COMPARATIVE EVALUATION OF THE EFFECTS OF WHOLE ESSENTIAL OILS AND THEIR ACTIVE CONSTITUENT COMPOUNDS ON THE BIOHYDROGENATION OF POLYUNSATURATED FATTY ACIDS AND FERMENTATION CHARACTERISTICS OF RUMEN MICROBES *IN VITRO*

By

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ABSTRACT

Effects of whole essential oils (EOs) and their constituent compounds (EOCs) on the fermentation activities of rumen microbes and the biohydrogenation (BH) of *n*-3 polyunsaturated fatty acids (PUFA) were evaluated in four *in vitro* experiments and one feeding trial. In all the *in vitro* experiments, rumen fluid was collected from Hartline × Texel cross cull ewes. A basal feedstock comprising of 70:30 grass hay and concentrate was formulated, milled (1 mm screen) and then supplemented with 32.5 g oil/kg (40% oil from ground whole linseed and 60% from fish oil). In the first experiment using 15 EOCs, anethole and 4-allyanisole which were the most effective EOCs reduced the BH of 18:3 *n*-3 by 22.2% and 26.4%, respectively. But, at 300 mg/L there was a concomitant substantial inhibition of total volatile fatty acids (VFA). In the second experiment, out of 10 whole EOs, anise and cassia oils which were the most effective EOs reduced the BH of 18:3 *n*-3 by 58.2% and 54.3%, respectively. However, protection was accompanied with significant suppression of VFA at 300 mg/L. In the third experiment using varying doses (0, 100, 200 and 300 mg/L) of 4-allylanisole, anethole, anise oil and cassia oil, it was observed that at 200, 4-allylanisole, anethole and anise oil maintained best balance between satisfactory protection of *n*-3 PUFA and minimal disruption to VFA concentration. In the fourth experiment, six Hartline × Texel cross lambs were used. Three of the lambs were randomly assigned to the untreated basal diet (BDG) and the remaining three lambs were offered diet with anise oil (AOG). Rumen fluid collected from each of the BDG and AOG was used in *in vitro* batch culture system. The AOG maintained higher concentrations of PUFA and lower concentration of stearic acid. Results of these studies indicate that dietary addition of selected EOs and EOCs represent a potential effective strategy to optimize the fatty acid composition of ruminant food products (to be confirmed). However, whole EOs are more effective than EOCs.

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LIST OF ABBREVIATIONS

AACC: American Association of Cereal Chemists

ADF: acid detergent fiber

ALA: 4-allylanisole

ANE: anethole

ANO: anise oil

ANOVA: analysis of variance

AOAC: Association Official Analytical Chemist

AOG: anise oil group

BCVFA: branched chain volatile fatty acid

BDG: basal diet group

BEO: blend of essential oil

BH: biohydrogenation

BHT: butylated hydroxyl-toluene

BIS: bisabolol

BOR: borneole

CALA: conjugated linolenic acid

CAR: 3-carene

CHD: coronary heart disease

CIN: cinnamaldehyde

CIT: citronellol

CLA: conjugated linoleic acid

CLO: clove oil

CMO: cornmint oil

CON: control

CPY: caryophyllene

CSO: cassia oil

CTO: citronella oil

CVD: cardiovascular disease

CYM: *p*-Cymene

DHA: docosahexaenoic acid

DM: dry matter

DPS: deproteinising solution

EAFUS: everything added to food in the US

EFA: essential fatty acids

EOCS: essential oil compounds

EOS: essential oils

EPA: eicosapentaenoic acid

ETO: eucalyptus oil

EUC: eucalyptol

FA: fatty acid

FAMES: fatty acid methyl esters

FAO: food and agricultural organization

FCC: food chemicals codex

FFA: free fatty acid

FFDCA: federal food drug and cosmetic act

FG: food grade

GC: gas chromatography

GRAS: generally recognized as safe

HAP: hyper ammonia producing bacteria

HIV/AID: human immunodeficiency virus

IUPAC: international union of pure and applied chemistry

JPO: juniper berry oil

LA: linoleic acid

LDL: low-density lipoprotein

LIM: limonene

LNA: linolenic acid

LSD: least significant difference

LVO: lavender oil

MDO: mandarin oil

MEN: menthol

MIC: minimum inhibitory concentration

MUFA: monounsaturated fatty acid

MYT: myrtenol

NEFA: non-esterified fatty acids

NFD: neutral detergent fiber

P/S: PUFA/SFA ratio

PBC: plant bioactive compounds

PMF: proton motive force

PSM: plant secondary metabolites

PUFA: polyunsaturated fatty acid

RF: rumen fluid

RFA: remaining fatty acid

RMO: rosemary

SA: stearic acid

SFA: saturated fatty acid

TFA: total fatty acid

THU: thujone

TVFA: total volatile fatty acid

USFA: unsaturated fatty acid

VA: vaccenic acid

VAN: vanillin

VFA: volatile fatty acid

WHO: world health organization

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Introduction

Within the last few decades, remarkable changes in consumption of diets (shifts from the consumption of plant based products to mainly products of animal origin) took place in both the developed and the developing countries (World Health Organization, WHO, 2003). Increased human population, per capita income and urbanization are some of the driving factors of these changes (WHO, 2003). These changes have great direct implications for human health and indirect economic impacts. The direct health impacts result from the combined increase in consumption and the imbalance in the composition of ruminant animal fats, which are traditionally high in saturated fatty acids (SFA) and low in polyunsaturated fatty acids (PUFA) (Williams, 2000; Scollan *et al*., 2006). Increased occurrence of chronic diseases such as diabetes, obesity, compromised immune system and cardiovascular diseases (CVD) are some of these health challenges (Givens, 2005). These animal food products provide more than 30% of the total consumed energy and fat contributes about 50% of this energy (Givens, 2005). In 2003, WHO recommended less than 1% of *trans* FA, less than 10% of SFA and less than 15-30% of total fat to constitute the total consumed energy in human diets, because SFA is a risk factor of CVD. Unlike SFA, PUFA have among other positive health benefits, the potential to lower blood cholesterol. There is a positive relationship between increased intake of *n*-3 PUFA and reduced risk of coronary heart disease (CHD) (Abeywardena and Patten, 2011). These benefits, at least in part, explain why The Department of Health (1994) recommended increased intake of dietary *n*-3 PUFA over SFA. The economic impacts of these changes in consumption are due to the expensive nature (direct and indirect costs) of treating chronic diseases. The cost of treating CVD is more (over $400 billion) than the combine cost of HIV (about $29 billion) and cancers (about $200 billion) (Thom et al., 2006). In 1994, the Department of Health reported that 27% of all deaths recorded in the UK were caused by CHD. In about a decade after, WHO (2003) reported that approximately 60% of the total 56.5 million global deaths reported in 2001 were caused by chronic disease. The greater concern is the 75% of global deaths which is projected to result from chronic diseases by 2020 (WHO/FAO, 2003). Similarly, intake of animal food products (milk and meat) is projected to increase by 2030 (WHO, 2003). This situation is set to worsen the aforementioned health and economic impacts of these changes.

High content of SFA in ruminant derived food products is caused by hydrolysis of dietary lipids and the subsequent hydrogenation of the constituent PUFA by rumen microbes (Kim *et al*., 2009a). The process of biohydrogenation (BH) of C18:2 *n*-6 and C18:3 *n*-3 produces various intermediates such as conjugated linoleic acid (CLA) and Vaccenic acid (*trans-11* 18:1) (Harfoot and Hazlewood, 1997; Lee and Jenkins, 2011), which have been shown to have potential health benefits. The CLA (*cis-9 trans-11* 18:2 isomer) and its precursor (18:1 *trans*-11) have attracted considerable research attention as anti-carcinogenic, anti-inflammatory, anti-atherogenic and anti-diabetic agents (Kritchevsky, 2000, Kennedy *et al*., 2010). Identification of additives to reduce biohydrogenation of PUFA would lessen both the health and economic impacts of this seemingly inevitable change in consumption pattern.

A number of animal feeding strategies have been developed over the years to improve the PUFA content of ruminant food products through modification of rumen biohydrogenation, but these have been considerably inefficient (Fievez *et al*., 2007; Jenkins and Bridges, 2007). Formaldehyde treatment of free oil and protein mixtures has been described as effective in increasing the flow of 18:3 *n*-3 to the abomasum of goats (Scott *et al*., 1971), and in reducing the *in vitro* biohydrogenation of 18:2 *n*-6 (Gulati *et al*., 1997). However, beside the fact that the use of formaldehyde would potentially be criticized by regulatory authorities, the inconsistency of formaldehyde alone to reduce biohydrogenation of either 18:3 *n*-3 or 18:2 *n*-6 was reported by Sinclair *et al*. (2005). Recently, the potential of plant extracts such as essential oils (EOs) or their constituent compounds (EOCs) as rumen modifiers is receiving significant attention (Burt, 2004; Calsamiglia *et al*., 2007; Hart *et al*., 2008). Plants and their extracts are potential alternative because they are natural components of animal feed (Cowan, 1999). The effects of EOs or EOCs on methane production and ammonia nitrogen metabolism have been adequately investigated and recently reviewed (Patra, 2011). However, there is a scarcity of information on the effects of EOs or EOCs as modifiers of rumen biohydrogenation of PUFA. The aim of this PhD study was to compare the potential of EOs and EOCs as feed additives to reduce the extent of rumen biohydrogenation of *n*-3 PUFA.

CHAPTER 1

1.0. LITERATURE REVIEW

1.1. Definition and classification of lipids

Generally, lipids can be defined as a group of naturally occurring organic compounds or chemically heterogeneous substances, which are soluble in organic solvents such as benzene, hydrocarbons, ethers, alcohols and chloroform, but insoluble in water (Fahy *et al*., 2009; Fahy *et al*., 2011). A wide range of molecules, such as fatty acids, terpenes, phospholipids, sphingolipids, sterols, etc. possess these chemical characteristics. In functional terms, 'lipid' refers to a diverse group of compounds possessing different fundamental roles in the body such as storage of energy, metabolic fat and as structural components of cell membranes (Fahy *et al*., 2011). The variation in the structure of lipid molecule accounts for their diverse roles. The monocarboxylic (COOH) and aliphatic fatty acids are the structural components which are common to most lipids (Figure 1.1).

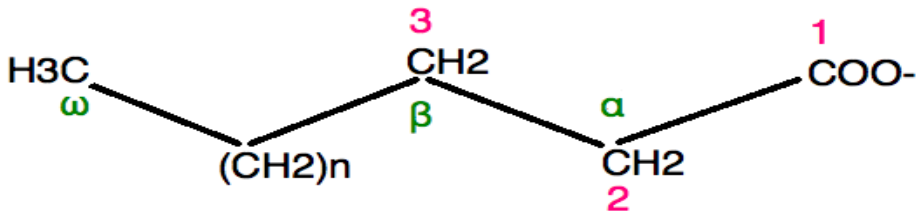


Figure 1.1 Fatty acid carbon atoms (Adapted from Stryer, 1988)

Lipid can be classified using different classification schemes. These differences are due to the fact that lipids are made of heterogeneous collection of molecules from both structural and functional perspectives. Lipids include oils, steroids, waxes, fats as well as compounds that are related to them, such as petrochemicals and soaps (Campbell, 1995; Fahy *et al*., 2005). A neutral lipid, such as triacylglycerol is formed from a mixture of one molecule of glycerol and three molecule of fatty acids attached together (International Union of Pure and Applied Chemists, 1978). The chemical structure of the attached fatty acid determines the variation in the physical characteristics of triacylglycerol (Campbell, 1995). A lipid

containing phosphoric acid as di-ester or mono-ester is known as a phospholipid (IUPAC, 1978), which is the main building block of cell membranes (Webb and O'Neill, 2008). Lipids are further divided into simple and complex classes for the convenience of chromatography analysis (Christie, 1983; Christie, 2007; Fahy *et al.*, 2009). Detailed and more complex classification of lipids is beyond the scope of this review, however, comprehensive update of lipid classification can be found in the review by Fahy *et al.* (2009).

Simple lipids are those types of lipids that yield at most two kinds of different primary products on hydrolysis (Christie, 1983). Acylglycerols (glycerol and fatty acids) are common examples of simple lipids (Figure 1.2). Almost all available oils and fats of both plant and animal origin are made completely of triacylglycerol (Mu and Hoy, 2004). More details about triacylglycerol is found under functions of lipids (section 1.1.1)

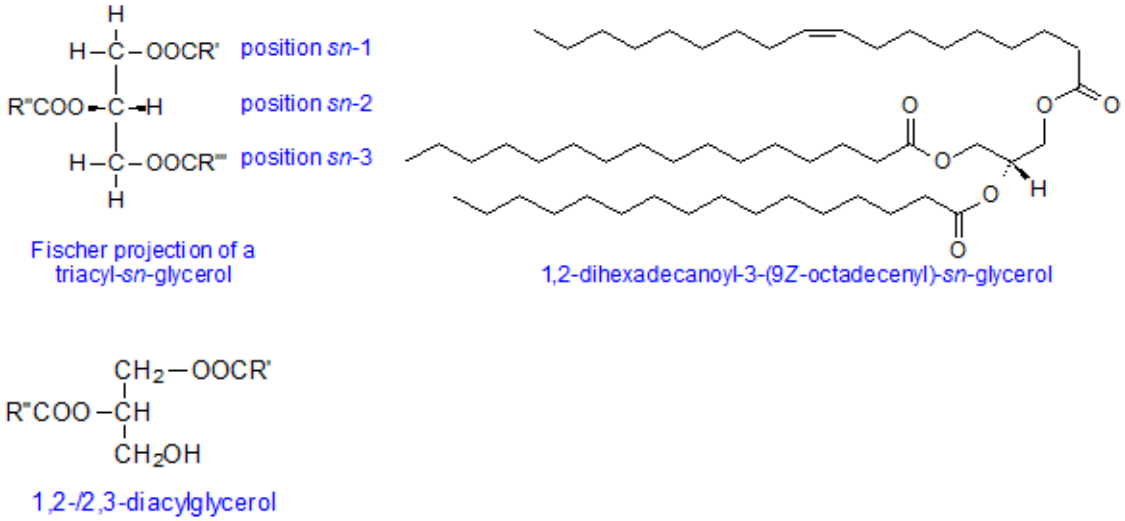


Figure1.2 Example of simple lipids (Adapted from Christie (2007)).

