isomer*	0.27	0.28	0.60	-0.0002	0.64	0.26	0.28	0.12
C18:2*n*6	2.26	2.49	0.23	0.002	0.74	2.43	2.33	0.30
ΣC20:2	0.02	0.02	0.24	0.0005	0.07	0.01	0.03	0.07
C18:3*n*6	0.14	0.12	0.19	0.0005	0.11	0.12	0.14	0.01
C18:3*n*3	0.34	0.33	0.77	-0.001	0.41	0.33	0.34	0.57
CLA*c*9*t*11	0.39	0.46	0.03	0.002	0.04	0.38	0.47	<0.01
CLA*t*10*c*12	0.02	0.06	0.01	-0.0001	0.85	0.04	0.04	0.64
CLA*c*11*t*13	0.03	0.04	0.55	0.0002	0.44	0.03	0.04	0.05
CLA*c*9*c*11	0.02	0.03	0.09	0.0004	0.07	0.02	0.03	0.01
CLA*c*10*c*12	0.01	0.02	0.18	0.0000	0.47	0.01	0.02	<0.01
CLA*t*9*t*11	0.02	0.05	<0.01	0.0002	0.46	0.04	0.04	0.95
C18:4*n*3	0.01	0.01	0.36	0.00003	0.78	0.01	0.02	0.06
C18:5*n*3	0.01	0.01	0.31	-0.00002	0.65	0.01	0.02	<0.01
C20:3*n*6	0.03	0.03	0.67	0.0002	0.21	0.03	0.03	0.49
C20:4*n*6	0.23	0.20	0.13	0.0004	0.42	0.20	0.24	<0.001

* Isomer identifies by position in the chromatograms.

Table 4.7: Effects of rumen protected CLA supplementation (rpCLA), days in milk at the beginning of the trial (DIM), and of sampling period on milk fat content (%) and on the proportion of groups of Fatty Acids (g FA×100 g total FA) and some indices of milk of three sheep breeds autochthonous of the Veneto Alps.

	rpCLA			DIM		Sampling period		
	Control	CLA	*P-value*	Slope[2]	*P-value*	A	B	*P-value*
Milk fat content	5.25	5.23	0.98	-0.018	0.47	4.94	5.53	0.04
Milk protein content	6.04	5.30	0.18	0.004	0.85	5.61	5.72	0.44
Milk lactose content	5.23	5.18	0.79	-0.005	0.40	5.25	5.16	0.55
SFA	74.2	71.6	0.02	-0.01	0.81	72.2	73.5	0.03
MUFA	21.3	23.5	0.02	0.00	0.89	23.1	21.7	0.01
PUFA	4.59	4.97	0.66	0.00	0.66	4.68	4.88	0.29
FA_{unkwon}	0.07	0.05	0.14	0.00	1.00	0.05	0.07	0.03
ΣCLA	0.48	0.64	<0.001	0.00	0.12	0.51	0.62	<0.01
Ω6	2.66	2.86	0.33	0.00	0.59	2.79	2.74	0.69
Ω 3	0.36	0.35	0.62	0.00	0.21	0.35	0.37	0.34
Ω 6/ Ω 3	7.26	8.30	<0.01	0.04	<0.01	8.06	7.51	0.12
Branched	3.08	3.22	0.35	0.00	0.45	3.48	2.82	<0.001
Odd chain	6.44	6.38	0.84	0.00	0.88	6.76	6.06	<0.001
<C16	43.4	39.2	<0.01	0.04	0.37	38.5	44.1	<0.001
C16	25.8	26.2	0.58	0.03	0.13	26.6	25.5	0.04
>C16	30.8	34.6	0.02	0.07	0.15	34.9	30.5	<0.001
Desaturase indices:								
C14	0.02	0.25	0.81	0.00	<0.001	0.02	0.03	<0.001
C16	0.05	0.05	0.67	0.00	0.02	0.05	0.05	0.16
C18	0.71	0.70	0.88	0.00	<0.001	0.69	0.72	<0.01
CLA*c*9,*t*11	0.23	0.20	0.10	0.00	0.34	0.18	0.25	<0.001
Total	0.29	0.32	0.05	0.00	0.95	0.31	0.30	0.18
IT	2.04	1.90	0.10	0.00	0.16	1.96	1.97	0.81
IAT	3.24	2.91	0.14	0.00	0.43	2.91	3.24	0.02

Figure 1. Effect of rpCLA supplementation on the content of fat (*P-value* = 0.41; RMSE = 2.00 %), protein (*P-value* = 0.02; RMSE = 0.07 %), and lactose (*P-value* = 0.77; RMSE = 0.04 %) of milk of ewes belonging to three autochthonous breeds of the Veneto Alps.



CHAPTER 5

Quality traits and modeling of coagulation, curd firming and syneresis of sheep's milk of Alpine breeds fed diets supplemented with rumen protected conjugated fatty acid (CLA)

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5.1 ABSTRACT

The aim of this study was to test the modeling of curd-firming (CF) measures and to compare the sheep of three Alpine breeds supplemented with or without rumen-protected conjugated linoleic acid (rpCLA). Twenty-four ewes of the Brogna, Foza and Lamon breeds were allotted to six pens (two pens/breed) and fed a diet composed of corn grain, corn silage, dried sugar beet pulp, soybean meal, wheat bran, wheat straw and a vitamin-mineral mixture. The rpCLA supplement (12 $g \times d^{-1}$ per ewe plus 4 $g \times d^{-1}$ for each lamb older than 30 d) was mixed into the diet of one pen per sheep breed (three pens/treatment), to provide an average of 0.945 and 0.915 g/d per ewe of the C18:2*c*9,*t*11 and C18:2*t*10,*c*12 CLA isomers, respectively. The trial started at 38±23 d after parturition, and individual morning milk samples were collected on days 16, 23, 37, 44 and 59 of the trial. Milk samples were analyzed for composition, and duplicate samples were assessed for milk coagulation properties (MCPs). A total of 180 CF measures for each sample (one every 15 sec) were recorded. Model parameters were the rennet coagulation time (RCT), the asymptotic potential curd firmness (CF_P), the curd firming instant rate constant (k_{CF}), the syneresis instant rate constant (k_{SR}), the maximum curd firmness achieved within 45 min (CF_{max}) and the time at achievement of CF_{max} (t_{max}). The data were analyzed using a hierarchical model which considered the fixed effects of breed, diet, lambs birth, and initial days in milk (DIM), which were tested on individual ewe (random) variance; the fixed effect of sampling day, which was tested on the within-ewe sample (random) variance; and the fixed effect of instrument/cuvette position (only for MCPs), which was tested on the residual (replicates within samples) variance. The local Alpine sheep breeds displayed similar milk compositions, traditional MCPs, and CF modeling parameters. Supplementation with rpCLA triggered changes in milk composition and MCPs (e.g., delayed RCT, slower k_{CF}, and a doubling of k_{SR}), but did not influence CF_P. Overall, our results indicate that rpCLA supplementation reduced the actual maximum CF (CF_{max}) but did not modify the interval between rennet addition and CF_{max} (t_{max}).

Keywords: ovine milk, milk coagulation property, conjugated fatty acids (CLA), curd-firming modeling

5.2 INTRODUCTION

For decades, bovine milk coagulation properties (MCPs) have been evaluated using mechanical lactodynamographs (Bittante et al., 2012). Three single-point parameters are defined: the rennet coagulation time (RCT, min), which is the interval from the addition of the enzyme to the gelation of the milk; the curd-firming (CF) rate (k_{20}, min), or the time from gelation to a curd firmness of 20 mm; and the curd firmness measured 30 min after rennet addition (a_{30}, mm). Combinations of these parameters are used to categorize milk samples for their cheese-making properties. Computerized lactodynamographs can record continuous repeated measurements of CF. Bittante (2011b) and Bittante et al. (2013b) proposed a model that fully represents the temporal evolution of CF on the basis of rennet coagulation time (RCT), the asymptotical potential curd firmness at infinite time (CF_P, mm), the curd-firming instant rate constant (k_{CF}) from RCT to infinite time, and the syneresis instant rate constant (k_{SR}). Although MCPs have not been widely studied among small ruminants. Partial studies in this field have been made on sheep (Pellegrini et al., 1997; Jaramillo et al., 2008; Pazzola et al., 2013) and goats (Park et al., 2007; Pazzola et al., 2011; Pazzola et al., 2012). Notably, the traditional MCP procedure is sometimes considered inadequate for evaluating the milk of these animals (Bittante et al., 2012). Compared to bovine milk, the milk-coagulation process of small ruminants is typically much faster and of greater magnitude; thus, a_{30} often measures CF after the maximum value has been reached, and k_{20} measures only a limited tract of the steep increase in CF (Bittante et al., 2012). Thus, the use of the model proposed by Bittante (2011b) and Bittante et al. (2013b) could provide new insights into the coagulation properties of ewe's milk, and permit us to evaluate if these properties can be influenced by breed and/or different diets and feed additives.

Conjugated linoleic acid (CLA) has gained attention in recent years for its beneficial effects on human health (Dilzer and Park, 2012). Although these effects have largely been studied in animal models and *in vitro* (McCrorie et al., 2011), further research is needed (Gebauer et al., 2011). The main source of natural CLA for humans is the consumption of food from ruminant species, especially those fed on pasture or diets containing oil seeds (Nuernberg et al., 2005; Scollan et al., 2006; Woods and Fearon, 2009). However, supplementing the animals' diets with rumen-protected CLA (rpCLA; produced by the feed industry) is an effective way to increase the CLA content of beef (Gillis et al., 2004; Schiavon et al., 2011) and lamb (Terrè et al., 2011) meat. The rpCLA has shown favorable effects on the efficiency of energy and nitrogen utilization in growing young bulls, and appears to exert a limited effect on fat

deposition (Schiavon and Bittante, 2012; Schiavon et al., 2010 and 2012). In dairy ruminants, however, the most notable effect of rpCLA is its ability to decrease the fat content in milk from cows (Baumgard et al., 2000; Bauman et al., 2008; Glasser et al., 2010), goats (Lock et al., 2008; Shingfield et al., 2009; Ghazal et al., 2012) and sheep (Oliveira et al., 2012; Weerasinghe et al., 2012). However, while there are numerous studies regarding the effects of rpCLA on dairy ruminants, little is known about its influence on MCP.

The aims of the current study were to: (i) examine the effect of rpCLA supplementation of lactating ewes on their milk composition; (ii) model the CF process of sheep's milk; (iii) compare the effect of three different sheep breeds on milk quality and MCPs; and (iv) study the effects of rpCLA supplementation on the composition and MCPs of sheep's milk.

5.3 MATERIALS AND METHODS

Animals

The present study was carried out at the "Lucio Toniolo" Experimental Farm of the University of Padova (Legnaro, Italy), using a flock of sheep representing endangered Alpine breeds native to the Veneto Region (Northeast Italy). Animals were treated following the Guideline for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Consortium, 1988). The study involved 24 ewes; of them, 10, 9, and 5 ewes belonged to the Brogna, Foza and Lamon breeds, respectively. The 24 ewes were allotted to six pens of 3 m × 6 m (two for each breed) with their 31 suckling lambs (15, 10 and 6 respectively for Brogna, Foza and Lamon breed). At the start of the trial, the ewes were 38 ± 23 days in milk (DIM) and weighed 61 ± 13 kg (body weight, BW). The trial lasted 63 days (d). Ewes and lambs were individually weighed each week. Animals were monitored daily by a technician, and health status was controlled three times per week by a veterinarian, following the experimental protocol for animal care.

Feeds and Feeding

The basal diet was composed of corn grain, corn silage, dried sugar beet pulp, soybean meal, wheat bran, wheat straw and a vitamin mineral mixture (Table 1). Dietary ingredients were mixed and fed as total mixed ration (TMR), offered *ad libitum*, and prepared daily using a mixer-wagon equipped with a computer-assisted weighing scale that was calibrated monthly. Three pens, one for each breed, were top dressed, and then mixed with TMR, with an rpCLA product (SILA, Noale, Italy) equal to 12 $g \times d^{-1}$ per ewe plus 4 $g \times d^{-1}$ per lamb aged

30 d or more. This rpCLA dose was established to provide averages of 0.945 and 0.915 g×d^{-1} per ewe for the C18:2*c*9,*t*11 and C18:2*t*10,*c*12 CLA isomers, respectively. The composition of the utilized commercial rpCLA was previously reported by Schiavon et al. (2010). The amount of each feed ingredient loaded into the mixer-wagon and the weight of the mixture uploaded in the manger of each pen were recorded daily. The orts (uneaten residues of feed) remaining in the mangers were weighed weekly by pen. The average dry matter intake (DMI) was computed on a pen basis.

Samples of each feed ingredient were analyzed for their proximate compositions (AOAC, 2000), and their neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents (Van Soest et al., 1991). The metabolizable energy of the basal ration was computed from the actual ration ingredient composition and tabular values of each feed ingredient (NRC, 2007).

Milk Sampling and Analyses

The ewes were separated from their lambs for at least 2 h and then hand milked on days 16, 23, 37, 44 and 59. After collection, milk samples (without preservative) were immediately divided into subsamples A (35 mL) and B (20 mL), stored in portable refrigerators (4°C) and transported to the Cheese-Making Laboratory at the DFANAE Department of the University of Padova (Legnaro, Padova, Italy) for analyses. All samples were processed within 5 h after collection.

For each ewe, milk subsample A was analyzed for fat, protein, lactose, total solids, and solid non-fat contents using a MilkoScan FT2 (Foss, Hillerød, Denmark). In addition, somatic cell counts were performed using a Fossomatic FC counter (Foss, Hillerød, Denmark). Each somatic cell count (SCC) was converted to the somatic cell score (SCS) by means of logarithmic transformation, as follows: SCS = ($\log_2$SCC×100,000^{-1})-3. The energy of milk was calculated using the values proposed by the National Research Council (2007) and converted to kJ×g^{-1} (fat = 38.89 kJ×g^{-1}; protein = 23.90 kJ×g^{-1}; lactose = 16.53 kJ×g^{-1}).

Analysis of MCPs

The B subsamples were assessed for MCPs using two mechanical lactodynamographs (Formagraph; Foss Electric, Hillerød, Denmark). All experimental conditions (milk temperature, rennet concentration, and rennet type) were applied as described in detail by Cipolat-Gotet at al. (2012). In brief, a rack containing 10 cuvettes (one rack per instrument) was prepared. Two milk sub-samples (10 mL) for each ewe were randomly allotted to the two

racks, heated at 35°C, and mixed with 200 μL of rennet solution (Hansen Standard 215 with 80±5% chymosin and 20±5% pepsin; Pacovis Amrein AG, Bern, Switzerland) diluted to 1.2% (w/v) in distilled water (to yield 0.051 IMCU×mL^{-1}). The instruments recorded the width (in mm) of the oscillatory graph during the test every 15 s. The observation period lasted for 45 min after rennet addition. Traditional MCPs (RCT, k_{20} and a_{30}) were provided directly by the instrument. Recording was prolonged to 45 min after enzyme addition, to achieve an additional measure of curd firmness (a_{45}, mm). Relatively few samples (8 of 206 samples) failed to coagulate within the 45-min duration of the test.

Modeling Curd Firmness and Syneresis

As CF was measured every 15 s for 45 min, a total of 180 CF values were recorded for each sample. The comparison of the much shorter RCT values of ovine milk to those of bovine milk, and the prolongation of recording to 45 min enabled the use of the four-parameter model described by Bittante et al. (2013b):

where CF_t is the curd firmness at time t (mm), CF_P is the asymptotic potential maximum value of curd firmness (mm), k_{CF} is the curd-firming instant rate constant (%×min^{-1}), k_{SR} is the curd syneresis instant rate constant (%×min^{-1}), and RCT is the rennet coagulation time (min).

This model uses all available information to estimate the four parameters, so (unlike the traditional MCPs) these are not single-point measurements. The CF_P parameter is conceptually independent from test duration and (unlike a_{30}) is not intrinsically dependent on RCT. The parameter k_{CF} is assumed to increase CF toward the CF_P asymptotic value, whereas k_{SR} is assumed to decrease CF toward a null asymptotic value. In the initial phase of the test, the first rate constant prevails over the second, so CF_t increases to a point in time (t_{max}) at which the effects of the two parameters are equal but opposite in sign and CF_t attains its maximum level (CF_{max}). Thereafter, CF_t begins to decrease, tending toward a null value because of the effect of curd syneresis and the corresponding expulsion of whey. The RCT parameter is still a traditional measure, but it is now estimated using all available data.

Statistical Analyses

The CF_t observations available for each sample were fitted with curvilinear regressions using the non-linear procedure (PROC NLIN) of SAS (SAS Inst. Inc., Cary, NC, SAS, 2001). The parameters of each individual equation were estimated employing the

Marquardt iterative method (350 iterations and a 10^{-5} level of convergence). In some late-coagulating samples (6 of 206 samples), the data did not converge. Samples in which the CF_P exceeded the replicate (3 cases) or the mean (3 cases) by three SD were considered outliers and excluded from our analysis of all equation parameters. No data editing was performed for the other parameters.

The data regarding milk analyses, traditional MCPs and CF modeling were analyzed using a linear mixed model employing the MIXED procedure of SAS. The statistical model used to analyze the traditional MCPs and the parameters of the CF_t model included the fixed effects of breed (Brogna, Foza and Lamon), dietary treatment (control *vs.* rpCLA addition), number of lambs suckling (single or twin lambing), a linear covariate of days in milk (DIM) at the start of the trial, sampling day (16, 23, 37, 44 and 59 d from the start of the trial), and the cuvette location within the two instruments (18 levels). The random effects included in the model were the individual animal (24 ewes, 16 df), the milk samples within each ewe (77 df), and the replicates within each milk sample (residual, 70 df). The significances of breed, diet, number of lambs and DIM were tested on the error line of animal variance; sampling day was tested on the error line of milk sample variance within animals; and cuvette location within instruments was tested on the residual variance. For milk quality traits, the two replicates were averaged and the within-ewe samples were assumed to coincide with the residual variance.

In the case of each ewe's live weight (one observation per animal, data not shown), the model was simplified because it did not include the effects of sampling day or the cuvette location within the instrument, and the only random effect included (residual) coincided with the animal. In the case of DMI (one observation per pen, data not shown), the model included only the effect of diet and breed, and the residual coincided with their interaction.

5.4 RESULTS

Brogna ewes presented a lower BW compared to the Foza and Lamon ewes (51.8 *vs.* 71.1 and 71.2 kg, respectively; $P < 0.01$), and Brogna and Foza ewes consumed less DM than Lamon ewes (2.45 and 2.50 *vs.* 3.00 $kgDM \times d^{-1}$, respectively; $P < 0.05$). Supplementation with rpCLA did not influence the DM intake of ewes and lambs (expressed per unit of ewe present; 2.63 *vs.* 2.67 $kgDM \times d^{-1}$, respectively, for control and supplemented diets), or influence the BW and average body gains of ewes and lambs, regardless of breed.

However, rpCLA supplementation did affect the milk composition, reducing the protein and solid non-fat contents, while increasing the SCS (Table 2). All of the other factors included in the model, with the exception of sampling day (which was significant for almost all traits), had limited effects: the Lamon breed yielded milk with a lower solid non-fat content compared to Foza ewes; and ewes that had lambed twins and/or had longer intervals DIM at the beginning of the trial produced milk with greater protein and solid non-fat contents.

The only breed effect observed among the traditional traits used to depict MCPs was a lower a_{45} for Lamon *versus* Foza sheep (Table 3). MCPs were significantly worse when rpCLA was added to the basal diet: RCT was delayed, curd firming was slowed, and curd firmness was lower after 30 and 45 min from rennet addition. Twin lambing had positive effects on both measures of curd firmness. Days in milk at the beginning of the trial did not affect any MCP, while sampling day and cuvette location influenced all traits except RCT.

For the CF_t model parameters, milk from Lamon ewes was characterized by slower curd firming and faster syneresis compared to the Foza breed (Table 4). Similar to the traditional MCPs, the parameters obtained from CF_t modeling showed that rpCLA supplementation had negative effects on the parameters of coagulation and curd firming, except for the asymptotic CF value and the interval between the addition of the enzyme and the moment of maximum curd firmness. In milk sampled from rpCLA-supplemented ewes, the expulsion of whey from the curd (i.e. the syneresis rate) was much faster and the number of samples showing no detectable syneresis within 45 min from rennet addition was halved.

The birth type affected the CF modeling, as samples from ewes with twin lambs showed more rapid decreases of CF after reaching the maximum (i.e., more rapid syneresis). A higher initial DIM tended to be associated with increased CF and fewer samples that failed to undergo syneresis, and appeared to anticipate the reaching of maximum curd firmness. The sampling day and instrument/cuvette location affected all modeling parameters except for RCT (both factors) and CF_P (sampling day).

5.5 DISCUSSION

Traditional Coagulation Properties of Sheep's Milk

The enzymatic coagulation of milk, the firming of curd, and the subsequent expulsion of whey (syneresis) are the key processes in cheese-making, and thus affect cheese yield and quality (Cipolat-Gotet et al., 2013; Bittante et al., 2013a). However, the mechanical and

optical NIR-lactodynamographs (Cipolat-Gotet et al., 2012) that are generally used to measure these traits are non-automated and time-consuming. In the bovine dairy industry, the Fourier-transform infrared (FTIR) spectrum of milk, which is heritable (Bittante and Cecchinato, 2013), was recently introduced as a method for predicting the parameters traditionally measured by lactodynamographs. The FTIR prediction is very rapid and inexpensive, does not need enzymes or mechanical tools, and does not require milk coagulation to occur. Such indirect predictions may also prove useful for breeding programs (Cecchinato et al., 2009). To our knowledge, however, these techniques have not previously been tested on milk from small ruminant species and more research is needed on this topic.

The data obtained in the present study confirmed that there are large differences between ovine and bovine MCPs as reported in Table 3. The average RCT measured from control ewes (6.5 min) was much shorter than that commonly found in bovine species (10 to 20 min), as reviewed by Bittante et al. (2012). The average k_{20} revealed that there is a much steeper increase in the CF of ovine milk (1.5 min) compared to bovine milk (5 to 10 min). The average CF after 30 min was also greater for sheep's milk (61 mm) than cow's milk (25 to 42 mm). Notably, the rennet concentration used in the present study (0.051 IMCU×mL^{-1}) was smaller than those in all but one of the papers on bovine MCP reviewed by Bittante et al. (2012) (0.061 to 0.150 IMCU×mL-1), and this disparity widens if the amount of rennet is expressed per unit protein instead of per unit of milk. Moreover, the milk of cows and ewes reacts differently to acidification, temperature changes, calcium addition, and variation in rennet concentration (Bencini, 2002).

Modeling the Coagulation, Curd Firming and Syneresis of Sheep's Milk

Two other shortcomings of the traditional MCP parameters are the increasing percentage of bovine milk samples that do not coagulate (NC) within the commonly used time interval of 30 min from rennet addition (Ikonen et al., 1999; Cecchinato, 2013), and the increasing proportion of milk samples that do not allow computation of the k_{20} trait. These increases reflect the worldwide spread of the Holstein breed, which are known for having inferior MCPs compared to breeds of Alpine origins (Cecchinato et al., 2011). A similar problem has been noted in small ruminants (Pazzola et al., 2012 and 2013). The traditional MCP analysis uses only three data points, while computerized rennet meters (lactodynamographs) use continuous repeated measurements. Bittante (2011b) modeled a dataset recorded from individual bovine milk samples using computerized rennet meters over 30 min (120 records, one every 15 sec, in the case of Formagraph lactodynamographs) and

proposed to use the obtained CF curve to estimate the RCT, the asymptotical potential CF at infinite time (CF_P, mm), and the CF instant rate constant (k_{CF}, %×min^{-1}) from RCT to infinite time. Later, Bittante et al. (2013b) expanded the CF model to account for the decrease in curd firmness often recorded after (though sometimes before) 30 min from rennet addition, using a forth parameter called the syneresis rate constant (k_{SR}, %×min^{-1}).

In the case of sheep's milk, the traditional MCP traits are considered even less reliable in depicting the process of curd firming, largely because the process is much faster and of greater magnitude compared to bovine milk, such that a_{30} often measures CF after the maximum value has already been reached and k_{20} measures only a limited tract of the steep increase in curd firming (Bittante et al., 2012).