Complex lipids are those lipids that yield three or more primary products on hydrolysis. Examples of complex lipids are glycerophospholipids (Figure 1.3). More details about glycerophospholipids are found in section 1.1.2.

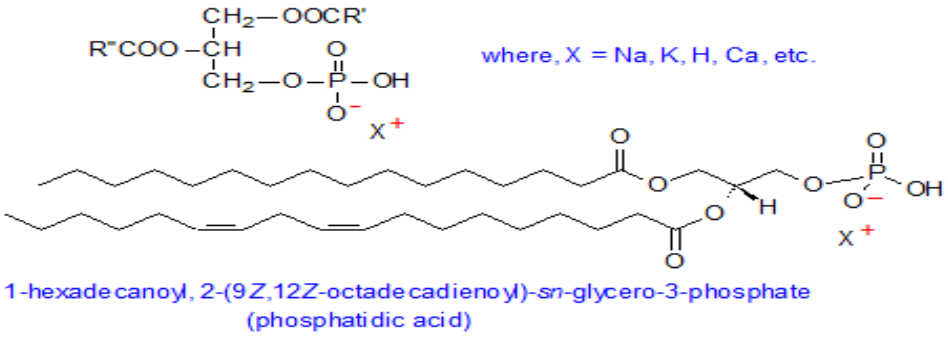


Figure 1.3 Example of complex lipids (Adapted from Christie (2007)).

1.1.1. Functions of lipids in food

Dietary lipids have a number of fundamental functions such as structural components of cells, as sources of energy, supply of fat-soluble vitamins and sources of essential fatty acids. Lipids in food also enhance the palatability of foods (Gurr and Harwood, 1991).

Lipids serve as storage of energy such as triacylglycerol (Stryer, 1988). In man and other mammals, triacylglycerol constitute the major source of fuel (Gurr and Harwood, 1991). Although the ultimate nutritional benefit of triacylglycerol is the provision of metabolic energy, the availability of fat can also contribute considerably to diet palatability (Gurr *et al.*, 2002). Adipose tissue is the largest source of fatty acids to provide the long-term energy requirements of human beings. When energy demand of the body is limiting, fatty acids are mobilized from adipose tissue through β-oxidation to meet the energy requirement (Gurr *et al.*, 2002). A number of factors such as types of dietary components and their amounts and hormones, whose secretion is also partly regulated by the diet, are responsible for the release of stored energy in the body (Gurr and Harwood, 1991). Although other tissues such as the liver can store fat in the form of small globules for short term needs, only adipose tissue can provide fatty acids to meet the energy requirement of the body in the long-term. Fatty acids such as stearic, palmitic (saturated) and monounsaturated fatty acids such as oleic acid are synthesized by adipose tissue (Gurr and Harwood, 1991; 2002).

Structural lipids (cholesterol, phospholipids and glycolipids) form an integral part of biological cell membranes, acting as barriers between environments (Doege and Stahl, 2006). Example of such

protection in the form of barrier is the skin, which is covered with a layer of protective lipids such as mono-, di- and triglycerols, sterol and sterol esters, unsaturated fatty acids (USFA), hydrocarbons and wax esters (Gurr and Harwood; 1992). The ability of fat to exclude water is partly one of the roles of fat in biological barriers. The chemical feature of part of the fat molecule which is hydrophobic (water hating) is the physical characteristic feature which accounts for their insolubility in water (Gurr and Harwood, 1991). The physical properties of fat are mainly determined by the nature of the fatty acid chain. Therefore, fat is softer when the melting point is low due to larger number of double bonds (degree of unsaturation) in the fatty acid chain. Because fats provide the framework on which much of the complex chemistry of the cell takes place, fats also occur within the cell (Stryer 1988; Gurr and Harwood, 1991). Phospholipids and glycolipids are also found in animal cell membranes. Some of the lipids required for the growth and development of tissue can be synthesized by the cells. However, FAs such as α-linolenic and linoleic acids (known as essential fatty acids) must be supplied in the diet as they cannot be synthesized by the cells (Gurr *et al*., 2002). Once these essential fatty acids are incorporated into cells, their elongation or desaturation can lead to the synthesis of long chain PUFA such as C20:5 *n*-3 and C22:6 *n*-3.

Lipids such as eicosanoids, steroid hormones and prostaglandins participate in signalling pathways and have recently been shown to have the potential for the exhibition of molecular roles such as regulation of local hormones and intracellular cell signal transduction (Eyster, 2007). In the structural and storage functions of lipids, their ability to associate and interact with each other, such as amphiphilic sheets in membranes and as hydrophobic globules in stored fat is their characteristic feature (Gurr and Harwood, 1991). Stored fats must undergo biochemical transformation for them to become physiologically useful. For instance, specific types of unsaturated FAs which are stored in membrane phospholipids can be transformed to hormone-like substances (such as eicosanoids) and cholesterol is metabolized in the adrenal gland to vitamin D in the skin, to a variety of steroid hormones and to bile acids in the liver (Gurr and Harwood, 1991). Fat soluble vitamins which are involved in metabolic processes can also be called metabolic lipids. Fat soluble vitamins which are present in fatty foods are vitamins A, D, E and K. It has

long been established that vitamins are key requirements in the diet to maintain health, and sustain normal growth and reproductive efficiency of the body (Gurr *et al*., 2002).

1.2. Nomenclature of fatty acids

The nomenclature of FA falls into two principal classes: the common or trivial names and the systematic names. The former uses abbreviations as the convenient way of defining fatty acids. According to the International Union of Pure and Applied Chemistry (IUPAC, 1977), the nomenclature of *n*-9, *n*-6 and *n*-3 polyunsaturated fatty acids can be determined by the position of the first double bond on the carbon chain. Example, α-linolenic acid (LNA; C18:3 *n*-3) is an essential fatty acid with *n*-3 and 18-carbon atoms. The *n*-3 is the position where the first double bond is located on the carbon backbone from the methyl end, 3 is the number of double bonds on the chain, and 18 refers to the number of carbon atoms present in the structure of the fatty acid. In a similar way, C18:2 *n*-6 (linoleic acid; LA) is an essential fatty acid with the first double bond located at position 6, with two number of double bonds and 18 number of carbon atoms (Figure 1.4). Oleic acid (C18:1 *n*-9) has just one double bond located at position 9. The structure and nomenclature of some polyunsaturated, monoenoic and saturated fatty acids are shown in Table 1.1.

Unsaturated fatty acids can undergo either geometrical or positional isomerization (Gurr and Harwood, 1996). Geometrical isomers occur when there is a chance of having either *trans* or *cis* at the configuration of the double bond. In the *trans*-configuration, the two hydrogen substituents are on the opposite sites of the molecule relative to the *cis*-configuration where they are on same side (Figure 1.5). Therefore, to show that C18:2 *n*-6 is an 18 carbon fatty acid having *cis* double bonds at carbons 9 and 12 counting from the carboxyl end; it is written as *cis* (Δ) 9, *cis* (Δ) 12- 18:2 or *cis* 9, *cis* 12 18:2-octadecadienoic acid (Wahle and James, 1993, Table 1.1). When the double bonds are located at different positions in the carbon chain, a positional isomer occurs. For instance, positional isomeric forms could occur in palmitoleic, a monounsaturated fatty acid (C16:1) at C7 and C9 which could be written as Δ7 and Δ9.

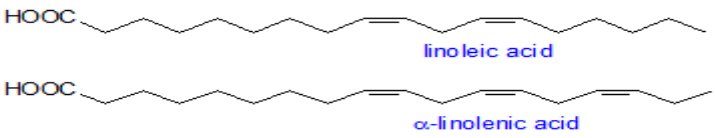


Figure 1.4 Examples of the C18:3 PUFAs and the position of double bonds (Adapted from Christie (2007)).

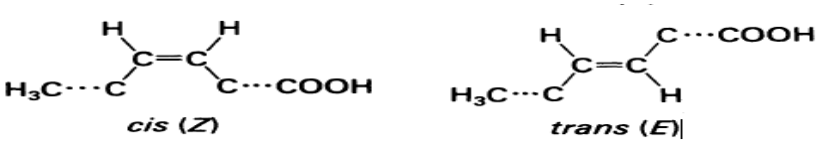


Figure 1.5 Example of geometric isomerism in PUFA (Adapted from Christie (2007)).

Table 1.1 Nomenclature of Some fatty acids (SFA, MUFA and PUFA) found in plant and animal tissues

Shorthand name	Systemic name	Common name	Melting point (°C)	Occurrence
Saturated fatty acids				
C2:0	Ethanoic acid	Acetic	16.7	Plants and animals
C6:0	Hexanoic acid	Caproic	-8.0	Milk fat
C8:0	Octanoic acid	Caprylic	12.7	Part of milk and seed triacylglycerols
C10:0	Decanoic acid	Capric	29.6	Part of milk and seed triacylglycerols
C12:0	Dodecanoic acid	Lauric	42.2	Part of some seed fats e.g coconut
C14:0	Tetradecanoic acid	Myristic	52.1	widespread
C16:0	Hexadecanoic acid	Palmitic	60.7	Common SFA in plants and animals
C18:0	Octadecanoic acid	Stearic	69.6	Main part of animal fats
C20:0	Eicosanoic acid	Arachidic	75.4	Widespread minor constituents
C22:0	Docosanoic acid	Behenic	80.0	Fairly widespread, minor
Monoenoic fatty acids				
C16:1(*n*-7)	Cis-9-hexadecenoic acic	palmitoleic	1	Main part of some seed oils
C18:1(*n*-12)	Cis-6-Octadecenoic acid	Petroselinic	33	Part of umbelliferous seed oils
C18:1(*n*-9)	Cis-9-Octadecenoic acid	Oleic	16	Animals and plants
C18:1(*n*-7)	Cis-11-Octadecenoic acid	*trans*-vaccenic	44	Rumen fats via BH of PUFA
C22:1(*n*-9)	cis-13-docosenoic acid	Erucic	24	Mustard and rapeseed oils
C24:1(*n*-9)	cis-15-tetracosenoic acid	Nervonic	-	-
Polyunsaturated fatty acids				
C18:2(*n*-6)	9,12-Octadecadienoic acid	Linoleic	-5	Main part of plant lipids
C18:3(*n*-6)	6, 9, 12- Octadecatrienoic acid	ɣ-linolenic	-	Minor part of algae and animals
C18:3(*n*-3)	9, 12, 15-Octadecatrienoic acid	α-linolenic	-11	Part of higher plants
C20:4(*n*-6)	5, 8, 11, 14-Eicosatetraenoic acid	Arachidonic	-49.5	Main part of animal phospholipids
C20:5(*n*-3)	5, 8, 11, 14, 17-Eicosapentaenoic acid	-	-	Abundant in fish
C22:6(*n*-3)	4, 7, 10, 13, 16, 19-Docosahexaenoic acid	-	-	Abundant in fish

SFA=Saturated fatty acids; BH=biohydrogenation; MUFA= monounsaturated fatty acids; PUFA= polyunsaturated fatty acids; Compiled from Christie, 1983; Gurr *et al*., 2002).

1.2.1. Fatty acids

Fatty acids, whether of animal or plant origin, consist of even number of carbons (from C4-C24), hydrogen (H) and oxygen (O). These elements are arranged in straight chains with a terminal carboxyl group. Fatty acid could be classified on the basis of two fundamental concepts: the degree of saturation and the chain length. On the basis of the former, FA could be unsaturated or saturated depending on the presence or absence of double bonds in the carbon chain. Unsaturated fatty acids (UFA) have double bonds in their chain structure. The number of double bonds in the structure of UFA also classifies fatty acids into monounsaturated and polyunsaturated FA. Based on the number of double bonds present within the carbon chain, fatty acids in mammalian tissues are categorized as polyunsaturated fatty acids (PUFA), monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA). On the basis of chain length, FA may have as many as thirty carbons.

1.2.2. Saturated fatty acids

A saturated fatty acid is a FA in which the carbon chain is completely hydrogenated (zero double bonds). Majority of SFA have straight chain structures with an even number of carbon atoms (Table 1.1). Although shorter (from 4-14 carbon atoms) chains of SFA occur in milk, the most common fatty acids in animal tissues are those containing between C12-C24 (Enser *et al*., 1984; Table1.1). Myristic acid (C14:0) which is a major component of seed oil from *myristicaceae* family is a minor constituent of the animal lipids (Christie, 1983). The commonest SFA in both plant and animal is C16:0 (palmitic acid), whilst C18:0 (Stearic acid) is less common compared to C16:0, but may be more abundant than palmitic in some cases (Christie, 1983). Although in fish and bacterial species, large amounts of longer chain FAs may occur but are less frequent in animal tissues. SFA longer than 10 carbon atoms are comparatively inert chemically and can be subjected to vigorous chemical conditions because of the absent of functional groups except the carboxyl group (Christie, 1983; Enser *et al*., 1984).

Although SFA are generally considered to be risk factors of coronary heart disease and cancers, stearic acid is a neutral fatty acid (Webb and O'Neill, 2008). It has been recommeneded that saturated fatty acids should contribute about 10% of the total 30% of lipid energy intake that is considered as normal for humans (World Health Organization; WHO, 2003). The ratio of polyunsaturated fatty acids to

saturated fatty acid (P:S) in human diets was recommemded to be above 0.4 (WHO, 2003), whilst the recommended ratio of *n*-6/*n*-3 is a value less than 4 (Webb and O'Neill, 2008). Nowadays ruminant meats have been considered to be causing the imbalance in the consumption of fatty acid as they have a P:S ratio of about 0.1 (Enser *et al*., 1996). Both P:S and *n*-6:*n*-3 in ruminant meat can be potentially improved through dietary manipulation (Wood *et al*., 2003).

1.2.3. Monounsaturated fatty acids

Unlike PUFA, MUFA are the group of FA with just one double bond in the carbon chain. The double bond is usually present between carbon atoms 9 and 10 counting from the alpha carbon, but in some cases, the double bonds could be between 7 and 8 carbon atoms. Unlike SFA, monoenoic fatty acids are more susceptible to chemical attack by oxidising agents (Wahle and James, 1993).

Palmitoleic acid (C16:1) is a constituent of most animal fats and may be found in significant concentrations in some seed oil and fish oils. The most abundant monoenoic acid is oleic acid (C18:1*n*-9), and is present in almost all plant and animal lipids. Although *trans* vaccenic (C18:1 *trans* 11) acid is an intermediate product of rumen biohydrogenation of PUFA, it is found in low concentrations in ruminant lipids (Enser *et al*., 1984). In a single natural lipid, many different positional isomers of MUFA may be present. For example, in bovine milk triacylglycerides, eleven different *trans*-octadecenoic acids and five different *cis*- octadecenoic acids have been reported (Christie, 1983). Monoenoic acids of long chain fatty acids (C20 and C22) are present in large amounts in fish oils and seed oils such as rapeseed oil. Shorter chain monoenoic acids are found in milk but rarely present in appreciable concentrations in other tissues. In bacterial and fish oil lipids, odd chain monoenoic acids are found in considerable concentrations but are minor constituents of animal lipids (Enser *et al*., 1984; Table1.1).

1.2.4. Polyunsaturated fatty acids

Polyunsaturated fatty acids are a category of FA with more than one double bond, and could have up to six (maximum) double bonds in their carbon chain structures. According to their biosynthetic derivation, PUFA of plant and animal lipids can be sub-grouped as *n*-3, *n*-6, *n*-7 and *n*-9 (Brenner, 1989). Although the focus of review in this area is on the *n*-3 and *n*-6, brief information on *n*-7 and *n*-9 would be provided. The fatty acids in each sub-group have the same terminal structure (-COOH) and the carbon chain structures contain two or more *cis*-double bonds, which are generally separated by a single methylene group ($-CH_2$). The higher the double bond in polyunsaturated fatty acids the more the susceptibility to oxidative attack (Gurr and Harwood, 1991).

1.2.4.1. *n*-3 and *n*-6 PUFA

Alpha-linolenic acid (C18:3 *n* -3) or *cis*-9, *cis*-12, *cis*-15-Octadecatrienoic acid (Table 1.1), which is a C-18 PUFA, is rarely found in large amounts in animal lipids but is a major component of plant lipids (Christie, 1983). Linolenic acid is an essential fatty acid (EFA) and a precursor of the long chain *n*-3 fatty acids such as C20:5 *n*-3 or eicosapentaenoic acid (5, 8, 11, 14, 17-eicosapentaenoic, EPA) and C22:6 *n*-3 or docosahexaenoic acid (4, 7, 10, 13, 16, 19-Docosahexaenoic acid) in animal lipids (Enser, 1984; Barcelo-Coblijn and Murphy, 2009; Figure 1.6). The long chain *n*-3 PUFA are present in significant concentrations in fish oils and are major components of phospholipids in many animal tissues (Brenner, 1989).

Essential fatty acids perform vital functions in immune response and they are known carriers of the fat soluble vitamins such as vitamins A, D, E and K (Webb and O'Neill, 2008). Linoleic acid (*cis*-9, *cis*-12-octadecadienoic acid, LA) and LNA are the two EFA with 18-carbon atoms, whereas EPA and arachidonic acid (C20:4 *n*-6) which are formed by the desaturation and elongation of linolenic and linoleic acids respectively, are the two most important C20 EFA (Smith, 2007). The importance of these FAs in humans was first demonstrated in the early 1980s (Holman *et al*., 1982). Since it is established that some long chain *n*-3 fatty acids such as EPA and DHA are metabolically synthesized from C18:3 *n* -3 (Figure 1.6), it has been considered as the important precursor of the group of fatty

acid commonly known as omega-3 fatty acids. The family of fatty acids called the *n*-3 fatty acid is known by the location of a double bond at the position 3 of the carbon atom chain starting from the methyl end. Due to the lack of the desaturase enzyme (Δ-9 desaturase) necessary to insert a double bond more proximal to the methyl end than the 9th carbon atom, mammals cannot synthesize the *n*-3 fatty acids *de novo*. Hence, omega-3 fatty acids have to be provided in the diets.

As in the case of omega-3 fatty acids, mammals cannot also synthesize another group of fatty acids, the *n*-6 (omega-6) fatty acids, which are metabolically synthesized from linoleic acid (LA; 18:2 *n*-6). Linoleic acid or C18:2 *n*-6 (*cis*-9, *cis*-12-octadecadienoic acid) is the simplest and commonest FA found both in animal and plant tissues (Gurr and Harwood, 1996). Arachidonic acid (*cis*-5, *cis*-8, *cis*-11, *cis*-14-eicosatetraenoic acid) or C20:4 *n*-6 is synthesized from C18:2 *n*-6, low in plant tissues but present as major constituent of animal tissues. In animals, the main precursor for biologically active C20 compounds called eicosanoids is arachidonic acid.

In terms of their synthesis, the omega-6 fatty acids shared the same characteristics to the *n*-3 fatty acids (Barcelo-Coblijn and Murphy, 2009). The omega-6 fatty acids are known by the location of the first double bond at position 6 of the carbon chain starting from the methyl end. Due to the fact that both omega-3 and omega-6 fatty acids cannot be synthesized *de novo* by mammals, they are both referred to as EFA which must be present in the diet for mammals. This means that LA and LNA are the two dietary sources from which the synthesis of the *n*-6 and *n*-3 fatty acids can be made through elongation and desaturation (Figure 1.6).

1.2.4.2. *n*-7 and *n*-9 PUFA

Oleic acid can also be the primary precursor of 5, 8, 11-eicosatrienoic acid, whose concentration in animal lipid can only be in high amounts when the animal lacks essential fatty acids (Brenner, 1989). There is another subclass of PUFA which is derived from C16:1*n*-7 (Enser, 1984).

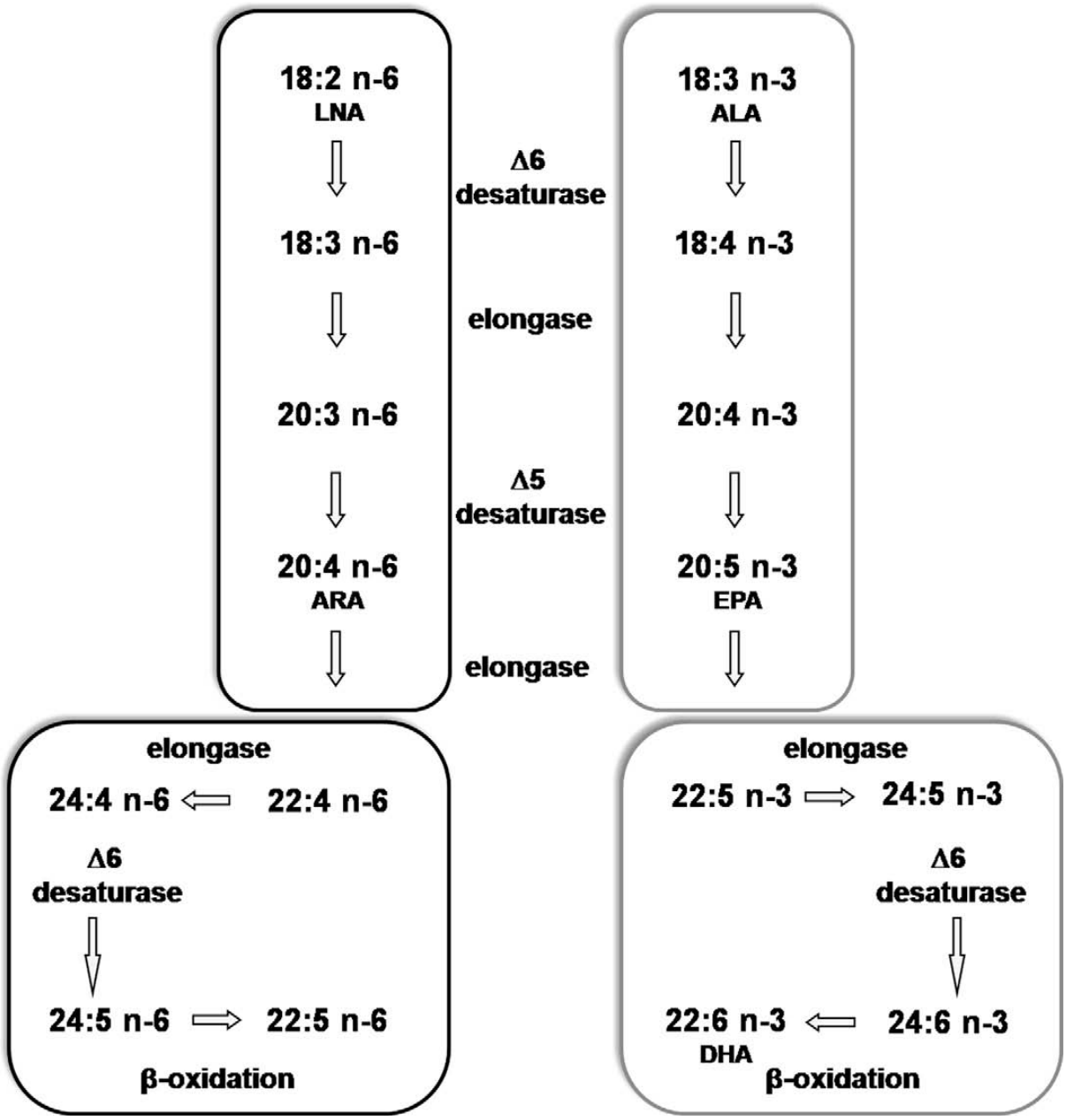


Figure 1.6 Elongation and desaturation pathways of the *n*-3 and *n*-6 fatty acids in the cytoplasm (Adapted from Barcelo-Coblijn and Murphy, 2009). LNA=linoleic acid, ALA=alpha linolenic acid, ARA=arachidonic acid, EPA=eicosapentaenoic acid, DHA=docosahexaenoic acid

1.2.5. Contribution of ruminant food products to global food security

The main source of milk for human consumption is from ruminant livestock. Milk is rich in energy, protein, fat and minerals such as calcium and iron including essential vitamins (National Research Council, 1998; Newman *et al*., 2003; Givens, 2005). Milk contains 3-5% fat, which is responsible for many of the manufacturing, sensory and physical properties of dairy products (Kaylegian and Lindsay, 1995; Jensen, 2002). In the milk fat composition, the proportion of PUFA is insignificant (5%) relative to the levels of SFA (70-75%) and 20-25% of MUFA (Lock and Shingfield, 2004). The fatty acid composition of milk has both positive and negative implications for the health of consumers (Williams, 2000). The high content of SFA which is directly related to the risk of cardiovascular disease accounts for the negative implications of milk fat composition (WHO, 2003). In addition, increased intakes of SFA may be associated with a higher fasting plasma glucose and insulin concentrations and higher risk of impaired glucose tolerance (Parker *et al*., 1993; Feskens *et al*., 1995).

Similarly, beef is enriched with high quality protein, energy, a wide range of minerals such as Fe, Zn and a number of essential vitamins including B12, B6, B2 and B1 (De Smet, 2012). In countries with limited access to sea and where the consumption of sea food products is reduced, the intake of beef can be a direct source of omega-3 PUFA such as 18:3 *n*-3, 20:5 *n*-3 and 22:6 *n*-3. These *n*-3 fatty acids have a number of health benefits (see section 1.2.6). However, the concentrations of SFA (45-55% of total FA) and *trans*-fatty acids (3-10%) in beef are higher than the levels of PUFA (Mapiye et al., 2015). The consumption of high SFA has been associated with increased risk of several diseases such as cardiovascular disease and cancer (Gurr and Harwood, 1991; Salter, 2013).

In both the developed and developing countries, the roles of milk and beef to the food and nutrition security are distinct. In developed countries such as the UK and the US, there is an abundant supply of milk and beef and, their accessibility and affordability is not a problem. However, there are concerns about the health implications of these foods because they are relatively rich sources of SFA which are known to increase the risk of the so called “diseases of affluence” such as cardiovascular diseases and obesity (McNeill & Van Elswyk, 2012). By contrast, in the developing countries, the intake of beef

provides essential nutrients to complement the nutritionally poor plant food sources (based on one type of cereal or root crop) (Mapiye *et al*., 2015). Therefore, in these countries, the consumption of beef is seen as essential in terms of reducing the so called "diseases of poverty" (McNeill & Van Elswyk, 2012). These diseases of poverty which are responsible for impaired mental development, increased exposure to infection and impairment of growth are the results of deficiencies in bioavailable micronutrients such as Zn, Fe, B-vitamins, *n*-3 PUFA and protein (McNeill & Van Elswyk, 2012). In the developing countries, urbanisation, increasing population growth and high purchasing power are expected to strongly increase the demand for milk and beef in the coming decades (FAO, 2009).

The intake of ruminant food products makes a substantial contribution to meeting food and nutrition security through the provision of the above mentioned nutrients. However, research to find dietary strategies to increase the ratio of PUFA to SFA in beef and milk is necessary as a useful means of reducing the risk of 'diseases of affluence' and "diseases of poverty" in the developed and the developing countries, respectively.