The present study showed that the model proposed by Bittante et al. (2013b) overcomes the concerns linked to traditional MCP measures, yielding results that can depict the evolution of CF over time for sheep's milk. In fact, the large majority of individual samples converged, allowing us to estimate the values for all four parameters of the model. Compared to the bovine milk studied by Bittante et al. (2013b), the control sheep's milk samples analyzed in the present study showed on average a much earlier gelation (RCT: 7.0 *vs.* 19.3 and 20.7 min compared to Brown Swiss and Holstein Friesian cows, respectively), a greater asymptotic potential CF (CF_P: 67 *vs.* 54 and 36 mm, respectively), a much steeper increase in CF (k_{CF}: 49.5 *vs.* 12.0 and 13.0 %×min^{-1}, respectively), and a slower decrease in CF due to syneresis (k_{SR}: 0.5 *vs.* 1.4 and 1.7 %×min^{-1}, respectively). Regarding this last parameter, ~ 30% of milk samples from control ewes did not exhibit any apparent decrease of CF during the 45 min after rennet addition, meaning that the k_{SR} could not be estimated and was assumed to be null. If we excluded these samples, the average k_{SR} of the remaining samples was 0.6 %×min^{-1}. In practice, the CF equation of the samples characterized by non-estimable k_{SR} values coincided with the three-parameter model that was initially proposed by Bittante (2011b) to depict the CF trends of lactodynamograms generated over a short observation interval (30 min for cow's milk). It is probable that prolonging the observation interval beyond 45 min would have allowed us to estimate k_{SR} for the samples that failed to show any significant decrease within the test period. Bittante et al. (2013b) prolonged their recording interval to 90 min and observed that almost all of their bovine milk samples presented an inflection, allowing them to compute k_{CF} for all samples. In any case, the samples with late CF decreases were characterized by very slow syneresis rate constants.

For each milk sample, knowing the four parameters of the CF curve allowed us to calculate: the maximum CF value (CF_{max}), which reflects the potential CF attainable and the

two opposite effects of curd-firming rate and syneresis rate; and the time interval from rennet addition to the attainment of the maximum CF (t_{max}), which also incorporates the RCT. Compared to the milk of Brown Swiss cows tested by Bittante et al. (2013b), the sheep's milk examined in the current study evidenced a greater CF_{max} (64 *vs.* 35 mm, respectively) and reached t_{max} earlier (24 *vs.* 41 min, respectively). The average t_{max} of the sheep's milk samples presented in Table 4 includes samples that failed to show any decrease within 45 min from rennet addition; for these last samples, t_{max} was assumed to be 45 min. When these samples were excluded from the analysis, we obtained an average t_{max} of 15 min. We cannot compare these parameters to others in the literature, because the present study is the first to model the output of computerized rennet meters when examining ovine milk.

Comparison among the Sheep Breeds of the Veneto Alps

Brogna, Foza, Lamon, and Alpagota are the only autochthonous sheep breeds of the Italian Alps still present in the Northern part of the Veneto region (Bittante, 2011a). Brogna sheep, which are reared in the province of Verona, are of medium size (similar to Alpagota) and characterized by red spots on a white coat (Pastore, 2002; Pellattiero et al., 2011). Of the three breeds studied herein, Brogna is the only breed that was often used (and in some cases is still used today), as a dairy ewe; its milk can be used to produce a local pecorino (Pegorin) cheese. The other two local breeds, which are both in danger of extinction, are reared in very low numbers in the provinces of Vicenza (Foza) and Belluno (Lamon). Both are large breeds with high growth rates among their lambs; they have long ears and small black spots (especially on the head) against a white coat (Pastore, 2002). These breeds are traditionally reared for meat production (mainly from weaned lambs and castrated yearlings). The Lamon breed has been studied in the past decades (Bittante et al., 1988 and 1996; Ramanzin et al., 1991), but no previous study has examined the quality and coagulation properties of milk from the Veneto sheep breeds.

The contents of fat, protein and lactose in the Veneto sheep breeds are close to the average values reported by Barłowska et al. (2011) for ovine milk in their literature review on the composition of ruminant milk, and are very good if compared to milk traits recorded for specialized dairy ewes, such as the Sarda sheep (Mura et al., 2012; Vacca et al., 2013). The milk compositions of the three local Alpine breeds were very similar, with the sole exception of a higher non-fat solid content in Foza milk compared to Lamon milk (Table 2).

The two larger breeds also presented some differences in terms of the traditional MCPs, as Foza milk samples tended to have greater a_{30} ($P = 0.07$) and a_{45} ($P = 0.01$) values. A

picture of the difference between the two breeds, given in Figure 1, shows that milk from Foza ewes was characterized by a steeper increase (k_{CF}) and a slower decrease (k_{SR}) in curd firmness compared to the milk of Lamon ewes. The graphical representation of CF evolution over time provides a much clearer picture of the pattern of coagulation, curd firming and syneresis than we would obtain using only the three points considered by the traditional MCP procedure (RCT, a_{30} and k_{20}). From the shapes of the curves, we see that traditional k_{20} measures only about one third of the increase of the CF_t curve, and that a_{30} is in the decreasing tract of the curve, and not in the increasing one like for bovine milk, and it is influenced by all the other parameters. Finally, the differences in the milk CF_t curves due to single or twin lambings (Fig. 2) were mainly caused by differences in syneresis.

Effect of rpCLA on the Composition of Sheep's Milk

We found that rpCLA supplementation strongly modified both milk composition in sheep, even though the ewes received less than 1 g of each CLA isomer per day.

The rpCLA-induced decrease in milk fat content was only nominal (-0.62 percentage points; not significant), but it was similar in magnitude to that found in dairy cows that received similar CLA dosages expressed per unit of metabolic weight (Baumgard et al., 2000; Selberg et al., 2004; Castaneda-Gutierrez et al., 2005). The rpCLA-induced decrease in milk fat observed herein was much lower than that previously observed in sheep (~ -2.57 and -2.26 percentage points; $P < 0.001$) (Weerasinghe et al., 2012). However, while the authors of the prior paper used sheep (breed unspecified) of similar BW and DIM compared to those used in the present study, their sheep were milked twice a day and received a restricted diet (DMI: 1.8 *vs.* 2.6 $kg \times d^{-1}$ in the prior and present studies, respectively) that had a lower dietary energy concentration (ME: 10.9 *vs.* 11.4 $MJ \times kgDM^{-1}$, respectively) and a greater dietary crude protein (CP) content (CP: 163 *vs.* 130 $g \times kgDM^{-1}$, respectively). In addition, while the CLA source was the same, the daily supply in the prior study was about twice that used in the current study. In dairy cows, the effect of rpCLA supplementation on milk fat content was lower in animals fed high-concentrate diets compared to those fed low-concentrate diets. The available energy supply may influence the response of the mammary gland to CLA isomers, particularly C18:2*t*10,*c*12, as suggested by Glasser et al. (2010). Oliveira et al. (2012) too recorded a large reduction (-1.76 percentage points) of milk fat content in CLA-supplemented ewes of the Lacaune breed (a specialized dairy sheep breed), but the authors used 10-fold more (not rumen-protected) CLA isomers compared to the current study.

Supplementation with rpCLA decreased the protein and non-fat solid contents, of sheep's milk (-0.34, -0.40, and -2.7 percentage points, respectively) compared to the control. The above-cited studies found inconsistent effects of CLA on milk protein content. In sheep, Weerasinghe et al. (2012) found a negative effect while Oliveira et al. (2012) found a positive effect. In cows, the effect of CLA on milk protein content generally appears to be low or null (Maxin et al., 2011; Hötger et al., 2013).

In terms of other possible effects on milk quality, we observed that rpCLA supplementation tended to increase the SCS compared to the control. In bovine milk, the observed changes in milk composition (especially those in protein content) are generally considered negative for MCP because of their well-known phenotypic and genetic correlations (Cecchinato et al., 2011; Bittante et al., 2012).

Effect of rpCLA on Curd Firmness and Syneresis

In the literature, there is no information regarding the effects of rpCLA administration on coagulation, curd firming and syneresis in both bovine and sheep's milk. In the present study almost all parameters of the CF model were affected by rpCLA supplementation of ewes (Table 4). Supplementation did not affect the potential curd firmness; however, it delayed milk gelation (RCT: 7.0 *vs.* 9.8 min for control and rpCLA, respectively), slowed curd firming (k_{CF}: 49.5 *vs.* 34.8 %×min^{-1}, respectively) and doubled the rate of whey expulsion (k_{SR}: 0.51 to 1.00 %×min^{-1}, respectively). Furthermore, the incidence of samples that did not show any CF decrease during the test period was halved in ewes receiving rpCLA supplementation (no k_{SR}: 30.8 *vs.* 15.7%, respectively). When we excluded the samples with an apparent lack of syneresis, the average values of k_{SR} increased to 0.6 and 1.2 %×min^{-1}, respectively, for control and rpCLA-treated ewes. The effect of rpCLA on the CF curve is shown in Figure 3. The rpCLA-induced changes in the two first parameters would be considered negative for cheese-making, whereas the impact of the change in the third parameter on cheese-making is not yet known.

5.6 CONCLUSIONS

The present study provides new insights into the complex processes of coagulation, curd firming and syneresis in ovine milk, and shows that rpCLA supplementation can influence these processes. Despite the phenotypic and genetic diversity of the three local sheep breeds tested herein, the ewes produced milk with similar compositions and technological properties. Ovine milk is very different from bovine milk, not only in terms of

composition, but also because of a faster gelation after rennet addition, a steeper increase of CF, and a slower decrease of curd firmness caused by syneresis. These trends are not effectively captured by the single-point analysis of traditional coagulation traits (RCT, k_{20}, and a_{30}), but the present study showed that they can be fully captured by modeling the entire CF curve over time. This modeling requires the estimation of only four parameters, which can be achieved by prolonging the observation time up to 45 min from rennet addition.

In sum, we herein show that rpCLA supplementation of sheep can change the composition and the cheese-making properties of their milk (i.e., by delaying gelation, slowing curd firming, and accelerating syneresis). Future studies are warranted to examine the effects of CLA on cheese yield/quality; assess the relationships with milk coagulation, curd firming and syneresis; and identify causal mechanisms.

5.7 ACKNOWLEDGMENTS

The authors wish to thank the Veneto Region (BIONET project) for supporting our sampling and recording activities, the RISIB SMUPR project (no. 4145) for giving us access to the instruments of the NIRS-Food Lab; and Drs. Alberto Simonetto, Nicola Tormen, Giorgia Stocco, and Cinzia Ribeca of DAFNAE department of the University of Padova for their cooperation in sample collection, MCP measurements, and initial data analyses. We also thank SILA s.r.l. (Noale,VE, Italy) for providing the rumen-protected CLA used in this trial.

Table 5.1: Ingredients, chemical composition, dry matter (DM) and metabolizable energy (ME) of total mixed ration (TMR) and of its ingredients

	TMR	DM	Chemical composition (g×kgDM^{-1}):				ME[1]
	g×kgDM^{-1}	g×kg^{-1}	PG	NDF	ADF	Starch	MJ×kgDM^{-1}
TMR ingredients							
Corn grain, ground	373	884	89	127	25	686	13.1
Corn silage	260	351	77	402	228	365	10.6
Dried sugar beet pulp	111	897	93	439	252	0	11.4
Soybean meal	110	891	491	139	90	0	13.3
Wheat bran	64	881	169	375	113	0	10.7
Wheat straw	66	917	23	810	491	0	6.0
Vitamin mineral mixture[2]	16	920	-	-	-	-	-
TMR	1000	504[3]	130	293	146	347	11.4

[1]Values taken from NRC (2007).

[2] Content per kilogram of DM: 12.4 g of Ca, 1.7 g of P, 2.5 g of Na, 100 mg of Cu, 300 mg of Zn, 1.0 mg of Co, 3 mg of I, 1 mg of Se, 200 mg of Mn, 22,000 IU of vitamin A, 83 IU of vitamin E, and 2750 IU of vitamin D3.

[3]Including water added to the mixer wagon to increase moisture of the TMR.