1.2.6. Nutritional importance of PUFA, CLA and MUFA in human diets

Alpha linolenic acid, C20:5 *n*-3 and C22:6 *n*-3 are the most important *n*-3 fatty acids. As mentioned previously, C18:3 *n*-3 is synthesized in plants whilst C20:5 *n*-3 and C22:6 *n*-3 are predominantly in marine products. Interest in the potential benefits of C18:3 *n*-3 has been on the increase as it is the metabolic precursor of EPA and DHA, which have a number of benefits in human health. Physiologically, C18:3 *n*-3 is as important as its metabolites such as C20:5 *n*-3 and C22:6 *n*-3 (Barcelo-Coblijn and Murphy, 2009). Alpha linolenic acid is converted to C22:6 *n*-3 through a multi-step process of desaturation and elongation (Figure 1.6). Through Δ-6 desaturase, C18:3 *n*-3 is desaturated to C18:4 *n*-3 as the initial step, followed by a carbon chain elongation step i.e. C18:4 *n*-3 to C20: 4 *n*-3, and then Δ-5 desaturase converts C20: 4 *n*-3 to C20: 5 *n*-3 (Figure 1.6). The next step is the elongation of EPA to form C22: 5 *n*-3 through elongase-2 (Wang *et al.*, 2005). This is followed by the formation of C24: 5 *n*-3 which is then desaturated by Δ-6 desaturase to produce C24: 6 *n*-3

(Sprecher, 2000). These reactions show that any of the omega-3 fatty acid synthesized in the endoplasmic reticulum undergoes either elongation or desaturation to eventually produce C22: 6 *n*-3.

Few decades ago, medical specialists recommended that the intake of total fat should be reduced (Webb and O'Neill, 2008). This recommendation was made in attempt to control the potential adverse effects of fats on coronary heart disease, obesity as well as other health disorders associated with excessive intake of fat. Presently, there is a significant shift from fat quantity to fat quality. For instance, with regard to reducing the risk of cardiovascular disease (CVD) in middle-aged men, the intake of *n*-3 PUFA and MUFA is more important as a regulator than the intake of total fat (Laaksonen *et al*., 2005). This group of PUFA has potential to reduce the risk of cardiovascular disease by regulating the concentrations of blood cytokine (Von Schacky *et al*., 1999; Von Schacky, 2007; Laaksonen *et al*., 2005; Coates *et al*., 2009). In addition, the *n*-3 PUFA have been reported as stimulators of endothelial relaxation which leads to anti-arrhythmic and cardioprotective effects on the heart (Raheja *et al*., 1993).

The development of carcinogenesis has also been shown to be regulated by the consumption of PUFA. The intake of long chain *n*-3 fatty acids has been shown to reduce the risk and inhibit the development of cancer (Larsson *et al*., 2004; Palmquist, 2009), through the following processes: regulation of signal transduction pathways, gene expression, transcription factor activity, membrane fluidity and insulin sensitivity. In addition, PUFA also cause suppression of eicosanoid biosynthesis from arachidonic acid, decreased or increased production of reactive oxygen species and free radicals and modification of oestrogen metabolism (Palmquist, 2009). Following the review of available data on clinical results of supplementing fish oil and cancer prevalence, Colomer *et al*. (2007) inferred that it is beneficial to administer *n*-3 fatty acids particularly C20:5 *n*-3 and C20:6 *n*-3 at 1.5 g/d to patients with advanced cancer. The benefits include improvement in quality-of-life and both clinical and biological parameters.

In recent times, it is considered that the anti-atherosclerotic effect of *n*-3 PUFA through decreasing the concentrations of tumour necrosis factor-α and proinflammatory cytokines (e.g. interleukins -6,

interleukins-Iβ), is attributed to their actions on growth and cytokines factors (Palmquist, 2009). The *n*-3 fatty acids are also known to offer a protective effect on the risk of atherosclerosis by altering the concentration of plasma lipids i.e. reducing the concentration of triacylglycerols (Harris, 1997; Sirtori and Galli, 2002). EPA and DHA have been reported to possess high potential to lower serum triglycerides (WHO, 2003), which suggests that they are capable of reducing coronary heart disease. PUFA such as C18:2 *n*-6 (LA) is a useful structural component required for the synthesis of lipids in the tissue and a component of cellular membranes (Simopoulos, 2000; Palmquist, 2009). The benefits of *n*-3 fatty acids on increased glucose metabolism include the potential to reduce low density lipoprotein cholesterolemia, positive effects on high density lipoprotein and decreased hyperglycaemia (Raheja *et al*., 1993; Sirtori and Galli, 2002).

Several reports (Funahashi *et al*., 2006; Kim *et al*., 2009) have repeatedly indicated that *n*-3 fatty acids may be responsible for inhibition of tumours growth rate by decreasing the number and size of tumours, as well as delaying its appearance. Long chain *n*-3 PUFA have also been reported to regulate chronic inflammatroy disorders through inhibition of the expression of adhesion molecules and reduction of the production of cytokines, reactive oxygen species and inflammatory eicosanoids (Calder, 2006).

EPA and DHA are required as key fatty acids in the development and growth of the central nervous system in humans (Innis, 2003). The *n*-3 PUFA, especially C22:6 *n*-3 (DHA) play an important function in the development of the liver and brain in infants (Martinez and Ballabriga, 1987), hence, they are needed as basic requirement for normal development and growth of humans (Simopoulos *et al*., 1991; Salem *et al*., 1996).

In addition to the above mentioned health benefits of long chain *n*-3 PUFA, they also have beneficial effects such as antithrombotic and immuno-suppressive effects (Kremmer *et al*., 1995, Gonzalez, 1995; Narayan *et al*., 2006), management and prevention of hypertension (Morris *et al*., 1994), renal disease (Donadio *et al*., 1994), type 2 diabetes (Connor *et al*., 1993), rheumatoid arthritis (Kremer, 1996), and chronic obstructive pulmonary disease (Shahar *et al*., 1994). Omega-3 PUFA are also

implicated in cyclic adenosine monophosphate signal transduction pathways where they function as regulators of specific gene transcription, and are also involved as second messengers in gene expression (Clark and Jump, 1994; Graber *et al*., 1994).

The main dietary source of conjugated linoleic acid (CLA) in the human diet is milk fat (Lawson *et al*., 2001). CLA are known as the collective term for a mixture of geometric and positional isomers of C18:2 *n*-6 having conjugated double bonds. Although there are many isomers of CLA, *cis*-9 *trans*- 11 is the major isomer (approximately 90%) in milk (Parodi, 1977). A range of health benefits such as antimutagenic, antidiabetic, antiobesity, antiatherogenic, immunomodulation and modulation of bone growth and anticarcinogenic effects have been attributed to *cis*-9 *trans*- 11 CLA in different biological models (Banni *et al*., 2001; Corl *et al*., 2003). In a Swedish Mammography cohort where records of 60 000 women were analysed, the intake of CLA from dairy products was shown to be inversely related to the risk of colorectal cancer (Larsson *et al*., 2005). For more information about CLA refer to section 1.7.3.

The main MUFA in milk fat are vaccenic acid (C18:1 *trans* 11) and oleic acid (C18:1 *n*-9). The former is the major *trans* fatty acid in milk and has been linked with improvement in cholesterol levels, lower rates of cancer and cardiovascular disease (Kris-Etherton *et al*., 1999; Banni *et al*., 2001). Based on endogenous conversion of vaccenic acid to *cis*-9 *trans*-11 18:2 CLA through the delta 9 desaturase in the mammary gland, C18:1 *trans* 11 has been reported to have anticarcinogenic properties (Banni et al., 2001). The endogenous conversion of C18:1 *trans* 11 to *cis*-9 *trans*-11 18:2 CLA is as important for prevention of cancer as the dietary supply of CLA (Corl *et al*., 2003).

Despite the above nutritional benefits of PUFA, their concentration in ruminant food products is low; hence, there is a need to device a means of increasing the content of PUFA in ruminant food products. This would lessen the negative effects of high SFA and improve the accessibility of other nutritional benefits derivable from ruminant food products.

1.3.0. Dietary sources of lipids for ruminants

The main dietary sources of lipids for ruminants are forage, oilseeds and fish oils. The lipid content and fatty acid composition are significantly different among the different sources of dietary lipids. In this section these main dietary sources of lipid for ruminants would be discussed.

1.3.1. Forage and grass lipids

Generally, ruminants are provided with considerable amounts of lipids from the forage component of their dietary daily allowance. These lipids which consist of high content of glycolipids and phospholipids are mainly of leaf origin, comprising between 6-8% of the dry matter content of the leaf (Harfoot, 1978). Glycolipids are the most abundant form of lipids, consisting between 70-80% of the available lipid content, whilst phospholipids, cholesterol esters and triacylglycerides represent the less (20-30%) abundant lipids (Christie, 1978).

Plant lipids are comprised of high levels of USFA, predominantly C18:2 *n*-6 (linoleic acid) and C18:3 *n*-3 (linolenic acid) and some smaller amounts of oleic acid (Palmquist and Jenkins, 1980). Majority of these lipids, which account for about 22-25% of the lipid on dry matter basis, are found in the chloroplast of leaf tissue (Harfoot, 1978). Of this lipid content, about 80% is monogalactosyldiglycerides and digalactosyldiglycerides (Harfoot, 1978). The fatty acid composition of monogalactosyldiglycerides from Lucerne (alfalfa) was reported to contain C18:3 *n*-3 (95%), C16:0 (2.7%) and C18:2 *n*-6 (1.7%) on a weight basis (O'Brien and Benson, 1964). The C18:3 *n*-3 concentrations in other legumes such as clover depend on both the species and the season of growth (Collins *et al.*, 2002; Loor *et al*., 2003; Table 1.2). Consequently, glycolipids and phospholipids are the predominant lipids present in the forage consumed by ruminants.

Alpha linolenic acid, the precursor for the long chain *n*-3 PUFA, is high in fresh grass, consisting between 0.50-0.75 g/g of the total fatty acids (Hawke, 1973). However, the concentration of C18:3 *n*-3 in fresh grass depends on grass species (Dewhurst and Scollan, 1997) and the stage of maturity (Bauchart *et al*., 1984). Drying and storage of forages can result in significant losses in the content of

C18:3 *n*-3 and corresponding increases in the concentration of palmitic acid (Noble, 1981). Therefore, it can be inferred that fresh grass or Lucerne would have higher content of C18:3 *n*-3 than grass hay.

Table 1.2 Profile of fatty acids of common fatty acid sources (g/100 g total fatty acids)

Fatty acid sources	**C16:0**	**C18:0**	**C18:2 *n*-6**	**C18:3 *n*-3**	**References**
Grass Silage	24.0	2.90	14.5	46.2	French *et al*. (2000)
Grass	20.8	3.29	14.0	49.2	French *et al*. (2000)
White Clover	33.0	4.1	28.2	34.7	Collins *et al*. (2002)
Red Clover spring	24.2	4.35	19.1	45.9	Loor *et al*. (2003)
Red Clover autumn	31.1	4.81	21.4	33.6	Loor *et al*. (2003)
Linseed	5.0	2.5	15.0	63.5	Karleskind (1996)
Cottonseed	24.0	2.0	47.0	1.0	Karleskind (1996)
Soybean	11.0	3.5	56.0	7.5	Karleskind (1996)
Rapeseed	3.5	1.2	13.8	9.1	Karleskind (1996)
Corn	11.0	2.0	58.5	2.0	Karleskind (1996)

1.3.2. Oilseeds

Concentrates, which are feedstuffs with high energy content, are conventionally added to ruminant diets to improve the energy density of the diet, especially for lactating animals (Palmquist, 1984). These concentrate feeds which are processed to prevent negative effect on digestion of fibre in the rumen, contain large amounts of crushed cereal grains, crushed oil seeds or their extracts (Palmquist, 1988). The addition of concentrate supplements to ruminant feed can also help to manipulate the digestion and absorption of nutrients, in addition to increasing the energy value of the diet (Palmquist, 1984; Palmquist, 1988). Rumen acidosis or reduced milk fat content due to low fibre diet or high carbohydrate diets can be limited by addition of fats to the diet (Palmquist, 1984). In addition, the fatty acid composition of ruminant food products can be manipulated to increase their desirability by food industries and for human consumption through addition of concentrates (Grummer, 1991).

Although grass and forages contain high concentration of C18:3 *n*-3 as a proportion of the total fatty acids, the content of C18:2 *n*-6 is higher in most commonly used oil seeds (Christie, 1983; Enser, 1984; Gurr and Harwood, 1996; Table 1.2). However, a significant concentration of C18:3 *n*-3 is found in a few number of oil seeds such as linseed oil, where C18:3 *n*-3 accounts for more than 50% of the total fatty acids (Sim, 1990; Table 1.2).

The long chain *n*-3 fatty acids such as C20:5 *n*-3 and C22:6 *n*-3 are completely absent in oils of higher plants. However, there is some evidence that substantial concentration of C20:5 *n*-3 can be found in lower land plants such as ferns and mosses (MAFF, 1997). Certain technologies such as the development of transgenic plants which expresses the desaturase gene could be used to produce higher concentration of C20:5 *n*-3 and C22:6 *n*-3 in higher plants (Kyle *et al*., 1990). Similar technology has been successfully used with tobacco to cause accumulation of gamma-linolenic acid (Reddy and Thomas, 1996).

1.3.3. Fish oil

There is a marked difference between the fatty acid composition of higher plants and marine organisms, with the latter containing significant concentrations of C20:5 *n*-3 and C22:6 *n*-3 (Nettleton, 1991). Relative to land animals, the unsaturated fatty acid content of fish oil is higher because fish like monogastric animals, absorb and deposit a wide range of available fatty acids from their diet (Sargent, 1997). The fatty acid composition of marine zooplanktons is substantially determined by the fatty acid composition of the phytoplanktons which serve as their main source of food (Sargent, 1996). Triacylglycerides or wax esters (fatty alcohols esterified to fatty acids) represent the oil reserves of zooplanktons. Marine zooplanktons in the polar latitudes deposit wax esters as an adaptation to extreme light regimes because the condition is characterized by limited supply and seasonal availability of phytoplanktons (Sargent, 1997). Under such extreme weather conditions, more than 50% of the dry weight of zooplanktons is oils, composed of wax esters, whereas zooplanktons in lower latitudes accumulate much lower oils with high levels of phospholipids and insignificant concentration of wax esters.

In the lower latitude where the zooplanktons contain negligible levels of waxes, fish such as sardines, anchovy and pilchards, consuming the zooplanktons have higher proportion of *n*-3 PUFA and lower body triacylglycerides than high altitude fish oils (Sargent, 1997). By contrast higher altitude fish such as herring, sprats, capelin and mackerel consuming zooplanktons containing high levels of wax esters can digest, absorb and transform the waxes to triacylglycerol. Thus, high altitude fish contains low levels of *n*-3 PUFA and relatively high concentration of C20:1 *n*-9 and C22:1 *n*-11 (Sargent, 1997).

1.4.0. Fatty acid metabolism in animals

1.4.1. Metabolism of lipid in non-ruminants

The pattern of lipid digestion by the animal, whether ruminant or non-ruminant, substantially regulates the transfer of fatty acids from the diet to the products derived from that animal. In non-ruminants, unlike ruminant animals, the small intestine is the main site for the digestion of ingested dietary lipids. The dietary lipids (triacylglycerols) are broken down by pancreatic lipase to mainly free fatty acids and 2-monoacylglycerols with absorption aided by the formation of micelles (Woods and Fearon, 2009). Then, lipoprotein lipase enzyme which is distributed widely around the body mediates the uptake of lipid in the jejunum. In the non-ruminant animal, relative to the ruminant animal, lipids in the diet are absorbed without transformation before their integration into the tissue lipids. Because dietary lipids are absorbed unchanged in non-ruminant animal, sources of dietary lipids have a determining and predictable effect on the fatty acid composition of human food products from poultry and pigs (Chesworth *et al*., 1998). The supply of UFA to tissue in non-ruminants could therefore be increased by simply increasing their dietary supply.

1.4.2. Hydrolysis and biohydrogenation of PUFA in ruminants

It is well established that lipolysis is a prerequisite for the occurrence of biohydrogenation of unsaturated fatty acid in the rumen (Dawson *et al*., 1977). Lipolysis is the process which hydrolyses ester linkages of esterified lipids to non-esterified fatty acids in the rumen and occurs rapidly after ingestion (Garton *et al*., 1958; Dawson *et al*., 1977). Lipolysis precedes biohydrogenation because the availability of a free carboxyl moiety is an important requirement for biohydrogenation to proceed (Harfoot and Hazlewood 1997; Jenkins and Bridges, 2007).

The non-esterified unsaturated fatty acids which are the products of lipolysis have a relatively short half-life, hence, they are rapidly hydrogenated after being adsorbed unto bacterial surfaces in the rumen (Keeney, 1970). The rational for the saturation of unsaturated fatty acid is still controversial. It has been suggested that ruminal biohydrogenation of UFA would be predictably manipulated if the reason as to why microbial population saturate unsaturated fatty acid is established (Jenkins *et al*., 2008). This implies

that understanding the basis for biohydrogenation of unsaturated fatty acid is the principal starting point for the development of suitable nutritional approaches to regulate its extent. To-date, two main hypotheses have been put forward to explain the rational for the biohydrogenation of unsaturated fatty acid. Initially, since biohydrogenation serves as hydrogen sink, it was thought that saving hydrogen from been re-channelled into other processes is the reason why this undesirable process occurs (Lennarz, 1966). However, the weakness of this hypothesis is that an inconsequential amount of hydrogen has been estimated to be saved during the process of biohydrogenation of unsaturated fatty acid in the rumen (Czerkawski, 1972). Kemp and Lander (1984) have also suggested another reason for biohydrogenation as the process of detoxifying unsaturated fatty acid. The suggestion by these authors is based on the understanding that polyunsaturated fatty acids are toxic to some rumen bacteria. More recently, studies have observed that the growth of pure bacterial strains recognized to be producers of C18:0 was inhibited by PUFA (Maia *et al*., 2007; 2010). These studies are confirming that PUFA are indeed toxic to some microbes, especially, cellulolytic bacteria. The production of *trans*-18:1 intermediate from incomplete biohydrogenation has been suggested to be a ruminal microbial mechanism to deal with stressor such as excess lipid (Bessa *et al*., 2000).

1.4.2.1. Ruminal Pathways of lipolysis and biohydrogenation

Previous studies have clearly established the pathways of biohydrogenation of linolenic and linoleic acids (Harfoot and Hazlewood, 1997; Palmquist *et al.*, 2005; Jenkins *et al.*, 2008; Figure 7). In those studies, the pathways for the biohydrogenation of C18:2 *n*-6 and C18:3 *n*-3 indicating how esterified dietary lipids are hydrolysed, followed by isomerization of non-esterified FA, and hydrogenation of free fatty acids to saturated FA was established. Triglycerides, galactolipids and phospholipids are the main dietary lipids entering the rumen (Jenkins *et al*., 2008). As mentioned previously, long chain omega-3 PUFA, particularly DHA (C22:6 *n*-3) and EPA (C20: 5 *n*-3) are the major fatty acids in fish oil and algae (marine products). By contrast, forages, oil seeds and cereals contain α-linolenic acid (C18:3 *n*-3) and linoleic acid (C18:2 *n*-6) as their major fatty acids (Chillard *et al*., 2007). In the rumen, these fatty acids are extensively biohydrogenated by microorganisms and a number of primary and secondary intermediates are produced (Harfoot and Hazlewood, 1997; Palmquist *et al*., 2005). Recently, it has been indicated that

great variety of monounsaturated and polyunsaturated isomers, particularly conjugated and *trans*-fatty acids are formed before the final product of biohydrogenation (C18:0) is produced in the rumen (Mosley *et al*., 2002; Chillard *et al*., 2007; Figure 1.8). In these studies, other intermediates of biohydrogenation that were not considered in the pathways reviewed by Harfoot and Hazlewood (1997) are discussed. Figure 1.8 is therefore a confirmation of earlier report by Keeney (1970) who recognized that a great variety of *trans*- and *cis*-18:1 isomers are contained in rumen bacteria and digesta. However, the pathways of the biohydrogenation of C20:5 *n*-3 and C22:6 *n*-3 and the respective intermediates products are not indicated in Figure 1.8 because their pathways are not yet clearly established. It would not be assumed that all inter-conversions among the isomers of C18:1 and all putative fatty acids are shown in Figure 1.8 because metabolism of a specific polyunsaturated fatty acid under some dietary conditions can produce certain intermediates (Chilliard *et al*., 2007).

As discussed previously, lipolysis hydrolyses the ester linkages resulting in the release of free fatty acids (FFA). This is the first process to occur when dietary lipids enters the rumen (Kim *et al*., 2009; Buccioni *et al*., 2012). This leads to the availability of free carboxyl moieties which is an important requirement for biohydrogenation to proceed (Jenkins and Bridges, 2007). Following the production of FFA, isomerization reaction converts *cis*-12 double bond to a *trans*-11 isomer producing conjugated linolenic acid (CALA) and conjugated linoleic acid (CLA) depending on whether linolenic acid or linoleic acid is involved (Jenkins *et al*., 2008; Kim *et al*., 2009; Buccioni *et al*., 2012). The next step after isomerization is the hydrogenation of unsaturated double bond leading to the production of single bond. For the LNA and LA, this involves the action of a microbial reductase. In the case of linoleic acid, linoleic acid isomerase (LA-I), which is the lipase that is involved in the first biohydrogenation step of linoleic acid, converts linoleic acid to rumenic acid, called CLA (Figures 1.7; 1.8). This is followed by the activity of the rumenic acid enzyme (reductase enzyme) to hydrogenate the rumenic acid to *trans*-11 18:1 (vaccenic acid; VA), which is then subsequently hydrogenated to stearic acid (C18:0) as the last step of C18:2 *n*-6 biohydrogenation. The biohydrogenation of α-linolenic acid starts with its isomerization to *cis*-9 *trans* 11 *cis*-15 C18:3. The hydrogenation of *cis*-9 *trans* 11 *cis*-15 C18:3 leads to the formation of *cis*-15, *trans*-11 C18:2 by the reduction of *cis*-9 double bond. This is then followed by the hydrogenation of *cis*-15 *trans*-

11 18:2 to produce either *cis*-15 18:1 or *trans*-11 *cis*-13 18:2, or *trans*-11 18:1 (vaccenic acid). The production of C18:0 results from further hydrogenation of *trans*-11 18:1, as the final step in the ruminal biohydrogenation of C18:3 *n*-3.

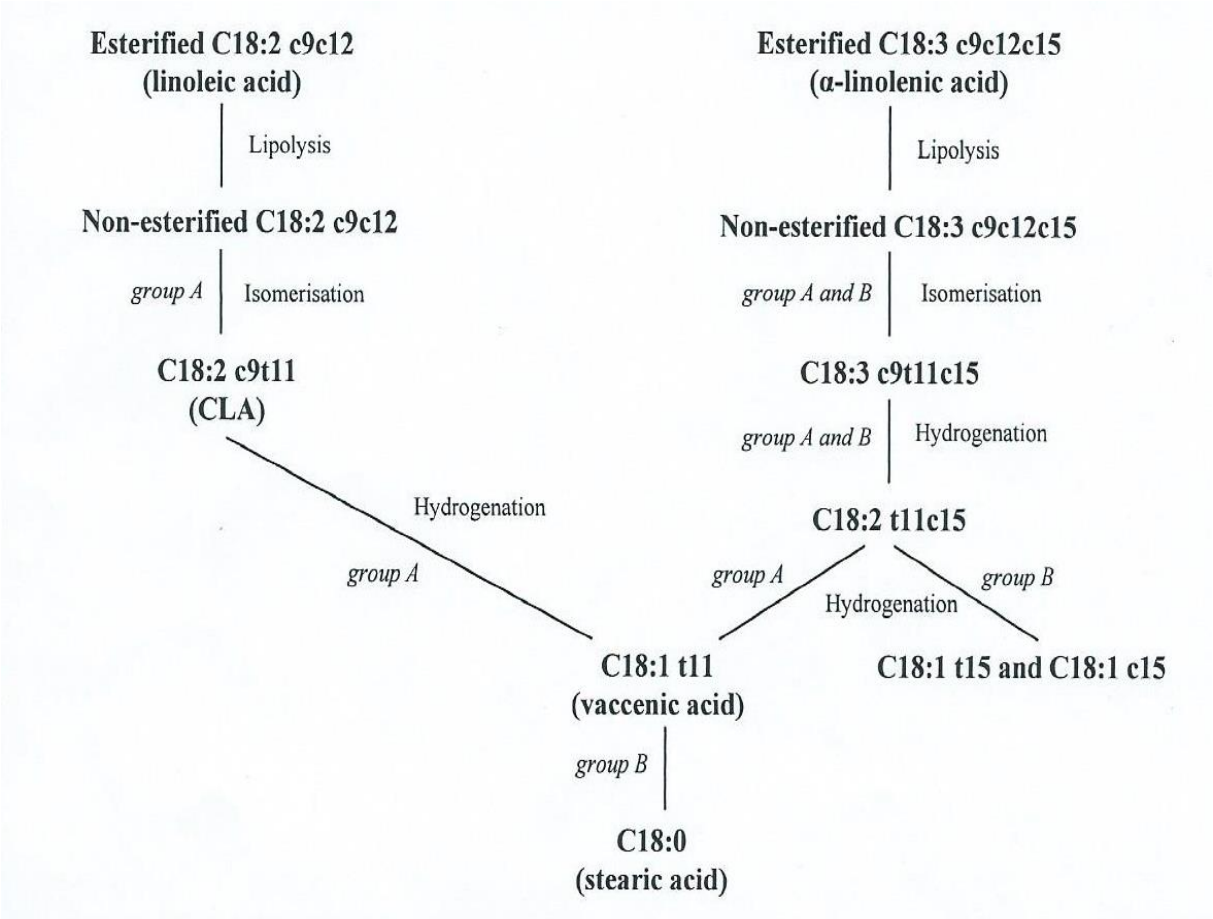


Figure 1.7 Biohydrogenation pathways of linolenic and linoleic acid in the rumen (Adapted from Harfoot and Hazlewood, 1997); CLA= conjugated linoleic acid