Table 5.2: Effect of breed, rumen-protected conjugated linoleic acid (*rp*CLA) supplementation, birth type, days in milk at first sampling, and date of sampling on quality traits of ewe's milk

	Fat	Protein	Lactose	Total solids	Solids non-fat	Energy	SCS
	%	%	%	%	%	MJ/kg	Units
Breed:							
Brogna (Br)	6.60	5.74	5.06	17.9	12.0	4.77	4.89
Foza (Fo)	5.45	5.71	5.28	17.0	12.1	4.35	4.35
Lamon (La)	6.69	5.51	5.08	17.7	11.7	4.75	5.96
Contrasts, P-value							
Br vs (Fo+La)/2	0.52	0.34	0.40	0.45	0.47	0.50	0.74
Fo vs La	0.21	0.22	0.24	0.41	0.05	0.29	0.09
Diet:							
Control	6.56	5.82	5.12	18.0	12.1	4.79	4.37
rpCLA addition	5.94	5.48	5.16	17.1	11.7	4.47	5.76
P-value	0.41	**0.02**	0.77	0.21	**0.007**	0.29	0.06
Birth type:							
Single	6.25	5.46	5.12	17.3	11.7	4.58	5.22
Twin	6.24	5.85	5.16	17.7	12.1	4.68	4.90
P-value	0.99	**0.01**	0.79	0.56	**0.01**	0.77	0.69
Days in milk:							
<50d	6.68	5.60	5.16	17.9	11.9	4.79	5.18
50-75d	6.39	5.42	5.12	17.4	11.7	4.63	4.57
>75d	5.67	5.93	5.14	17.3	12.2	4.47	5.44
P-value	0.75	**0.04**	0.95	0.82	0.05	0.83	0.59
Ewe RMS[1]	1.88	0.05	0.06	1.53	0.06	0.29	1.89
Sampling day, *P*-value	**<0.001**	0.08	**0.01**	**<0.001**	**0.02**	**<0.001**	0.13
RMSE[2]	2.00	0.07	0.04	1.44	0.08	0.28	0.84

[1]Ewe RMS = ewe root mean square
[2]RMSE = root mean square error
P-value < 0.05 are highlighted in bold print

Table 5.3: Effect of breed, rumen-protected conjugated linoleic acid (*rp*CLA) supplementation, birth type, days in milk at first sampling, date of sampling and instrument/cuvette position on traditional milk coagulation properties of ewe's milk[1]

	RCT min	k_{20} min	a_{30} mm	a_{45} mm
Breed:				
Brogna (Br)	8.68	1.62	59.3	54.8
Foza (Fo)	7.18	1.57	58.7	55.2
Lamon (La)	8.05	1.67	54.6	47.8
Contrasts, P-value				
Br vs (Fo +La)/2	0.36	0.99	0.17	0.16
Fo vs La	0.51	0.41	0.07	0.01
Diets:				
Control	6.50	1.46	60.6	56.9
rpCLA addition	9.44	1.78	54.5	48.3
P-value	**0.01**	**0.004**	**0.003**	**<0.001**
Lambs born:				
Single	7.66	1.66	55.2	50.0
Twin	8.29	1.58	59.9	55.2
P-value	0.59	0.44	**0.02**	**0.03**
Days in milk:				
Linear regression, b	-0.028	-0.004	-0.006	-0.113
P-value	0.38	0.17	0.91	0.08
Ewe RMS[2]	1.78	0.16	2.10	2.14
Sampling day, *P*-value	0.56	**0.02**	**<0.001**	**0.002**
Sample RMS[3]	2.46	0.20	n.e.	n.e.
Instrument/position, *P*-value	0.11	**<0.001**	**<0.001**	**<0.001**
RMSE[4]	1.08	0.16	7.86	10.46

[1]RCT = rennet coagulation time; k_{20} = time interval between coagulation and attainment of a curd firmness of 20 mm; a_{30} (a_{45}) = curd firmness after 30 (45) min from rennet addition.
[2]Ewe RMS = ewe root mean square.
[3]Sample RMS = sample root mean square.
[4]RMSE = root means square error.
P-value < 0.05 are highlighted in bold print

Table 5.4: Effect of breed, rumen-protected conjugated linoleic acid (*rp*CLA) supplementation, birth type, days in milk at first sampling, date of sampling and instrument/cuvette position on modeling of coagulation, curd firming and syneresis of ewe's milk[1]

	RCT min	CF_p mm	k_{CF} % $\times min^{-1}$	k_{SR} % $\times min^{-1}$	no k_{SR} %	CF_{max} mm	t_{max} min
Breed:							
Brogna (Br)	9.1	68.6	42.8	0.70	25.2	63.2	25.3
Foza (Fo)	7.6	66.8	45.9	0.58	28.2	62.7	24.6
Lamon (La)	8.4	67.5	37.8	0.99	16.4	59.1	22.5
Contrasts, P-value							
Br vs (Fo+La)/2	0.33	0.35	0.77	0.53	0.69	0.19	0.37
Fo vs La	0.54	0.69	0.04	0.01	0.17	0.08	0.37
Diets:							
Control	7.0	67.3	49.5	0.51	30.8	64.0	24.1
rpCLA addition	9.8	68.0	34.8	1.00	15.7	59.3	24.2
P-value	**0.01**	0.64	**<0.001**	**<0.001**	**0.03**	**0.007**	0.96
Birth type:							
Single	8.1	67.6	41.6	0.90	26.7	60.4	24.6
Twin	8.7	67.7	42.7	0.61	19.9	63.0	23.7
P-value	0.58	0.99	0.73	**0.04**	0.35	0.15	0.66
Days in milk:							
Linear regression, b	-0.027	0.086	0.162	0.005	-0.409	0.038	-0.173
P-value	0.39	0.05	0.08	0.13	0.05	0.42	**0.003**
Ewe RMS[2]	1.75	1.14	4.28	n.e.	4.8	2.61	2.35
Sampling day, *P*-value	0.52	0.38	**<0.001**	**0.003**	**0.002**	**0.001**	**<0.001**
Sample RMS[3]	2.43	n.e.	3.88	n.e.	9.7	n.e.	2.77
Instrument/position, P-value	0.16	**<0.001**	**0.02**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
RMSE[4]	1.04	7.54	10.92	0.69	33.7	4.99	7.69

[1]RCT = rennet coagulation time; CF_P = asymptotic potential curd firmness; k_{CF} = curd firming instant rate constant; k_{SR} = syneresis instant rate constant; no k_{SR} = incidence of milk samples with not estimable k_{SR}; CF_{max} = maximum curd firmness achieved within 45 min; t_{max} = time at achievement of CF_{max}.
[2]Ewe RMS = ewe root mean square.
[3]Sample RMS = sample root mean square.
[4]RMSE = root means square error.
P-value < 0.05 are highlighted in bold print

Figure 1: Effect of sheep breed on coagulation, curd firming, and syneresis processes of ewe's milk.

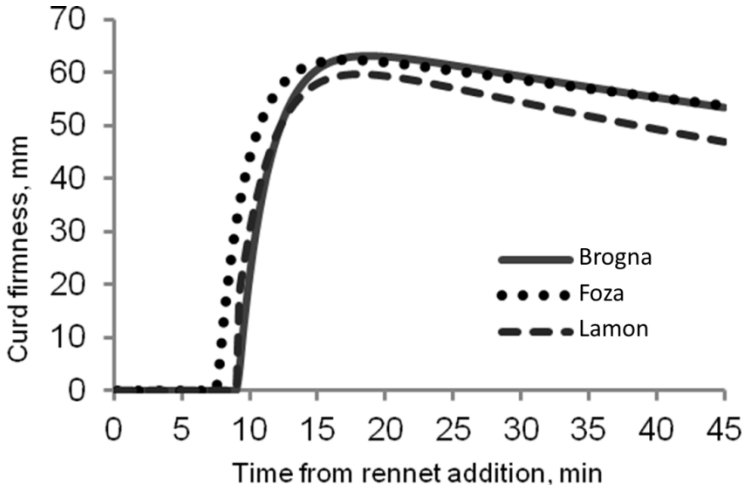


Figure 2 Effect of number of lambs born on coagulation, curd firming, and syneresis processes of ewe's milk.

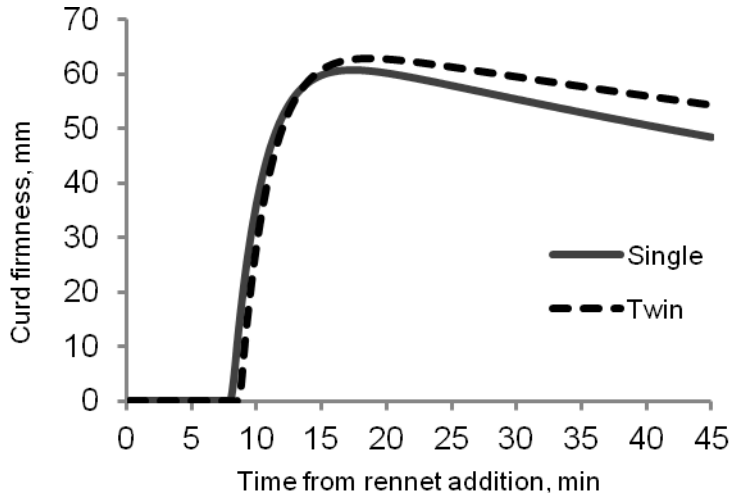
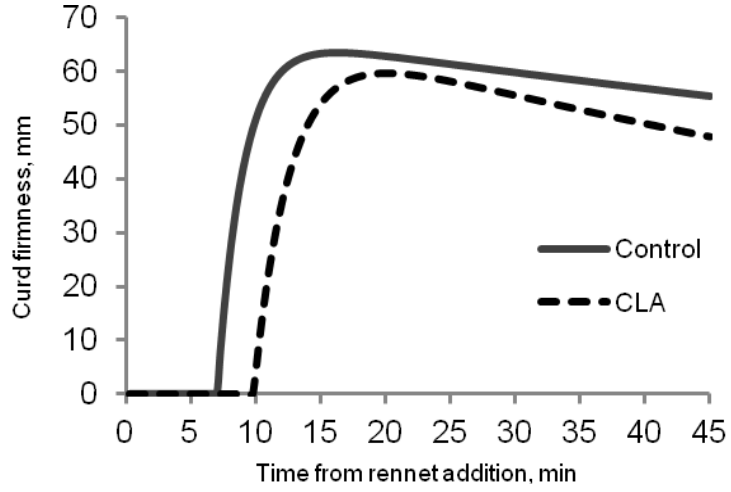


Figure 3: Effect of rumen-protected conjugated linoleic acid (*rp*CLA) supplementation to diet on coagulation, curd firming, and syneresis processes of ewe's milk.



CHAPTER 6

Growth rate, slaughter traits and meat quality of lambs of three Alpine sheep Breeds

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6.1 SUMMARY

A fattening and slaughter trial was carried out on 36 lambs of Alpagota, Brogna and Foza sheep breeds native of Veneto Region Alps (6 male and 6 female lambs per breed) divided in three groups depending on the type of feed used: pasture, hay and concentrate, hay and concentrate supplemented with conjugated linoleic acid. Lambs were slaughtered at 225 days (mean weight: 30 kg). *Infra-vitam* and *post-mortem* data were analyzed by using a linear model which included the cross-classified effect of breed, sex, feeding system and age of lambs as linear covariate. The three breeds shows some specificity: Foza lambs, of both sexes, were larger-sized and faster growing, with a lower incidence of gastro-intestinal tract and lower cooking losses of the hind-leg samples compared to the other two middle-sized breeds. Alpagota breed tended to be leaner, with heavier shin and greater cooking losses than Brogna breed. In conclusion the three Alpine breeds of the Veneto Region confirmed to be able to produce lamb carcasses and meat with valuable characteristics that can be exploited through typical products and food preparation in local markets and gastronomy, according to the tradition. The valorisation of these production can be an important instrument for *in situ* conservation of these breeds.

***Key words*:** lamb, meat quality, carcass traits, breed

6.2 AIM

Alpagota, Brogna and Foza, together with Lamon, are the sheep breeds originated in the mountain (Italian eastern Alps) of Veneto Region. Alpagota, Brogna and Foza have shown a genetic specificity that identify them as genetic resources to be protected (Bittante, 2011; De Marchi et al., 2005; Dalvit et al., 2008, 2009). All these breeds are endangered because of the low number of existing animals. They are reared in small farms at pasture, according to tradition, even if some researches to increase their productivity have been carried out (Bittante and Pastore, 1988; Bittante et al., 1996; Bonsembiante et al., 1988). These sheep populations are still used for some traditional conserved meat preparation (Paleari et al., 2006; Bovolenta et al., 2008). The aim of this study was to estimate the effect of breed and sex on growth rate, slaughter traits and meat quality traits of lambs of Alpagota, Brogna and Foza breeds.

6.3 MATERIAL AND METHODS

This experiment was conducted from July 2010 to, November 2010 at the "Lucio Toniolo" Experimental Farm of the University of Padova in Legnaro (Padova, Italy).The lambs used for this research belongs to two flocks undergoing an *in situ* conservation program (Legnaro – Villiago). In total 36 animals were used: 6 males and 6 female for each breed, Alpagota, Brogna and Foza. Lambs were divided in three groups (2 males and 2 females for each breed) with different feeding systems: a) pasture, b) penned in an open barn and fed with hay and concentrate, and c) penned in the open barn and fed with hay, concentrate and supplemented with 8.0 g/d of a rumen protected conjugated linoleic acid (*rp*CLA) product (Sila, Noale, Italy). Each month lambs were weighed and measured, recording live weight, height at withers, thoracic circumference and body condition score (BCS). At the age of 225 days and mean weight of 30 Kg lambs were slaughtered. At the slaughterhouse the following weighs were recorded: live weight, skin, feet, head, gastro-intestinal tract, offal (trachea, lungs, heart and spleen), liver, and genitals. Carcasses were divided in two halves, weighed and cold stored at 4 °C. The day after slaughter, halves were weighed again and the right half-carcasses were measured and dissected into five cuts (hind-leg, fore-leg and shoulder, ribs-loin**,** withers, brisket). On the hind leg and the rib-loin muscle pH and temperature were measured. The pH and temperature were measured using a Crison PH 25 pH-meter equipped with a penetrating electrode. Each cut was weighed and stored under vacuum at 4 °C. After 6 days the packages were open, the cuts were dried and weighed and the drip loss were computed.