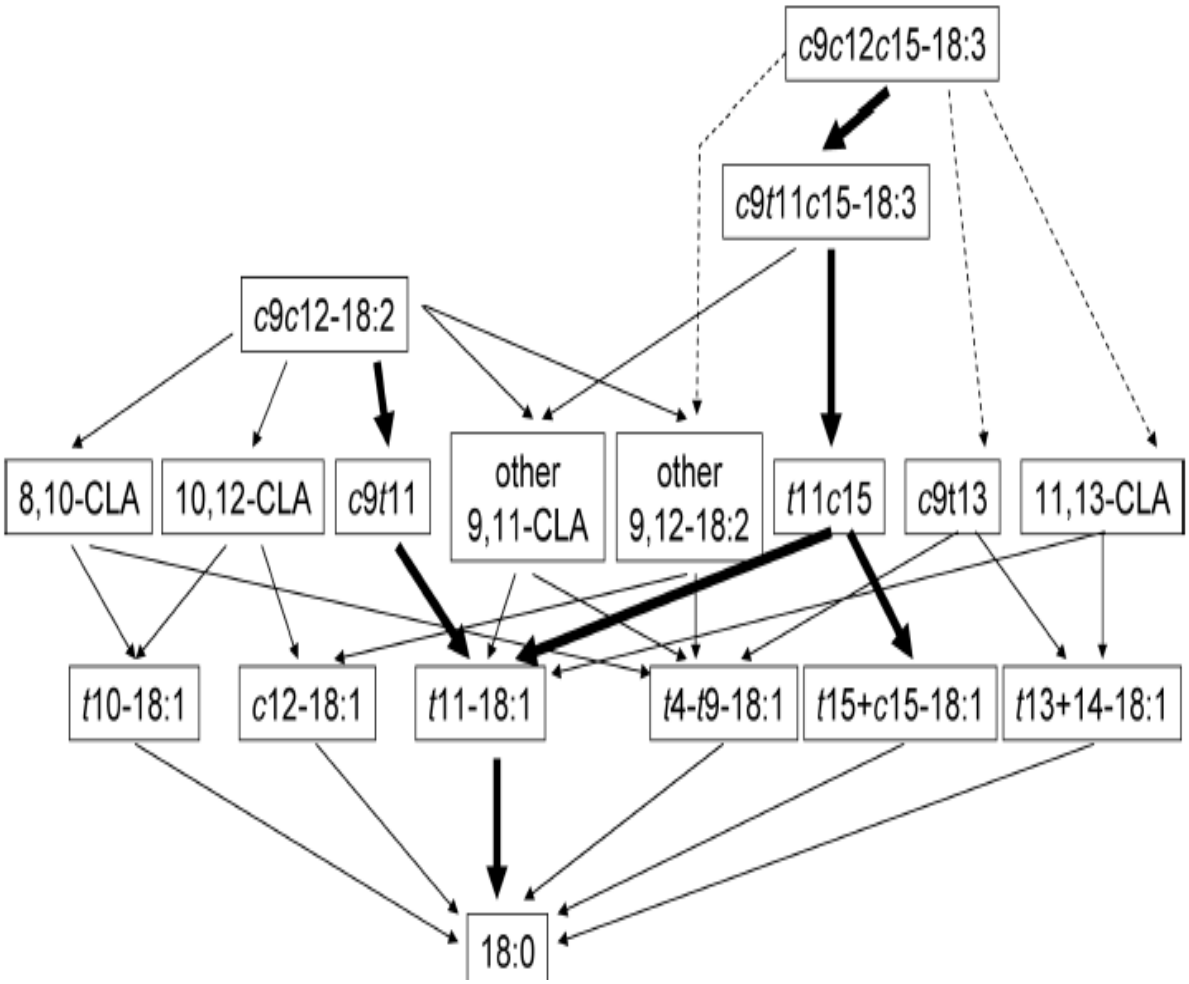


Figure 1.8 Biohydrogenation pathways of the main secondary and primary intermediates of C18:2 *n*-6 and C18:3 *n*-3 in the rumen (Adapted from Chilliard *et al.*, 2007). Thin arrows represent other possible secondary pathways whereas thick arrows represent the primary pathways.

1.4.2.2. Biohydrogenation intermediates

Although major intermediates of biohydrogenation have been discussed previously (section 1.4.2.1), it is important to emphasis some of the key intermediates, particularly those with known human health benefits. The process of biohydrogenation produces various intermediates; the most important of these intermediates recognized in human health are conjugated linoleic acid (CLA) and its precursor, vaccenic acid (VA, 18:1 *trans*-11). *Cis-9 trans-11* CLA isomer is one of the most important transient intermediates of RBH of UFA because of its anti-carcinogenic, anti-atherogenic, anti-diabetic and anti-inflammatory effects (Kritchevsky, 2000, Jenkins *et al*., 2008; Kennedy *et al*., 2010; Crumb, 2011). However, a wide range of other positional and geometrical fatty acid isomers including modified FA (keto and hydroxyl derivatives) are produced during the biohydrogenation of C18:2 *n*-6 in the rumen (Jenkins *et al*., 2008; Figure 1.8). Absorption and incorporation of these BH intermediates into milk fatty acid differs depending on their concentrations. But, their production can be influenced under experimental conditions using specific diets (Harvatine and Bauamn, 2006).

Conjugated linoleic acid is the collective name for a series of geometric and positional isomers of 18:2 *n*-6 possessing conjugated double bonds. There has been a surge in interest in the study of *cis-9 trans* 11 CLA in the last decade. This increased interest is due to the health benefits of *cis-9 trans* 11 CLA as discussed previously (Belury, 2002; section 1.2.6). Ruminant food products are the natural and richest dietary sources of CLA available for human consumption. The isomer, *cis-9, trans-11* CLA accounts for more than 90% of the total CLA present in ruminant milk fatty acid composition (Piperova *et al*., 2002; Kay *et al*., 2004; Savoini *et al*., 2010). The *cis-9, trans-11* CLA in milk fatty acid can possibly result from two sources: firstly, it is the product of ruminal incomplete biohydrogenation of 18:2 *n*-6 by microorganisms. Secondly, *cis-9, trans-11* CLA originates endogenously from the mammary gland by the action of Δ-^{9} desaturase enzyme which converts vaccenic acid (VA, *trans*-11 18:1) to *cis-9, trans-11* (Griinari *et al*., 2000). The latter represents more than 70% of the total CLA present in milk fat of ruminant origin (Bauman *et al*., 2006; Santora *et al*., 2000; Piperova *et al*., 2002). This suggests that major proportion of the total CLA content of milk results from desaturation of VA and means that increasing the ruminal concentration of VA would have a proportional effect on the level of CLA.

Although CLA in the rumen is synthesized only during the ruminal biohydrogenation of linoleic acid, biohydrogenation of both linolenic and linoleic acids generates VA (Bauman *et al*., 2003; Figure 1.7& 1.8).

It is a paradox that ruminant products which have long been blamed for their high contents of saturated fatty acids, and as such, potential risk factors of chronic disease, are also rich sources of nutraceutical component such as CLA. Nevertheless, as conjugated linoleic acid is a fatty acid, there is reduced concentration of this fatty acid in foods due to the renewed interest in the production of reduced-fat or fat-free ruminant products (Webb and O'Neill, 2008). The established importance of CLA and its reduced concentration in ruminant products has necessitated exploration of dietary approaches to modify ruminal biohydrogenation so as to enhance its amounts in ruminal products. Studies have indicated that the concentration of CLA in ruminant derived products can be increased through dietary manipulation of ruminant feeding (Lawson *et al*., 2001). See section 1.4.2.1 for other major intermediates of biohydrogenation.

1.4.2.3. Factors affecting biohydrogenation

The major sources of lipids used by ruminant nutritionists in the formulation of diets are marine origin (algae and fish oil) and plants (Kim *et al*., 2009). Lipolysis of unprotected oils has been suggested to be in the range of 85-95% (Doreau and Ferlay, 1994; Dewhurst *et al*., 2006). By contrast, lower lipolysis has been proposed for the structural plant lipids because of the requirement for the removal of the surrounding matrices before the occurrence of lipolysis (Doreau and Ferlay, 1994; Dewhurst *et al*., 2006). This indicates that lipids in oils are more degraded than forage lipids and suggest that a number of factors regulate the extent of ruminal biohydrogenation of PUFA. Lipolysis is a rate limiting step for biohydrogenation (Harfoot and Hazlewood, 1997). Forage maturity (Gerson *et al*., 1986), particle size (Gerson *et al*., 1988) and diet composition (Gerson *et al*., 1983; Gerson *et al*., 1985) are among the different factors responsible for altering *in vitro* rate of lipolysis.

Ruminal biohydrogenation of unprotected linoleic and linolenic acids across a range of different types of diets is in the mean range of 85 and 93% respectively (Fievez *et al*., 2007; Jenkins *et al*., 2008). Glasser *et al*. (2008) concluded from a meta-analysis of the ruminal metabolism of C18 fatty acids with database of 294 treatments in 77 experiments that a number of factors are responsible for the extent of biohydrogenation in the rumen. These factors are the forage: concentrate ratio, fish oil supplementation, ruminal pH and level of feed intake. When the forage content of the diet is higher than the proportion of concentrate, higher extent of biohydrogenation is more likely because of the likelihood of cellulolytic bacteria to dominate such diets. However, when concentrate is higher, other alternative pathways of biohydrogenation occur, with the formation of some *trans* fatty acids (Chilliard *et al*., 2007). It was observed that C18:3 *n*-3 and C18:2 *n*-6 were considerably protected at low ruminal pH than at high pH (Glasser *et al.*, 2008). Increased feed intake causes a corresponding increase on the extent of linoleic and linolenic acid biohydrogenation, especially if the forage proportion of the diet is high.

The extent of biohydrogenation of PUFA in the rumen can also be determined by the amount and type of fat added to the diet. For instance, the extent of biohydrogenation of constituent PUFA in diet supplemented with marine oils which contain EPA and DHA as the main fatty acids would be lower than the biohydrogenation of C18 PUFA found in oilseeds such as linseed, sunflower or soybean. This

inability of microbes to hydrogenate the fish oil fatty acids is not due to the difference in the lipase activities but because microbes lack the enzymes necessary to hydrogenate the long chain *n*-3 PUFA (Ashes *et al*., 1992). Advance maturity of forage and forage which has been thoroughly ground before added to the diet can also influence the biohydrogenation of PUFA in the rumen (Buccioni *et al*., 2012). In the latter, it can be speculated that grinding forage to produce fine particles would reduce the adherence of bacteria to feed particle and increase the rate of passage of food material through the rumen. Increased passage rate would consequently reduce the resident time and microbial activities, hence, decreased biohydrogenation of PUFA. Biohydrogenation has also been shown to be influenced by plant secondary metabolites such as tannins, protein bound phenols (PBP) and fatty acid oxidation (Cabiddu *et al.*, 2010).

1.4.2.4. Microorganisms involved in lipolysis and biohydrogenation

Lipases responsible for lipolysis are from both microbial and dietary origin. However, the actual contribution of dietary and microbial lipases to these processes is not clear (Lourenco *et al*., 2010). About 74 strains of microbial lipases in the rumen have been reported, all with varied lipolytic activity (Fay *et al*., 1990). The lipolytic activity of *Butyrivibrio fibrisolvens* and *Anaerovibrio lipolytica* has been extensively studied and reported (Harfoot, 1978). Hespell and O'Bryan-Shah (1988) examined the lipolytic activities of 30 strains of *Butyrivibrio fibrisolvens* and observed that there is wide variation among strains of *Butyrivibrio fibrisolvens*. *Anaerovibrio lipolytica* hydrolysed triglycerides less rapidly than diglycerides; however, they did not hydrolyse galactolipids and phospholipids (Henderson, 1971). The production of saturated fatty acids require first, the hydrolysis of dietary esterified lipids by plant (Lee *et al*., 2004) and microbial (Harfoot, 1978) lipases to release the constituent fatty acids. A well-recognized ruminal lipolytic bacterium (*Anaerovibrio lipolytica*) (Hungate, 1966) produces an extracellular lipase that has the potential for hydrolysing diglycerides more readily than triglycerides. Galactolipids and phospholipids are not however, affected by these lipases (Kim *et al*., 2009). Latham *et al*. (1972) identified bacteria with the same morphology like those of the genus *Butyrivibrio* having the capacity to hydrolyse triglycerides. A *Butyrivibrio* strain known as LM8/1B and *Butyrivibrio fibrisolvens* have also been observed to carry out phospholipase activity (Hazlewood and Dawson, 1975).

Hydrolysis of ingested esterified plant lipids has also been linked with the activity of ciliated rumen protozoa. In the early 1960s and 1970s; the activity of protozoa in lipolysis was reported (Wright, 1961; Latham *et al*., 1972). Wright (1961) suggested that protozoa, particularly, *Epidinium spp*. could contribute about 40% of the total lipolysis occurring in the rumen. This observation emerged after it was observed that ruminal lipolysis was reduced when cultures were treated with penicillin. Harfoot and Hazlewood (1988) proposed that the engulfment of lipases in the chloroplasts by protozoa could be responsible for the lipolytic activity in protozoa. The ruminal fungi have not been linked with the hydrolysis of esterified lipids in the rumen; however, there is evidence that they are involved in biohydrogenation (Nam and Garnsworthy, 2007).

Biohydrogenation of FFA has been exclusively attributed to rumen bacteria (Lourenco *et al*., 2010). The main microbes which are responsible for biohydrogenating PUFA are the surface-associated bacteria (Lough, 1970). This is because the released PUFA are absorbed to the surface of plant material by means of hydrophobic interactions (Harfoot *et al*., 1973). Initially, the biohydrogenating bacteria were grouped into group A and group B (Kemp and Lander, 1984). The group A bacteria are generally seen as bacteria with the ability to reduce PUFA to form vaccenic acid, whilst, group B bacteria have the ability to biohydrogenate UFA to stearic acid. The main group A bacteria have been recognized as *Butyrivibrio fibrisolvens*, whereas *Fusocillus* spp has been known as the main group B bacteria (Kemp *et al*., 1975; Harfoot and Hazlewood, 1997). More recent studies have shown that the C18:0 producers are clustered together and strains are so similar to *Clostridium proteoclasticum* (van de Vossenberg and Joblin, 2003; Wallace *et al*., 2006). These biohydrogenating bacteria are generally recognized as cellulolytic bacteria (Kepler and Tove, 1967), particularly, the *Butyrivibrio* group which are the most active group (Durmic *et al*., 2008). Although the bacterium that was called *Clostridium proteoclasticum* (Kemp *et al*., 1975; Maia *et al*., 2007) which is now re-classified as *Butyrivibrio proteoclasticus* (Moon *et al*., 2008), is the only bacteria capable of converting VA to C18:0, all bacteria in the *Butyrivibrio* group are capable of producing CLA from linoleic acid. Hudson *et al*. (1998) observed that *Streptococcus bovis* has the ability to cause hydration of linoleic acid to 13-hydroxy-9-octadecenoic acid, hence diverting it from the biohydrogenation course. This early study provide evidence that other facultative ruminal bacteria

including *Lactobacillus*, *Staphyloccous*, *Pediococcus* and *Enterococcus* have the ability to hydrate linoleic acid (Hudson *et al*., 2000). In addition, a concurrent increase in *Megasphera elsdenii* within the rumen has been identified with increased concentrate feeding (Counotte *et al*., 1981). Biohydrogenation of linoleic acid to the *trans*-10, *cis*-12 CLA has been linked to the activity of *Megasphera elsdenii* (Kim *et al*., 2002). There is limited evidence whether there are many more bacteria involved in biohydrogentaion due to the time-consuming nature and the high cost of isolating such bacteria (Huws *et al*., 2006). Available evidence suggests that other bacteria such as ruminococcaceae, *Anaerovoax*, *Prevotella* as well as other clostridiales that have not been identified could also play a role in biohydrogenation pathways (Huws *et al*., 2006, Huws *et al*., 2011).