In the Meat Quality Laboratory of the Department of Animal Science of Padova University, two cuts (hind-leg and rib-loin) were weighed and dissected. Rib-loin was divided in ribs and loin sections. Ribs were dissected, and weighed, in meat and bones. Loins were dissected, and weighed, in bones, subcutaneous fat and muscle (*Longissimus lumborum*). From hind-legs, only the inner part of the round (*Quadriceps femoris*) was separated and weighed. From each muscle sample pH, temperature and drip loss were obtained.

All traits were analyzed with the following linear model, using the PROC GLM of SAS (2008):

$$y_{ijklm} = \mu + age_i + feeding_j + breed_k + sex_l + e_{ijklm}$$

where y = experimental observation, μ = overall mean, age_i: linear covariate of age , $feeding_j$: effect of feeding treatment (j = pasture; hay and concentrates; hay, concentrates and

*rp*CLA), $breed_k$: effect of breed (k = Alpagota, Brogna and Foza); sex_l: effect of sex (l = F and M); e_{ijklm}: random residual term ~ N (0, σ^2_e).

6.4 RESULTS AND DISCUSSIONS

Initial and final live-weight and growth rate of Alpagota, Foza and Brogna lambs are reported in Table 5.1. The initial and final live-weight of the lambs reflects the different size of the three Veneto sheep breeds. The Alpagota and Brogna breeds lies at the two extremes for genetic and geographic distances, but not in terms of size (P = 0.083 and n.s., respectively at the beginning and at the end of the trial). The Foza lambs, genetically and geographically intermediate between Alpagota and Brogna, were heavier than the lambs of the other breeds at both dates (P < 0.001). The differences in growth rate of the lambs of the three breeds were not significant, even if the contrast between the Foza breed and the other two breeds approached the statistical significance (P = 0.066). It was observed that when growth rate was expressed in relation to the initial size of animal - allometric coefficient – the growth rate of the three breeds was very similar. The differences among breeds of body condition score (BCS), i.e. of fatness of animals, were much more pronounced at the beginning than at the end of the trial (Table 5.1), reflecting, probably, different maternal milk production. The Brogna lambs were fatter than Alpagota lambs at both dates, while Foza lambs were fatter than the other two breeds at the beginning but not at the end of the trial.

Sex of lambs influenced only the final live-weight with the males heavier than females (+11.1%, P < 0.05). It should be considered that the majority of animals were in their pre-pubertal period. The effect of age (linear regression) was significant on initial and final live-weight (P < 0.001), on allometric coefficient (P = 0.012) and on final BCS (P = 0.011), but not on daily growth rate and initial BCS.

The effect of breed, sex and age of lambs on slaughter data are summarized in Table 5.2. Respect to Alpagota, Brogna lambs were characterized by a smaller incidence of the pelt and by a tendency for a higher dressing percentage, while Foza lambs were heavier at slaughter and yielded heavier carcasses with comparable dressing percentages respect to the lambs of the other two breeds. Moreover, they were characterized by a higher incidence of feet and by lower incidences of gastrointestinal tract and offal. The males were heavier at slaughter (P < 0.05) but yielded carcasses not significantly different because of their lower killing out percentage. The tendency for a lower skin and liver was counterbalanced, in males, by higher incidence of genitals and of gastro-intestinal tract. Age influenced significantly the

weight of lambs at slaughter and of their carcasses, but also of their dressing percentage and incidence of feet and gastro-intestinal tract.

Table 5.3 shows the effects of breed, sex and age of lambs on some physical quality traits of two meat sample joints.

While pH and drip loss were not significantly affected by breed, cooking losses were greater for meat samples from Alpagota than from Brogna lambs and smaller for Foza meat samples from hind-leg but not from loin. Female lambs were characterized by lower pH, greater drip losses and smaller cooking losses, but only on meat samples from hind-legs, also because of the greater residual variability found on loin samples. The only effect exerted by age of lambs was on the cooking losses of *Quadriceps femoris*. Several authors observed that the effect of breed is often associated with differences in muscle distribution and consequently in the proportions of the various joints in the carcass (Santos-Silva et al., 2001). Breed effects on meat quality seems to be not important when referred to pH, amount of pigments, physical color, instrumental hardness and sensorial characteristics (Santos-Silva et al., 2002) but is important for the evolution of sensory traits that were not reported in this paper (Solomon et al., 1980; Arsenos et al., 2002; Martìnez-Cerezo et al., 2005). Lind et al. (2011), results support the hypothesis that the difference for meat quality between different breeds could be small when lambs are slaughtered at equal degrees of maturity. The most important differences often found in literature are referred to color and texture and can be justified by differences precociousness or in the muscularity degree (Sanudo et al., 1998).

6.5 CONCLUSIONS

In conclusion the three Alpine breeds of the Veneto Region confirmed to be able to produce lamb carcasses and meat with valuable characteristics that can be exploited through typical products and food preparation in local markets and gastronomy, according to tradition. The valorisation of these productions can be an important tool for the *in situ* conservation of these breeds. As an example of that, Slow Food organization has recognized “Agnello Alpagoto” (lambs of Alpagota breeds) as a Slow Food Presidium. Moreover the three breeds confirmed also some differences among them. In particular Foza breed is characterized by a large size and growth rate, a lower incidence of gastro-intestinal tract and by lower cooking losses of the hind-leg samples compared to the other two middle-sized breeds. Alpagota breed tended to be leaner, with heavier shin and higher cooking losses than Brogna breed. These

differences evidenced some peculiarity of the three breeds that can be of value for their possible use in different segments of the food-service chain.

Table 6.1: Least square means (LSM) of breed and sex and significance of breed contrasts and of initial age covariate on average growth rate, allometric coefficient and BCS of Alpagota (A), Foza (F) and Brogna (B) lambs.

	Breed LSM			Breeds contrasts (*P*)		Sex LSM		Age (*P*)	RMSE
	A	F	B	A*vs*B	F*vs*(A+B)	M	F		
Live weight (kg)									
Initial	17.3	25.0	20.6	0.083	<0.001	21.7	20.3	< 0.001	3.60
Final	25.8	35.9	28.9	n.s.	<0.001	31.8 [b]	28.6 [a]	<0.001	4.17
Growth rate (g/d)	75.7	96.8	74.6	n.s.	0.066	90.9	73.7	n.s.	25.7
Allometric coef.	1.14	1.11	1.12	n.s.	n.s.	1.13	1.12	0.012	0.04
BCS (1-5)									
Initial	2.65	3.31	3.14	0.002	0.002	3.00	3.07	n.s.	0.28
Final	3.02	3.25	3.28	0.019	n.s.	3.23	3.14	0.011	0.22

a, b P<0.05

Table 6.2: Least square means (LSM) of breed and sex and significance of breed contrasts and of initial age covariate on slaughter traits of Alpagota (A), Foza (F) and Brogna (B) lambs.

	Breed LSM			Breed contrasts (P)		Sex LSM		Age (P)	RMSE
	A	F	B	A*vs*B	F*vs*(A+B)	M	F		
Slaughter-wt, kg	26.17	36.24	28.95	n.s.	<0.001	32.18[b]	28.73[a]	<0.001	4.41
Skin, %	13.84	12.80	11.48	0.009	n.s.	12.2[α]	13.2[β]	n.s.	1.67
Head, %	6.07	5.98	6.00	n.s.	n.s.	5.91	6.11	n.s.	0.36
Feet, %	2.24	2.62	2.20	n.s.	<0.001	2.37	2.34	0.023	0.17
Gastroint. tract, %	30.72	27.53	30.15	n.s.	0.006	30.48[b]	28.45[a]	<0.001	2.19
Offal[1], %	2.55	2.26	2.52	n.s.	0.024	2.37	2.51	n.s.	0.27
Liver, %	1.41	1.34	1.34	n.s.	n.s.	1.34[α]	1.38 [β]	0.097	0.06
Genitals, %	0.39	0.37	0.47	n.s.	n.s.	0.69[B]	0.13 [A]	n.s.	0.23
Dressing, %	41.08	43.65	42.64	0.083	n.s.	41.64[A]	43.28[B]	<0.001	1.63
Carcass wt:									
hot, kg	10.75	15.87	12.46	n.s.	<0.001	13.51	12.54	<0.001	2.05
cold, kg	10.09	14.60	11.74	0.090	<0.001	12.84	11.90	<0.001	1.98

α, β P<0.10; a, b P<0.05; A, B :P<0.01

[1]: trachea, lungs, heart and spleen.

Table 6.3: Least square means (LSM) of breed and sex and significance of breed contrasts and of initial age covariate on meat pH, drip and cooking losses of meat from Alpagota (A), Foza (F) and Brogna (B) lambs.

	Breed LSM			Breed contrasts (*P*)		Sex LSM		Age (*P*)	RMSE
	A	F	B	A*vs*B	F*vs*(A+B)	M	F		
pH:									
loin	5.61	5.63	5.64	n.s.	n.s.	5.67^{b}	5.58^{a}	n.s.	0.12
hind-leg	5.50	5.56	5.54	0.097	0.089	5.53	5.53	n.s.	0.05
Drip loss (%):									
loin	1.43	1.26	0.91	n.s.	n.s.	0.98	1.41	n.s.	1.28
hind-leg	0.47	0.53	0.63	n.s.	n.s.	0.32^{a}	0.76^{b}	n.s.	0.49
Cooking loss (%):									
Longissimus thoracis	26.7	24.1	23.1	0.006	n.s.	24.1	25.1	n.s.	2.39
Quadriceps femoris	40.7	37.8	39.0	0.034	0.005	39.9^{B}	38.4^{A}	0.012	1.53

a, b P<0.05; A, B :P<0.01

CHAPTER 7

Use of Ag^{+}HPLC for analyzing fatty acids including CLA isomers in beef fed *rp*CLA supplements

7.1 ABSTRACT

Aim of this study was to use the Silver Ion High Liquid Performance (Ag^{+}HPLC) to analyze CLA isomers content of 3 different tissues collected from young growing bulls (liver, subcutaneous fat and *Longissimus Thoracis*). The experimental design involved 9 crossbred young bulls fed with a supplementation of 0, 8 or 80 g/d of rumen protected CLA (*rp*CLA). Samples analyzed came from 3 tissues (liver, subcutaneous fat and *Longissimus Thoracis*). Data were analyzed using the MIXED procedure considering as fixed effects feeding system, tissue and repetitions. Considering the hierarchical structure of the experimental design, fixed effects was tested using different error lines: feeding system was tested using as error line, repetition (feeding system) and tissues on the residual error line. Effect of diet and tissue were important for CLA distribution suggesting that isomers has a tissue-depending distribution and CLA isomers composition can change in relationship to the *rp*CLA supplementation. In total 13 CLA isomers, belonging to the three regions (*cis*/*cis*, *trans*/*trans* and *cis*/*trans*), were identify. In muscle the most abundant are C18:2*cis*9,*trans*11, C18:2*trans*7,*cis*9 and C18:2*trans*10,*cis*12. In liver C18:2*cis*9,*trans*11, C18:2*trans*7,*cis*9, C18:2*trans*9,*trans*11 and CLA*trans10*,*cis*12. In subcutaneous fat are C18:2*cis*9,*trans*11, C18:2*trans*7,*cis*9 and C18:2*trans*10,*cis*12. The amount was different in relation to the diet and CLA supplementation, in muscle diet was significant for C18:2*trans*10,*cis*12 (P=0.02) and C18:2*trans*10,*trans*12 (P=0.07) with a higher amount in 80 g/d/animal. In liver diet influence C18:2*trans*7,*cis*9 (P=0.05) and C18:2*trans*10,*cis*12 (P=0.02) with a high amount of C18:2*trans*7,*cis*9 in 8 g/d/animal and of C18:2*trans*10,*trans*12 in 80 g/d/animal. Subcutaneous fat has a similar isomers distribution with a significant effect on C18:2*trans*10,*cis*12 (P<0.001) and the isomers of *trans*/*trans* region (C18:2*trans*9,*trans*11, C18:2*trans*10,*trans*12 and C18:2*trans8*,*trans*10) with an higher amount in 80 g/d/animal dose.

7.2 INTRODUCTION

Conjugated Linoleic Acid (CLA), is a collective term for indicate a mixture of geometric and positional isomers of Linoleic Acid with double bonds in different position on the carbon chain, known for their biological activity on human health and animal *performance* (Schmid et al., 2006; Park et al., 2007; Park et al., 1997; Pariza et al., 2001; Perfield II et al., 2002; Sinclar et al., 2007; Weerasinghe et al., 2011). The quantification of CLA isomers is of particular interest, but it is necessary make attention to evaluate a method that allows the

complete determination of CLA isomers. CLA determination is complex because of the presence of unsaturated double bonds that give an unstable nature, as easily subjected to epimerization and isomerization (Fuchs et al., 2011; Dance et al., 2010). The first critical step is the methylation because of base catalysis do not esterified free fatty acids (FFA) but avoid migration and isomerization of double bonds. On the contrary acid catalysis esterified all complex and simple forms of FA causing isomerization of conjugated double bonds. To overcome this problem of the methylation Jenkins (2010) proposed a modification of Sukhija and Palmquist (1988) method given by a combination of acid and base catalyst for shorter incubations times minimizing the problems given by other methods (Jenkins,2010; Sukhija and Palmquist,1988). Another problem is given by the chromatography technique used, because often the most common is not the one that gives the most optimal results. A number of methods have been developed for analysis of octadecenoic fatty acids (FA) in food such as infrared spectroscopy (FTIR), gas chromatography (GC) combined with flame ionization detector (FID) or mass spectrometry (MS) and silver ion high performance liquid chromatography (Ag^+HPLC) or reverse phase HPLC (Villegas et al., 2010). Considering the gas chromatography (GC), often the single GC, using the currently available columns, is not always the best option to identify components from natural samples. In fact, conjunction with mass spectroscopy (MS) detector permit more fatty acids and isomers to be separated, as happened with Linoleic Acid but sometimes is not possible to achieve the separation of all the fatty acid isomers as in the case of Linolenic Acid (Manzano et al., 2011). Gas Chromatography (GC) is the most popular and often the only methods used, for the analysis of fatty acids and for CLA, but the information on CLA isomeric composition gave by GC are incomplete. Normally the various types of geometrical isomers give distinct peaks but in this group, positional isomers are not completely resolved. GC well separate the two main isomers, but not the other positional isomers. The reason of this lack of capacity are that information on the double bonds position is usually not directly available and identifications are limited to comparison of retention times with a limited number of internal standard (Roach et al., 2002). Despite the long column, is difficult obtain a good separation and reproducibility of isomers and a single run give an approximate idea of the total content of CLA relative to other components. One of the reasons is that when CLA is added in nutritional experiments, other FA such as, C21:0 and C20:2 can be eluting in the same region of the chromatogram as the *cis*/*cis* and *trans/trans* isomers (Christie et al., 2001). The most important component of the GC, that allows a clear definition of the peaks, is the column used. Many authors observed that consider a long column is very important for require a

resolution of CLA isomers. In conclusion is possible say that the GC exhibits rapidity, high resolution and sensitivity however a complete separation of all CLA isomers is not yet achievable and it is recognized that the combination of methods will be required to provide the full isomeric distribution of sample (Aldai et al., 2005). The suitable separation of CLA isomers present in biological tissues is not possible by GC, for this reason the complementary use of Ag^{+}HPLC is currently the most effective way to separate and quantitate individual isomers of CLA in beef (Roach et al., 2002; Nuernberg et al., 2007). Ag^{+}HPLC has been one of the most important techniques available to lipid analysis for the separation of molecular species of lipids since 1974. It can separate FA according to the configuration and the number of their double bonds and also according to the position of the double bonds (Fritsche and Steinhart, 1998).CLA FAME were detect thanks to their characteristic UV absorbance at 233nm. The identities of the isomers in HPLC chromatograms are based on co-injections of known reference materials obtained from commercial sources or synthesized. The problem of this technique is that only a limited number of CLA isomers were available and the pure isomers are C18:2*cis*9,*trans*11, C18:2*trans*10,*cis*12, C18:2*cis*9,*cis*11 and C18:2*trans*9,trans11. Also in this technique the column is very important, the use of three columns increase the resolution of the peaks but in the contrary more than three columns in series provide a decrease of the benefits (Roach et al., 2002). Considering the importance of CLA and the problems related to their determination, the aim of this study was to analyze through Ag^{+}HPLC, CLA isomers in three different tissues (liver, subcutaneous fat and *Longissimus Thoracis*) taken from beef fed with "low protein" diet and three different *rp*CLA dose.

7.3 MATERIAL AND METHODS

The present project followed the Guideline for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Consortium, 1988) and all the experimental procedure were approved by the Ethical Committee for the Care and Use of Experimental Animals of the University of Padova (CEASA, Legnaro, Italy).

Animal breeding and diets

The trial was carried out at "Lucio Toniolo", the Experimental Farm of the University of Padova in Legnaro (Padova, Italy). Fifty-four crossbred young bulls and heifers were used. For the first 28 days of adaption animals were fed with an adaptation diet, composed only of

hay but after this period animals of each group were progressively fed with one of 3 experimental diets.

Experimental diets were fed *ad libitum* and total mixed ration containing 108 g/kg DM of CP, 35 g/kg of FA and supplemented with 0, 8 or 80 g/d of rumen protected CLA (*rp*CLA) supplement from 5 to 16 months of age (18 animals for each *rp*CLA dose) and they consumed 9.3 kg/d of DM on average. The total mixed ration was composed, on DM basis, of corn meal (400 g/kg), corn silage (276 g/kg), soybean meal (33 g/kg), dried sugar beet pulp (113 g/kg), wheat bran (70 g/kg), wheat straw (66 g/kg), vitamin and mineral mixture (26 g/kg), calcium soap (9 g/kg), and hydrogenated soybean oil (7 g/kg). The *rp*CLA supplement consisted of methyl esters of CLA bound to a silica matrix and coated with hydrogenated soybean oil. The lipid-coated *rp*CLA was composed of 800, 178, and 22 g/kg of lipid, ash, and moisture, respectively, and 456 g/kg of palmitic and stearic acids, 79.2 and 76.8 g/kg of C18:2*cis*9,*trans*11 and C18:2*trans*10,*cis*12, respectively, and 91 g/kg of other FA (SILA, Noale,Italy). A detailed description of the chemical composition of the *rp*CLA used is given in Schiavon et al. (2011).

Muscle, fat and liver collection

The trial started in July 2010 ended in March 2011 for heifers and May 2011 for bulls, when all animals were slaughtered at the mean weight of 512.4 ± 64.3 Kg for heifers and 646.5 ± 47.46 Kg for bulls. At the end of fattening the calves were fasted for one day and then slaughtered. According to the reference meat market, heifers or bulls were slaughtered when they reached an estimated *in vivo* fatness score around 3 or 2 points on a scale from 1 (very lean) to 5 (very fat), respectively (Schiavon et al., 2013). Animals were slaughtered outside the faculty in a slaughterhouse located in Pergine (Province of Trento, Trentino Alto Adige Region, North Italy). Immediately after slaughter, liver was collected from each animal and stored at -20°C in the Department of Agronomy, Food, Natural Resources, Animals and Environment (DAFNAE) for further analysis on fatty acids profile. Twenty-four hours after slaughter from the left half part of the carcass the whole cut of the 5th rib was collected (from the cranial edge of the 5th rib to the cranial edge of the 6th rib). The entire rib was vacuum packed, moved to the Laboratory and aged at 4 °C in a chilling room for 10 days. At the end of ageing, the rib cut was dissected into muscles (*Longissimus Thoracis*, and Other Muscles), fat (Subcutaneous Fat) and bones. Each fraction was weighted. Therefore samples used for these investigations were choosing randomly between 9 bulls belonging to each of the three

diets: 9 of *Longissimus Thoracis* samples, 9 of Subcutaneous Fat and 9 of Liver. Part of these samples collected in Italy was sent to the Leibniz Institute of Farm Animal Biology (Dummerstorf, Germany) for CLA isomers analysis.

Lipid extraction and transesterification (FAMEs) for Ag^+-HPLC

This part of the experiment was carried out at Leibniz Institute of Farm Animal Biology (Dummerstorf, Germany). Lipid extraction was made according to Folch (1957). Briefly, samples of *Longissimus Thoracis*, Subcutaneous Fat and Liver were thawed at 4°C. After homogenisation (Ultra Turrax, IKA, Staufen, Germany; T25, 3 x 15 sec, 12,000 rpm) and the addition of the fatty acid C19:0 as an internal standard, the total lipids were extracted in duplicate using chloroform/methanol (2:1, v/v) at room temperature. The detailed sample preparation procedure has been previously described by Nuernberg et al. (2011). The lipid extracts were re-dissolved in 300 µL of toluene, and a 25 mg aliquot was used for methyl ester preparation. Next, 2 ml of 0.5 M Sodium Methoxide in methanol (Fluka, Buchs, Switzerland) was added to the samples, which were shaken in a 60 ºC water bath for 10 minutes. Subsequently, 1 ml of 14% Boron Trifluoride (BF_3) in methanol (Sigma-Aldrich, Deisenhofen, Germany) was added to the mixture, which was then shaken for an additional 10 minutes at 60 ºC. Saturated $NaHCO_3$ (2 ml) solution was added, and the fatty acid methyl esters (FAMEs) were extracted three times in 2 ml of *n*-hexane. The solvent containing the FAMEs was reduced to dryness under an oxygen-free nitrogen stream, and the FAMEs were re-suspended in *n*-hexane and stored at -18 ºC until used for Silver-ion HPLC/DAD analysis. Identification and quantification analysis of the CLA isomers in Muscle, Subcutaneous Fat and Liver extracts of the bulls was performed by Ag^+-ion HPLC involved an HPLC system (LC 10A, Shimadzu, Japan) equipped with a pump (LC-10AD VP), auto sampler (SIL-10AF), 50 µL injection loop, a photodiode array detector (SPD-M 10Avp, Shimadzu, Japan), and a Shimadzu CLASS-VP software system (Version 6.12 SP4). Four ChromSpher 5 Lipids analytical silver ion-impregnated columns (4.6 mm i.d. × 250 mm stainless steel; 5 µm particle size; Agilent, USA) were used in series. The mobile phase (0.1% acetonitrile in *n*-hexane) was prepared fresh daily and pumped at a flow rate of 1.0 mL/min as described by Nuernberg et al. (2011). The injection volume varied between 20 and 50 µL, according to the content of minor CLA isomers in the different adipose tissues. The detector was operated at 233 nm to identify CLA isomers based the measurement of integrated area under the 233 nm peaks attributed to conjugated dienes. The identification of CLA isomers was made by the

retention time of individual CLA methyl esters (C18:2*cis*9,*trans*11, C18:2*trans*9,*trans*11, C18:2*trans*10,*cis*12, C18:2*cis*9,*trans*11, C18:2*cis*9,*cis*11 and C18:2*cis*11,*trans*13). The external calibration plots of the standard solutions were adapted to different concentration levels of individual CLA isomers in the lipid extracts, recently in detail described (Nuernberg et al., 2011; Shen et al., 2011).

Reagents

A reference standard 'Sigma-FAME mixture' was obtained from Sigma-Aldrich (Deisenhofen, Germany). Additionally, individual isomers of CLA methyl esters (CLA-ME), C18:2*cis*9,*trans*11, C18:2*trans*9,*trans*11, C18:2*trans*10,*cis*12, C18:2*cis*9,*cis*11 and C18:2*cis*11,*trans*13 (as a free fatty acid) were purchased from Matreya (Pleasnet Gap, United States). All solvents used for Ag^{+}-HPLC were HPLC grade from AppliChem (Darmstadt, Germany). The derivatization reagents, sodium methylate and borontrifluoride/methanol (14 % w:v), were respectively obtained from Fluka (Buchs, Switzerland) and from Sigma-Aldrich (Deisenhofen, Germany).

Statistical analysis

The statistical analysis was performed using the MIXED procedure (SAS, Institute Inc., Cary, NC). The main sources of variation of the 13 isomers were considered. Feeding system, tissue and repetitions were considered as fixed effects. Considering the hierarchical structure of the experimental design, fixed effects was tested using different error lines. The effect of feeding system was tested using as error line, repetition (feeding system). Besides, a tissue was tested on the residual error line.