Although BH of PUFA has been exclusively attributed to bacteria, biohydrogenating activity has been reported from both protozoal and bacterial fractions of rumen contents (Wright, 1959; 1960). This suggests a potential role of protozoa in the saturation of PUFA. However, further study suggests that the rumen protozoa act as hosts for bacteria in a commensal relationship (Dawson and Kemp, 1969). Therefore, the role of protozoa in biohydrogenation was at this time questioned. An experiment conducted at the Rowett Research Institute shows that the concentration of CLA in protozoa was higher than in bacteria (Devillard *et al*., 2006). Protozoa were not shown to have delta-9 desaturase activity, suggesting that they preferentially incorporate VA and CLA produced by bacteria. Or-Rashid *et al*. (2007) also carried out an *in vivo* study and the data showed that the concentrations of PUFA and CLA in protozoa were higher than their concentrations in bacteria. Nam and Garnsworthy (2007) reported that fungi also have the potential to biohydrogenate PUFA, but at a rate lower than bacteria, even though there are not known to be involved in lipolysis.

1.5.0. Methods for protecting lipids from biohydrogenation in the rumen

Various technologies have been developed over the years to either protect ruminal fermentation against the negative effects of dietary lipids or to protect the constituent PUFA against biohydrogenating microorganisms in the rumen. These technologies include hydrogenation or saponification of fatty acids and crystallization of fat (Jenkins and Palmquist, 1984), formaldehyde treatment of whole oilseeds and encapsulation of lipids in a formaldehyde-treated protein sources (Scott *et al*., 1970). Natural protection of dietary fat from ruminal biohydrogenation can also be achieved through inclusion of whole oilseeds with intact seed coat in ruminant diets instead of oils (St John *et al*., 1978; Solomon *et al*., 1991; Ekeren *et al*., 1992).

A biologically effective technology to protect lipid from biohydrogenation is expected to have some essential features such as:

a. The technology should have minimal adverse effect on general fermentation
b. The protection mechanism should be inert in the rumen
c. The flow of unsaturated fatty acids to the duodenum should be predictable and consistent
d. There is sufficient release and absorption of unsaturated fatty acids in the duodenum by the technology.

A number of methods are available to test the effectiveness of protected lipid mechanisms (Ashes *et al*., 1979). For *in vitro* and *in vivo* testing mechanisms, a 60% and 70% protection respectively is considered satisfactory (McDonald and Scott, 1977).

1.5.1. Formaldehyde protein-lipid supplements

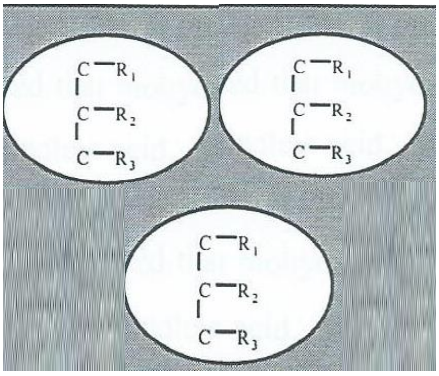
These mechanisms which were first developed over four decades ago involved the emulsification of polyunsaturated vegetable oils with plant proteins or casein at 70°C with pH 6.8 (Scott *et al*., 1970; Figure 1.9a). After forming emulsion, formalin was introduced as a fine mist during the drying process of the spray dried emulsion. The interaction of formaldehyde and amino groups resulted to inter and

intramolecular methylene linkages which reduced the susceptibility of the protein to ruminal degradation. When the supplements were fed, the proteins were solubilized in the abomasum and pancreatic lipase hydrolysed the lipids before absorption in the small intestines (Scott *et al*., 1970).

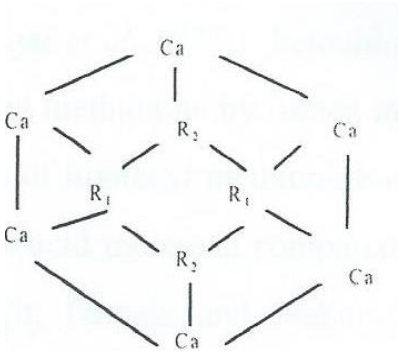
It was observed that high amount of dietary fats in the form of protected lipids did not have any deleterious effects on rumen fermentation (Palmquist and Jenkins, 1980). This observation stimulated extensive study into lipid utilization by ruminants (Palmquist and Jenkins, 1980). The proportion of protected lipid supplement *in vitro* was observed to be closely related to the levels of formalin added (Ashes *et al*., 1979). Similarly, the concentration of formalin in protected lipid supplement fed to goats was also proportional to the milk fatty acid response in the animals (Ashes *et al*., 1979). A significant correlation between resistance to ruminal hydrolysis and resistance to ruminal biohydrogenation was reported (n=6, r^2=0.96). The formaldehyde treatment of vegetable oils was modified by Scott and Cook (1973) to enable the use of natural oilseeds as the source of both protein and oil. However, protein which is required for efficient emulsification and subsequent protection of polyunsaturated oils was low in some oilseeds such as sunflower. Therefore, the addition of small amounts of casein (5-10%) or the mixture of soybean meal or seeds and sunflower (about 30 -70 parts, respectively) was used to overcome this deficiency. Knight *et al*. (1978) reported that oilseeds treated directly with formalin only offered partial protection from hydrolysis and biohydrogenation in the rumen. The physical breakdown of the product during mastication and insufficient control of the manufacturing process was suggested as the cause of this partial protection (Ashes *et al*., 1979). Notwithstanding the incomplete protection, the feeding of steers with formaldehyde protected canola and cotton seeds increased unsaturation in the subcutaneous adipose tissue (Scott and Ashes, 1993). In addition, the concentrations of C18:3 *n*-3, C18:2 *n*-6 and C18:1*n*-9 in milk was significantly increased after feeding protected canola supplements to lactating dairy cows (Ashes *et al*., 1992). The content of C18:2 *n*-6 in tissues of cattle (Garrett *et al*., 1976) and sheep (Faichney *et al*., 1973; Hogan and Hogan, 1976) was increased between 18-25% and 50-60, respectively, when lipids were treated with formaldehyde. To date, the only documented protection method with increased proportion of the absorbed PUFA is the formaldehyde treatment. Other encapsulation methods

have not been very effective, such as those that involve encapsulation using calcium alginate (Ekeren *et al*., 1992).

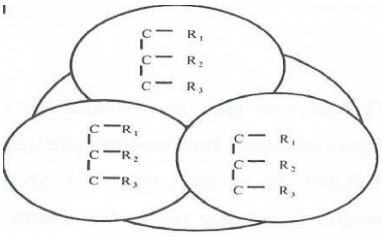
However, beside the fact that the use of formaldehyde would potentially be criticized by regulatory authorities, the inconsistency of this approach to reduce biohydrogenation of either C18:3 *n* -3 or C18:2 *n* -6 was reported by Sinclair *et al*. (2005). The use of linseed, a natural source of C18:3 *n* -3 (Givens *et al*., 2001), which was treated with formaldehyde and fed to sheep produced an insignificant increase in the flow of C18:3 *n*-3 to the lower digestive tract (Chikunya *et al*., 2004). The result of this investigation led Sinclair et al. (2005) to assume that pre-treatment with sodium hydroxide and formic acid prior to treating with formaldehyde could improve the flow of C18:3 *n*-3 to the small intestine. Therefore, linseed was pre-treated with sodium hydroxide and formic acid then treated with formaldehyde in an *in vitro* batch culture (Sinclair *et al*., 2005). This approach offered a better protection (about 45% and 31% for sodium hydroxide and formic acid respectively) of C18:3 *n*-3 from biohydrogenation compared with treatment with formaldehyde alone (8%). However, the use of formaldehyde may not be allowed by some regulatory bodies, therefore, this method has not been commercially accepted and used (Scollan *et al*., 2014).



a) aldehyde protein



b) calcium salt



c) Prilled fat

Figure 1.9 Techniques for protecting dietary fat (Adapted from Scott and Ashes, 1993). Ca=calcium, R1-R3= Methylene linkages, showing binding sites.

1.5.2. Saponification (Calcium soaps)

Saponification technique which involved the addition of calcium chloride to molten sodium salts of the lipid was proposed by Jenkins and Palmquist (1984; Figure 1.9b). After adding the calcium chloride, calcium salts precipitated and the soap was air dried before milling and added to diets. The authors proposed this means of protecting PUFA from ruminal biohydrogenation because calcium soaps dissociated in the acidic environment of the abomasun but were insoluble in the rumen.

Wu *et al*. (1991) reported 49% biohydrogenation (51% protection) by using calcium salt of palm oil to reduce microbial degradation in the rumen compared with 80% biohydrogenation for the control diets. Tallow calcium soaps allowed a normal fibre digestibility of 50%, whilst tallow fatty acids caused a decrease in fibre digestibility from 51% (control cows) to 45% (Jenkins and Palmquist (1984). The negative effects of calcium soaps based on palm oil were not observed by Elmeddah *et al.* (1991). This was attributed to the low dissociation of calcium salts at a normal rumen pH of 6-7 (Sukhija and Palmquist, 1991). However, subsequent studies reported extensive biohydrogenation of calcium salts, especially those of USFA at pH less than 6.0 (Ferlay *et al*., 1993; Enjalbert *et al*., 1994). The rapid decline in pH upon feeding the calcium soaps was suggested to be responsible for the extensive biohydrogenation. Subsequent *in vitro* studies confirmed the effects of rumen pH on calcium soaps (Van Nevel and Demeyer, 1996). The flow of unsaturated fatty acids to the duodenum was not affected when calcium linoleate was fed to sheep (Fotouhi and Jenkins, 1998). Therefore, it was suggested that protection of unsaturated fatty acids from ruminal microbial break-down is only possible if an insoluble matrix of saturated calcium salts is used for the encapsulation. This suggests that protection is only likely at low concentration of unsaturated fatty acid content, thereby limiting the extent to which alteration of the unsaturated fatty acid composition of milk and meat can be achieved (Jenkins and Bridges, 2007). The report by Enjalbert *et al*. (1997) supported this suggestion where the feeding of calcium salts of palm oil fatty acids resulted in higher flow of linoleic acid to the duodenum than the feeding of calcium salts of rapeseed fatty acid. When a commercial product based on calcium salts of soyabean oil was used to replace soyabean oil (Oliveira *et al*., 2012), the concentration of 18:2 *n*-6 was observed to increase in the

muscle of beef cattle. However, experimental application of this process to linseed oil did not protect PUFA from ruminal biohydrogenation (Oliveira *et al*., 2012).

1.5.3. Prilling (prilled fatty acids)

A dried prilled fatty acid supplement which is inert in the rumen environment is obtained by liquefying a mixture of fatty acids containing high levels of saturated fatty acid and passing the fatty acid mixture under pressure into a cooled atmosphere (Figure 1.9c). Prilling can therefore be defined as the process in which fatty acids are crystallized together in a matrix through a rapid cooling process resulting in the production of small spherical beads (Schauff and Clark, 1989; Figure 1.11). Because prilled fat supplements are inert in the rumen, nutrient digestion is not affected when the supplement is added at 3.5% or less in the diet. Calcium salts of palm oil fatty acids and a relatively saturated fat source (C18:1, 12.8%; C18:0, 35.1% and C16:0, 48.6%) were compared by Grummer (1988). When the rumen pH is maintained at above 6.0, hydrolysed saturated fatty acids react more readily with metal ions resulting in the formation of salts that are insoluble in the rumen (Palmquist, 1984). In *in vitro* experiment to assess the efficiency of fat supplements for ruminants, Gulati *et al*. (1997) observed that there was 90% and 65% biohydrogenation for the control and prilled fat supplement respectively. However, the potential of prilling to protect PUFA from biohydrogenation was not observed in other studies. For example, Harvatine and Allen (2006) reported that prilling did not affect milk fat concentration and the fatty acid profile of milk fat obtained from animals fed prilled fat supplement was similar to the control.

1.5.4. Fatty acyl amides

Fatty acyl amides which resist microbial breakdown in the rumen are formed by reacting fatty acids with amines (Fotouhi and Jenkins, 1992a; Jenkins, 1995). The amide bond which results from the primary amine group and the fatty acid carboxyl group is digestible in the small intestine, but undegradable in the rumen (Langar *et al*., 1978). Biohydrogenation of linoleic acid by rumen microorganisms was observed to be higher than that of linoleoyl methionine (Fotouhi and Jenkins, 1992b). The duodenal flow of linoleic acid increased when small quantities of linoleoyl methionine were added directly to the rumen of sheep compared with a free linoleic acid supplement (Fotouhi and Jenkins, 1992b; Jenkins, 1995). Addition of soybean oil amide derivates to a diet for lactating cows increased the concentration of 18:2 *n*-6 in plasma

from 54.3% (control) to 59.0% (Jenkins *et al.*, 1996). However, this protection technique is not commercially available yet. In addition, Jenkins (1998) reported that addition of oleamide to the diet for dairy cows negatively affected dry matter intake compared with the control.