7.4 RESULTS

Tables 1, 2 and 3 shows the distribution of the CLA isomers in the three tissues subjected to analysis (respectively, *Longissimus Thoracis,* Liver and Subcutaneous Fat). All the data are expressed in mg/g of tissue and the order in which they are report in tables is in accord to their amount in samples, from the most abundant to the least abundant. Firstly, was report the sum of CLA isomers identify and after that the individual isomers. In total 13 isomers are identify.

In Table 1 are report CLA isomers of *Longissimus Thoracis.* The most abundant isomers are C18:2*cis*9,*trans*11, C18:2*trans*7,*cis*9 and C18:2*trans*10,*cis*12 normally known to be the most important in beef and milk products from ruminants. C18:2*cis*9,*trans*11 amount is higher in diet with 8 g/d/animal (LSM=2.08) and C18:2*trans*10,*cis*12 in diet with 80 g/d/animal (LSM=0.30), besides C18:2*trans*7,*cis*9 is higher in diet with 0 g/d/animal (LSM=0.46). Observing the results of the statistical analysis and the column with the *P*-value of the feeding system (FS), no significant effect of the diet is observed. Therefore, two isomers are on the threshold of significance C18:2*trans*10,*cis*12 and C18:2*trans*10,*trans*12 that present a *P*-value respectively of P=0.02 and P=0.07. Observing the orthogonal contrasts between diets, C18:2*trans*10,*cis*12 and C18:2*trans*10,*trans*12 are on the threshold of significance with *P*-value respectively P=0.01 and P=0.05.

In Table 2 are report CLA isomers of Liver with a different order if compare to *Longissimus Thoracis*. The different order is due to the different isomers distribution and the tissues specific distribution of them. The most abundant are the C18:2*cis*9,*trans*11, C18:2*trans*7,*cis*9, C18:2*trans*9,*trans*11 and CLA*trans10,cis*12. Observing the diet (FS), no significant effects are report, but C18:2*trans*7,*cis*9 and C18:2*trans*10,*cis*12 are on the threshold of significance with respectively P=0.05 and P=0.02. Observing the orthogonal contrast there are only some isomers on the threshold of significance: C18:2*trans*7,*cis*9, C18:2*trans*10,*cis*12, C18:2*cis*12,*trans*14 and C18:2*trans*8,*trans*10. Normally, the effect of diet is observe in the contrast 8 *vs* (0.9thesis0+0.1thesis80) excluding C18:2*trans*10,*cis1*2 where significance is on both contrasts. From the contrast 8 *vs* (0.9thesis0+0.1thesis80) is observe an high amount of these isomers in the diets with 8 g/d/animal (C18:2*trans*7,*cis*9, C18:2*cis*12,*trans*14 and C18:2*trans*8,*trans*10), but for C18:2*trans*10,*cis*12 the higher amount is observe in the 80 g/d/animal supplementation, as one of the main components of the supplementation.

In Table 3 are report CLA isomers of Subcutaneous Fat. The order is different from the other two tissues because of the tissue's isomer dependency and the amount is higher than in the other two tissues. The major isomer is C18:2*cis*9,*trans*11 follow by C18:2*trans*7,*cis*9 and C18:2*trans*10,*cis*12. Diet results highly significant (*P*<0.001) for the C18:2*trans10*,*cis*12, significant (*P*<0.01) for the C18:2*trans*9,*trans*11 and on the threshold for C18:2*trans*10,*trans*12 and C18:2*trans*8,*trans*10 (P=0.01). Observing the contrast between the different supplementation, as expected C18:2*trans*10,*cis*12 is highly significant (*P*<0.001) and also C18:2*trans*9,*trans*11 and C18:2*trans*10,*trans*12 (*P*<0.01). From the contrast it has been deduced that C18:2*trans10*,*cis*12 is higher in 80 g/d/animal supplementation (LSM=2.55) as

consequence of supplementation and its composition. In fact, this isomer is one of the main components of the *rp*CLA supplementation that

consisted of methyl esters of CLA bound to a silica matrix and coated with hydrogenated soybean oil (Palmitic, Setaric Acid, C18:2*cis*9,*trans*11 and C18:2*trans*10,*cis*12). C18:2*trans*9,*trans*11 and C18:2*trans*10,*trans*12 are higher in 80 g/d/animal supplementation (respectively, LSM=0.48 and LSM=0.36). C18:2*trans*8,*trans*10 is higher in 80 g/d/animal supplementation (LSM=0.06).

7.5 DISCUSSION

According to the bibliography references, CLA isomers composition change in relation to different factors, such as, diet, tissue, methods of analysis, gender, breed and repetition. Significant effect of the tissue was observe and the tissues specific distribution between fat, liver and muscle has been demonstrated (Hoehne et al., 2012; Jiang et al., 2013). As report by Jiang et al (2013) and confirm from these results the concentration of CLA is higher in the Subcutaneous Fat (ΣCLA=29.4) if compared to *Longissimus Thoracis* (ΣCLA=3.01) and Liver (ΣCLA=1.39). Regarding the single isomers and in particular the main components of the supplementation, the amount of C18:2*cis*9,*trans*11 is higher than C18:2*trans*10,*cis*12 in all tissues. As reported by Cordero et al. (2010), pig feed with 1% supplementation involves in a higher accumulation of C18:2*cis*9,*trans*11 if compare to C18:2*trans*10,*cis*12 both in *Longissimus Thoracis* and Subcutaneous Fat. Nuerberg at al. (2002) report that Subcutaneous Fat show an higher concentrations of C18:2*cis*9,*trans*11 if compared to Intramuscular Fat ($P<0.01$). Whereas the opposite was observed for C18:2*trans*10,*cis*12 ($P<0.01$) (Nuerberg at al.,2002; Schiavon et al., 2011).The reason of the higher concentration of C18:2*cis*9,*trans*11 in lipid tissues is because fat tissue is rich in neutral lipids. Observing differences between muscle and fat was observe that in muscle the percentage of CLA is lower than in fat (7.72% subcutaneous fat *vs* 2.64% *Longissumus Thoracis* muscle). Not only between muscle and fat there are differences, but also between different fat tissues (for example kidney fat is totally different from subcutaneous fat) because of the anatomical location and its lipids composition (Jiang et al., 2013). No particular effect of liver has been reported in this study because of the amount of CLA isomers in liver is lower than in muscle. Regarding liver, Tous et al. (2013) observe that supplementation with *rp*CLA can increase the liver weight and the metabolic potential process. In general the different composition between tissues confirm that the lymphatic recovery of Linoleic acid (LA) is

higher than CLA and that not all the isomers are absorbed in the same amount, for example the most preferentially absorbed are C18:2*trans*9,*trans*11 and C18:2*trans*10,*trans*12 (Sugano et al., 1997). It was estimate that rumen CLA synthesis was 4 to 7% and the rest part derived through endogenous synthesis, despite, not many studies were report to investigate the importance of endogenous synthesis in the adipose tissue of ruminant muscles (Dannenberger et al., 2005). Effect of the diet is important because the CLA isomers composition can change in relationship to the *rp*CLA supplementation. The reason of the use of rumen protected CLA is linked to increase the passage of CLA into the duodenum, protecting against rumen biohydrogenation (Albertì et al., 2013). Scarce information are available about the influence of *rp*CLA on CLA content in beef, as example, in this trial it is observed, that not all the isomers are higher in the diet with supplementation 80 g/d/animal, but some are higher in the supplementation 8 g/d/animal. From results of this trial, *rp*CLA supplementation influences mainly the two main isomers C18:2*cis*9,*trans*11 and C18:2*trans*10,*cis*12, otherwise, Poulson et al.(2004) found that *rp*CLA mixture increase C18:2*trans*10,*cis*12 but not C18:2*cis*9,*trans*11. In Schiavon et al. (2011) was found that supplementation of *rp*CLA increased the C18:2*trans*10,c*is*12 isomer on average by +5.6 times with respect to HSO (Hydrogenate Soybean Oil) and when the CLA mixture was included in the rations the concentration of this isomer is increased with respect to control much more in muscles (+20.4 times), than in subcutaneous fat (CF) and intramuscular (IF) (where this increase respectively of +0.2 and +0.9 times). This shows that C18:2*trans*10,*cis*12 isomer present in the tissues seem to originate only from the gastrointestinal tract.

From the analytical point of view It has been demonstrated that the use of different extraction methodology results in different lipid recoveries in biological samples. The main problem in this step are due to the incomplete extraction which means an underestimation of CLA isomers and an increase of the proportions of *trans*/*trans* isomers. The analytical methodology for CLA isomers are important now that are recognized their various effect in biological system. To overcome isomerization and epimerization problems, in this study, is used Folch (1957) for the extraction and an acid-base methylation characterized by different mixture of solvents if compare to Jenkins, (2010). Regarding, the chromatography technique one dimensional GC and two dimensional GCxGC may have some limitation in CLA isomers analysis (Jover et al., 2005; Manzano et al.,2012) and they are considered not suitable for a complete separation. It is recognized that the combination of methods will be required to provide the full isomeric distribution (Aldai et al., 2005). With Ag^{+}HPLC CLA FAME are selectively detected by their characteristic UV absorbance at 233 nm and isomer identities in

HPLC chromatograms are based on co-injections of known reference materials obtain from commercial sources of synthesized. The quantification is based on the measurement of the integrated area under the peaks (Rodríguez-Castañedas et al., 2011). The use of three columns in series increase the resolution of this system allows a clearly separation of the four isomers C18:2*cis*9,*trans*11, C18:2*trans*10,*cis*12, C18:2*cis*11,*trans*13, and C18:2*trans*8,*cis*10 that are found in a commercial CLA preparation, plus the identification of the *trans*/*trans* and *cis*/*trans* groups. In our study the number of columns used are three because the use of more than three columns in series provide diminishing benefits (Roach et al., 2002; Kramer et al.,1998). As report by Sehat et al. (1999), the importance of use column in series is that in the two C18:2 11,13 geometric isomers are clearly resolved.

7.6 CONCLUSIONS

CLA content, as the fatty acid profile, can change according to many effects. Diets and tissue are probably the most important and as report in this trial CLA distribution is tissue-specific and highly dependent on the diet. *rp*CLA supplementation increase CLA isomers content but in particular increase the amount of the two main isomers that are also main components of the supplementation (C18:2*cis*9,*trans*11 and C18:2*trans*10,*cis*12). The different distribution among tissue is related to the different capacity of the tissue to absorb CLA isomers. Subcutaneous fat is the tissue with the high amount of CLA if compare to the others two and no differences where identify between muscle and liver. The knowledge obtained in this experiment are linked to the use of a different chromatography technique. The most common is the two dimensional GC (GCxGC) a technique with an high resolution power, able to separated fatty acids according to their carbon chain length but not able to identify more than the two main CLA isomers (C18:2*cis*9,*trans*11 and C18:2*trans*10,*cis*12). Actually this method permit to separate 5 CLA isomers according to the number of pure isomers use as references standards. Otherwise, Silver Ion High Performance Liquid Chromotography (Ag^+HPLC) identify 13 CLA isomer belonging to the three regions *cis*/*cis*, *trans*/*trans* and *cis*/*trans.* In this situation is possible separated not only the main isomers but also the minor with only a problem in the region of *cis*/*cis* due to a lack of references. In conclusion is possible to say that analysis of the complete fatty acids profile and CLA isomers are very important, because of the biological activity of the different groups (Saturated, Monounsaturated and Polyunsaturated) and CLA isomers. However, it is necessary linked the

common analytical gas-chromatography to others, such as, liquid-chromatography to have a complete knowledge of fatty acids profile and the content of CLA isomers.

Table 7.1: CLA isomers distribution in *Longissimus Thoracis* (LT). Effect of Feeding System (FS, 0, 8, 80 g/d/animal) on their distribution (CLA isomers are report according to the amount, from the most abundant to the least abundant). All the data are express in mg/g tissue.

	Feeding System			***P*[2]**		**FS *P***	**Animal Effect**	**RMSE**
	0[1]	**8[1]**	**80[1]**	**0 vs 80**	**8 vs (0.9 thesis0+0.1thesis80)**			
ΣCLA[3]	3.24	2.59	3.20	0.96	0.44	0.69	929837	141659
C18:2*c*9,*t*11	2.50	2.08	2.26	0.71	0.53	0.80	736	267
C18:2*t*7,*c*9	0.46	0.30	0.42	0.72	0.20	0.41	127	87.0
C18:2*t*10,*c*12	0.09	0.07	0.30	0.01	0.47	0.02	74.0	23.0
C18:2*t*11,*c*13	0.05	0.04	0.04	0.32	0.42	0.54	16.8	10.2
C18:2*t*9,*t*11	0.04	0.03	0.06	0.35	0.42	0.29	17.2	5.62
C18:2*t*8,*c*10	0.03	0.02	0.03	0.97	0.14	0.24	4.97	10.4
C18:2*t*10,*t*12	0.02	0.02	0.05	0.05	0.80	0.07	14.8	3.75
C18:2*t*7,*t*9	0.01	0.01	0.01	0.61	0.34	0.60	3.27	2.69
C18:2*c*11,*t*13	0.01	0.01	0.01	0.51	0.29	0.53	2.44	2.05
C18:2*t*11,*t*13	0.01	0.01	0.01	0.64	0.97	0.86	3.59	1.28
C18:2*c*12,*t*14	0.01	0.01	0.01	0.33	0.70	0.42	1.61	1.75
C18:2*t*8,*t*10	0.01	0.004	0.004	0.38	0.21	0.38	0.47	1.66
C18:2*t*12,*t*14	0.004	0.002	0.005	0.48	0.41	0.38	1.66	0.86

[1]:*rp*CLA supplementation (g/d/animal)

[2]: Orthogonal Contrast between diet.

[3]: ΣCLA= C18:2*c*9,*t*11+ C18:2*t*7,*c*9+ C18:2*t*10,*c*12+ C18:2*t*11,*c*13+ C18:2*t*9,*t*11+ C18:2*t*8,*c*10+ C18:2*t*10,*t*12+ C18:2*t*7,*t*9+ C18:2*c*11,*t*13+ C18:2*t*11,*t*13 + C18:2*c*12,*t*14+C18:2*t*8,*t*10+ C18:2*t*12,*t*14

Table 7.2: CLA isomers distribution in Liver. Effect of Feeding System (FS, 0, 8, 80 g/d/animal) on their distribution (CLA isomers are report according to the amount, from the most abundant to the least abundant). All the data are express in mg/g tissue.

	Feeding System			*P*[2]		FS	Animal	RMSE
	0[1]	8[1]	80[1]	0 vs 80	8 vs (0.9 thesis0+0.1thesis80)	*P*	Effect	
ΣCLA[3]	1.05	1.77	1.31	0.55	0.13	0.29	337	542
C18:2*c*9,*t*11	0.84	1.35	1.01	0.64	0.18	0.38	272	441
C18:2*t*7,*c*9	0.07	0.20	0.07	0.98	0.03	0.05	38.8	51.4
C18:2*t*9,*t*11	0.04	0.04	0.04	0.86	0.98	0.98	7.00	17.9
C18:2*t*10,*c*12	0.03	0.08	0.11	0.01	0.07	0.02	18.6	25.9
C18:2*t*11,*c*13	0.02	0.03	0.02	0.59	0.16	0.34	7.78	11.5
C18:2*t*8,*c*10	0.02	0.02	0.02	0.22	0.51	0.44	0.00	10.0
C18:2*t*7,*t*9	0.01	0.01	0.01	0.43	0.54	0.68	3.47	4.50
C18:2*t*10,*t*12	0.01	0.01	0.01	0.84	0.29	0.52	2.36	4.00
C18:2*c*11,*t*13	0.005	0.01	0.01	0.64	0.12	0.27	1.23	2.73
C18:2*c*12,*t*14	0.003	0.01	0.003	0.95	0.06	0.12	0.96	1.95
C18:2*t*11,*t*13	0.002	0.003	0.003	0.38	0.38	0.56	0.19	1.29
C18:2*t*8,*t*10	0.001	0.004	0.003	0.26	0.05	0.13	0.57	2.08
C18:2*t*12,*t*14	0.000	0.001	0.001	0.32	0.12	0.26	0.00	0.50

[1]: *rp*CLA supplementation (g/d/animal).

[2]: Orthogonal Contrast between diet.

[3]: ΣCLA= C18:2*c*9,*t*11+ C18:2*t*7,*c*9+ C18:2*t*9,*t*11+C18:2*t*10,*c*12+ C18:2*t*11,*c*13+ C18:2*t*8,*c*10+ C18:2*t*10,*t*12 + C18:2*c*11,*t*13+ C18:2*c*12,*t*14+C18:2*t*11,*t*13+C18:2*t*8,*t*10+ C18:2*t*12,*t*14.

Table 7.3: CLA isomers distribution in Subcutaneous Fat. Effect of Feeding System (FS, 0, 8, 80 g/d/animal) on their distribution (CLA isomers are report according to the amount, from the most abundant to the least abundant). All the data are express in mg/g tissue.

	Feeding System			*P*[2]		FS	Animal	RMSE
	0[1]	8[1]	80[1]	0 vs 80	8 vs (0.9 thesis0+0.1thesis80)	*P*	Effect	
ΣCLA[3]	21.8	33.6	32.8	0.16	0.16	0.24	5782	8769
C18:2*c*9,*t*11	17.4	26.6	24.8	0.26	0.19	0.33	5359	7063
C18:2*t*7,*c*9	2.74	3.91	3.49	0.20	0.07	0.16	0.00	908.3
C18:2*t*10,*c*12	0.42	0.68	2.55	<0.001	0.79	<0.001	161.0	245.2
C18:2*t*11,*c*13	0.38	0.47	0.36	0.85	0.46	0.64	0.00	199.1
C18:2*t*8,*c*10	0.26	0.49	0.36	0.47	0.14	0.30	131.5	136.8
C18:2*t*9,*t*11	0.22	0.31	0.48	<0.01	0.18	<0.01	0.00	82.6
C18:2*t*10,*t*12	0.09	0.18	0.36	<0.01	0.31	0.01	0.00	95.8
C18:2*c*11,*t*13	0.08	0.40	0.07	0.95	0.19	0.32	267.5	54.6
C18:2*t*7,*t*9	0.08	0.24	0.13	0.69	0.18	0.38	56.6	173.3
C18:2*c*12,*t*14	0.07	0.20	0.07	1.00	0.35	0.56	167.6	26.6
C18:2*t*11,*t*13	0.04	0.05	0.06	0.19	0.84	0.36	0.00	17.1
C18:2*t*8,*t*10	0.04	0.04	0.06	0.01	0.71	0.01	0.00	10.8
C18:2*t*12,*t*14	0.02	0.02	0.02	0.15	0.21	0.26	1.77	5.88

[1]: *rp*CLA supplementation (g/d/animal).

[2]: Orthogonal Contrast between diet.

[3]: ΣCLA= C18:2*c*9,*t*11+ C18:2*t*7,*c*9+ C18:2*t*10,*c*12+ C18:2*t*11,*c*13+ C18:2*t*8,*c*10+C18:2*t*9,*t*11+ C18:2*t*10,*t*12+C18:2*c*11,*t*13+C18:2*t*7,*t*9+C18:2*c*12,*t*14+C18:2*t*11,*t*13+ C18:2*t*8,*t*10+ *t*14C18:2*t*12,*t*14.

General Conclusions

As the title suggest the main purpose of this thesis was study Conjugated Linoleic Acid (CLA) content in animal, belonging to different breeds (beef and lambs), fed with rumen protected CLA supplementation (*rp*CLA). The study of CLA is very difficult, due to the presence of double bonds that make them unstable and more frequently subject to isomerization and epimerization. The topic of my thesis was thought considering the previous results on CLA for study in deep this important molecules paying attention to their effects on animal *performance* and the effects that can influences their amount in ruminant products (meat, milk and dairy products). As saw before the general objectives are divided as follow: the methodological approach, the study of the sources of variation on CLA isomers and fatty acids profile and the effect of *rp*CLA on animal performance.

Firstly was evaluate the importance of the method of extraction comparing three different methods in terms of mean, repeatability, and variance homoscedasticity, of the measures of single and groups of FA in 3 tissues collected from young growing bulls (liver, subcutaneous fat and muscle). In this first part only two dimensional gas-chromatography (GCxGC) was use for analyze fatty acids and it is found to be not the suitable method for CLA isomers. Initially, this technique identifies only the two main isomers by reference standards and one by position (C18:2*cis*9,*trans*11, C18:2*trans*10,*cis*12 by references standards and C18:2*trans*11,*cis*13 by position), but was not able to identify the minor isomers. After that, our purpose was to increase the power resolution of our GCxGC increasing CLA isomers identify by references standards. Actually, with five pure isomers as reference it is able to identify more than the two most important (C18:2*cis*9,*trans*11, C18:2*trans*10,*cis*12, C18:2*trans11*,*cis*13, C18:2*cis*9,*cis*11 and C18:2*trans*9,*trans*11). Despite, the importance and the high resolution power of our two dimensional GC, the best method for CLA isomers was the Silver Ion High Performance Liquid Chromatography (Ag^{+}HPLC). The second step was used Ag^{+}HPLC for analyzed CLA isomers with the objective to identify the 24 possible isomers normally present in matrix samples. Method, in particular extraction and methylation, was considered as a source of variation due to the potential effect that it can have on CLA content. As consequence, we try to increase the knowledge about the sources of variation, considering that method is not the only one. There are other many effects directly connected to animals that can change the FA profile and CLA content.

The main results obtain from my thesis are briefly report below. It was observe that many effects can influence FA profile, such as; methods and tissues. FA and CLA are tissues-

specific. Method should be chose in relation to the type of samples analyze and in my experiment is seen that many methods can give undetectable value. The number of this value is 5, 14 and 9 respectively in liver, subcutaneous fat and *Longissimus Thoracis*. The incidence of undetectable values on the total number of expected observation, which depend on the sensitivity of the method used was calculated for liver, fat and muscle, ranged 0.04 to 0.08, 0.05 to 0.06, and 0.05 to 0.12, respectively, with incidences greater for the J method compared to the other two for liver and muscle samples but not for subcutaneous fat. Regarding GC×GC comprehensive system revealed the presence of 76 peaks and 5 CLA isomers corresponding to the 5 pure isomers present like standards used as references. Using Ag^{+}HPLC, CLA FAME are detect thanks to their characteristic UV absorbance at 233nm and the CLA identify are in total 13 for each tissues and belonging to *cis*/*trans*, *trans*/*trans* and *cis*/*cis* regions. The only problem with Ag^{+}HPLC is the resolution power in the *cis*/*cis* region because of, very often, the identification of C18:2*cis*9,*cis*11 isomer is difficult and not accurate. Other effects considered in the second part of my thesis are still important for the influences that they can have on fatty acids profile. Among these factors diet and tissue are probably the most important. Diet is important and in particular the rule of pasture is very significant. Pasture can increase PUFA and MUFA content, in particular Ω3 and CLA decreasing SFA content and changing Ω6/Ω3 ratio. The supply of *rp*CLA increased the concentration of C18:2*trans*10,*cis12*. In some case there is also a relation between suckling lamb's tissues composition and ewe's milk. Tissues effect influence fatty acids composition and each tissue have a different composition, related to lipids composition and the type of lipids present. Tissue with the most different characteristic was liver, rich in polyunsaturated fatty acids and in particular in long chain fatty acids (LC-PUFA). Is known that in meat and milk samples different fatty acids can be found, as example, saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty (PUFA) and they can have different consequence (positive or negative) on human health. SFA are known to be a potential health risk but MUFA and PUFA have positive effect on health and in particular, LC-PUFA, CLA and Ω3.

Regarding animals performance, was observe that the *rp*CLA supplementation can have many effects such as increase the lean body mass and decrease fat deposition increasing as consequence the efficiency of energy. In young growing bulls CLA has favorable effects on nitrogen utilization and in ruminant *rp*CLA can reduce the fat content in milk from dairy cows, sheep and goats. In my thesis these effects are evaluate considering lamb reared with

different feeding system and the effects on milk and meat, considering the influences on animal growth and slaughter traits and the composition and the coagulation properties of milk.

In conclusion, is possible to say, that is important known which are the sources of variation because in this way is possible created meat and milk with an higher content of the fatty acid positive for health reducing fatty acid with negative effect. The idea is to change FA profile of meat, milk and dairy products, increasing PUFA, CLA and Ω3 but decreasing SFA. Regarding CLA content, it can increase with diet, especially pasture and *rp*CLA supplementation. The complex nature of these molecules introduces the necessity to identify new methods of analysis that not increase the isomers with *trans*/*trans* configuration that is considered man made and not naturally presents. Moreover, this geometric configuration is connected with an increase of negative effect on human health. Many new studies are necessary for identify which is the best method considering that it has been adapt to the sample matrix for obtain a real quantification of CLA isomers. Extraction methods and in particular methylation methods could have an important rule. Regarding, the chromatography technique CGxCG is the most important if the aim is the complete fatty acids profile however if the objective is analyzed CLA isomers, Ag^{+}HPLC is considered the most important and the most used.

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