1.5.5. Oil seeds

Polyunsaturated fatty acids in dietary fats can be naturally protected from ruminal biohydrogenation through the supplementation of whole oilseeds with intact seed coat in ruminant diets instead of oils. This has been observed to elevate the concentration of USFA in muscle tissue (St John *et al.*, 1978; Solomon *et al.*, 1991; Ekeren *et al.*, 1992). However, the efficiency of this technique can be reduced during processing and mastication, which disrupts the intact seed coat. For efficient post-ruminal digestion, it has been generally accepted that oilseeds or grains for ewes and cattle should be rolled or coarse ground before feeding. This form of processing would disrupt the seed coat consequently exposing the lipids to microbial transformation in the rumen. Orskov *et al.* (1974) suggested that there is no advantage in processing oil seeds because sheep masticate their feed efficiently.

In summary, comparison of the ruminal fatty acid protection potential for some of the available protection technologies was made by Lundy *et al.* (2003). In that study, the authors offered three equal amounts of soybean fatty acids to cows as amides, unprotected and as calcium salts. The C18:2 ruminal disappearance as a proportion of intake was 92.4%, 94.8 and 92.2 for the amides, the unprotected oil and the calcium salts, respectively. This clearly demonstrates a non-substantial difference between the control and the two protection technologies. In addition, de Veth *et al*. (2005) conducted a study to evaluate the effect of formaldehyde and calcium salts protected forms of a *trans*-10, *cis*-12 CLA. It was observed that the mean transfer of the CLA isomer to milk fat was 3.2 and 7.0 for the calcium salts and formaldehyde treatments, respectively. This compared with approximately 20% transfer effectiveness for abomasal infusion of the same CLA. This indicates less rumen protection efficiency by both calcium salts and formaldehyde treatments. However, protection was better with formaldehyde treatment than calcium salts.

As discussed above, inhibition of ruminal biohydrogenation is not consistent with any of the available protection technology and none of these technologies meets all the essential features of any supplement to protect ruminal biohydrogenation of PUFA. Therefore, in the recent times, considerable attention has been given to evaluation of plant secondary metabolites and how they influence ruminal biohydrogenation of PUFA. These compounds which possess antimicrobial activities are thought to inhibit lipases involved in hydrolysis of fat.

1.6. Plant Bioactive Compounds and rumen Biohydrogenation

Plant secondary metabolites (PSM) refer to a wide range of chemical compounds which are produced by plants but are not a requirement for the primary biochemical processes involved in growth, reproduction and development of the plant (Calsamiglia *et al.*, 2007; Patra, 2012). The primary functions of these PSM in plants are that they act as protective agents against invasion from a wide range of foreign particles such as pathogenic microorganisms (Calsamiglia *et al.*, 2007).

The use of plant bioactive compounds (PBC) such as tannins, saponins and essential oils to modify ruminal biohydrogenation is quite a recent development. Over many decades in the past, the main aim of evaluating plants (trees, bushes or forages) which are high in secondary compounds was to ascertain their suitability as feeds in livestock farming such as their effects on digestion of feeds, performance and growth, reproduction and health of animals (Vasta and Bessa, 2012). But, the interest in evaluating the influence of PBC on the fatty acid composition of meat was developed only in the last few years (Vasta *et al.*, 2013). In this review, only the effects of essential oils on rumen fermentation and biohydrogenation would be considered. However, the effects of other PBC on rumen fermentation and biohydrogenation of PUFA can be found in the recent review by Patra (2012). The effects of essential oils or their constituent compounds on VFA and methane production and ammonia nitrogen metabolism have been adequately investigated and recently reviewed (Patra, 2011; Vasta and Bessa, 2012). However, there is a scarcity of information on the potential of EOs or EOCs as modifiers of rumen biohydrogenation of PUFA and concentrations of BH intermediates such as CLA and VA. The aim of this PhD study was to compare the potential of EOs and EOCs as feed additives to reduce the extent of rumen biohydrogenation of *n*-3

PUFA. This would potentially enable the optimization of the fatty acid composition of ruminant food products with the use of either of these substances as feed additive.

1.7.0. Essential oils (the proposed technique)

1.7.1. Definition of Essential oil

Essential oils which are also known as volatile oils are aromatic (relating to the smell and taste) oily liquids (Guenther, 1948; Burt, 2004). The presence of EOs in plants determines the unique smell of plants due to the different smells and aroma produced by different EOs (Szumacher-Strabel and Cieslak, 2012). These complex compounds are produced as secondary metabolites from aromatic plants, particularly, spices and herbal plants (Bakkali *et al*., 2008; Patra, 2011). Contrary to what the name suggests, these oils are not oils or lipids, but mainly volatile aromatic compounds extracted from different parts of a plant such as the seeds, flowers, herbs, leaves, wood, bark, buds, roots and twigs (Burt, 2004; Hart *et al*., 2008). Naturally, the existence of EOs in a plant may provide protection for the plant against external invasion from organisms such as fungi, virus, bacteria and insects, as well as reducing the appetite of herbivores for such plant (Greathead, 2003; Bakkali *et al*., 2008). The term 'essential oil' is derived from 'Quinta essentia', a name which was first used by Paracelsus Von Hohenheim, a Swiss reformer of medicine, in the 16th century (Guenther, 1948). This term 'essential' coins from essence, is meant to relate to the property (smell or taste) of these materials in which the primary function is to provide specific odours or flavours to the parent plant (Calsamiglia *et al*., 2007). Although there are several methods of obtaining these oils from the plant, such as expression, extraction and fermentation, steam distillation is the most widely used method for the commercial production of EO (Gershenzon and Croteau, 1991; Van de Braak and Leijten, 1999). Steam distillation, which was initially developed and used in the Middle Ages by Arabs, is popularly used to extract EOs to prevent compositional changes due to the volatile nature of these oils (Simon, 1990; Greathead, 2003; Bakkali *et al*., 2008). Because of their volatile nature, EOs are usually stored in airtight jars in dark areas to prevent compositional changes. The quantity and quality of EOs may vary depending on the soil composition, climatic condition, vegetative stage, age and the plant organ from which the oil is extracted (Masotti *et al*., 2003; Angioni *et al*., 2006).

Naturally, EOs have a range of properties such as antifungal, insecticidal and antibacterial, and they have been used based on those properties (Thormar, 2011). Generally, about three thousand EOs are known,

but only around 300 have been commercially described by the fragrant and flavour companies (Van de Braak and Leijten, 1999). The usefulness of the described (referring to the 300 above) EOs have been shown in different industries like agriculture, sanitary, pharmaceutical, perfume, food, and cosmetic (Calsamiglia *et al*., 2007; Bakkali *et al*., 2008). They are also used as mixtures of vegetal oil used for massages, in aromatherapy and baths (Thormar, 2011). Some essential oils have been suggested to possess potential for the cure and prevention of systemic malfunction and organ dysfunction due to certain medicinal properties (Perry *et al*., 2003; Silva *et al*., 2003).

1.7.2. Composition of EO

EOs have a wide range of activities due to the diverse nature of their chemical composition. Compositional analyses of EOs have indicated that essential oils are a mixture of about 20-100 individual constituent compounds (EOCs) at different proportions (Senatore, 1996; Russo *et al*., 1998, Pengelly, 2004; Dung *et al*., 2008). The composition of the profile of EO can be determined either by gas chromatography or mass spectrometry (Juliano *et al*., 2000; Delaquis *et al*., 2002). The estimated proportion of the major components of EOs are around 20-85%, whilst trace amounts of other minor components account for about 15% of the total (Senatore, 1996; Bauer *et al*., 2001). For instance, the main components of *Coriandrum sativum* essential oil is linalool (68%); thymol (27%) and carvacrol (30%) are the main components of *Origanum compactum* essential oil; menthone (19%) and menthol (59%) are of *Menthe piperita*; and 1,8-cineole (50%) is the main component of the essential oil of *Cinnamomum camphora* (Calsamiglia *et al*., 2007; Bakkali *et al*., 2008). The antimicrobial properties of essential oils are predominantly determined by the major active compounds. However, other minor or trace compounds can also contribute either synergistically or antagonistically to the activities of the main components (Burt, 2004).

Structurally, EOs are complex mixtures of a diverse range of chemical compounds having terpenoids (monoterpenoids and sesquiterpenoids) and phenylpropanoids as the most active forms (Calsamiglia *et al*., 2007). A variety of low molecular weight aliphatic hydrocarbons, aldehydes, alcohols, lactones or acyclic esters and acids may also exist (Dorman and Deans, 2000). These two most active compounds, terpenoids and phenylpropanoids, are derived from different precursors of the primary metabolism, and

are synthesized from two separate pathways of metabolism (Gershenzon and Croteau, 1991; Calsamiglia *et al*., 2007). Based on these classifications, terpenoids are the most important and more abundant (about 15,000 compounds have been described) compounds in herbs and spices compared to the phenylpropanoids (Gershenzon and Croteau, 1991).

Terpenoids, which are synthesized from mevalonate metabolic pathways, are derived from a basic structure of C-5 isoprene units, and the number of these units in its skeleton determines the classification of terpenes (Calsamiglia *et al.*, 2007). Predominantly, terpenes occur as monoterpenes (C_{10}) and sesquiterpenes (C_{15}), but other categories such as diterpenes (C20), sesterterpenoids (C25), triterpenes (C30) and tetraterpenes (C40) may also exist (Gershenzon and Croteau, 1991; Thormar, 2011; Patra, 2012). The monoterpenes, which are the most abundant form of terpenes, are made from the linkage of two isoprene units; hence, they have 10 carbon atoms (Calsamiglia *et al*., 2007). As the most common form of terpenes, monoterpenes have a diverse range of structural composition, and constitute about 90% of EOs constituent compounds (Bodas *et al*., 2012). Types of monoterpenes (acyclic, bicyclic etc.) and their examples are shown in Table 1.3.

Sesquiterpenes are formed from the combination of three isoprene units (C_{15}). Different structures of sesquiterpenes are formed from the extension of the preceding chain, leading to increases in the number of cyclization (Thormar, 2011). In terms of functions and structures, sesquiterpenes are similar to monoterpenes (Bakkali *et al*., 2008; Thormar, 2011). Some types of sesquiterpenes and their examples are shown in Table 1.4. A number of plants such as Juniper, caraway, citronella, angelica, mandarin, pine, eucalyptus, lemon, thyme, bergamot, coriander, lemongrass, lavender, rosemary, mint, sage, peppermint, lavandin, celery, orange and geranium, contain these compounds (Bakkali *et al*., 2008).

The second most abundant form of EOCs is the Phenylpropanoids. Relative to the terpenoids, phenylpropanoids do not exist in high proportions in EOs, however, in some plants; their concentration may be significant (Calsamiglia *et al.*, 2007). Phenylpropanoids are derived from phenylalanine, and are synthesized through the shikimate pathway, a process which is only functional in plants and microorganisms (Sangwan *et al.*, 2001). Phenylpropanoids are compounds with a side chain of C3 which

is bound to an aromatic ring of six carbons (C6) (Calsamiglia *et al*., 2007). Types of phenylpropanoids and their examples are shown in Table 1.5. The main plant sources of this group of compounds are clove, sassafras, fennel, cinnamon, tarragon, nutmeg, anise, and some of the botanical families from which these plants belong are Rutaceae, Lamiaceae, Apiaceae and Myrtaceae (Thormar, 2011).

Some plants also contain sulphur and nitrogen containing compounds such as isothiocyanate or glucosinolates derivatives (mustard and garlic oils) (Dorman and Deans, 2000).

In terms of antimicrobial potency, essential oil constituent compounds can be grouped into the following in order of descending potential: phenols > cinnamic aldehyde > alcohols > aldehydes (ketones > ethers > hydrocarbons) (Kalemba *et al*., 2012).

As mentioned previously, although steam distillation is widely used for commercial production of EOs, obtaining these oils by means of liquid carbon dioxide under high pressure and low temperature is also available (Moyler, 1998). However, this CO_2 method is more expensive but produces oil with more natural organoleptic composition relative to steam distilled oils (Moyler, 1998). These two methods of extracting EOs are responsible for the difference in the composition of essential oil profile (Burt, 2004). The difference in the antimicrobial properties of EOs and their compounds is also attributed to the method used during extraction (Burt, 2004). This difference in antimicrobial properties have been shown where the steam distilled EOs exhibit lower antimicrobial activity compared to the antimicrobial potential of herb EOs extracted by hexane (Packiyasothy and Kyle, 2002).

The antimicrobial properties of EOs have been suggested to be influenced mainly by the phenolic components (Cosentino *et al*., 1999). In such phenolic compounds, the hydroxyl group and the dislocated electrons permeate the interaction with water through hydrogen bridges, enabling phenolic compounds to be very active against wide range of microorganisms (Griffin *et al*., 1999; Davidson and Naidu, 2000; Dorman and Deans, 2000; Cox *et al*., 2001). Table 1.6 shows the main components of a number of EOs and their individual proportion. The structural formulas of different major chemical components of EOs are presented in Figure 1.10. It has been indicated that minor components produce some synergistic

effect with other major components, suggesting their possible function in determining the antimicrobial potential of EOs (Burt, 2004). This suggestion has been confirmed in several studies for some species of *Thymus* (Lattaoui and Tantaoui-Elaraki, 1994; Marino *et al*., 1999), Oregano (Paster *et al*., 1995) and sage (Marino *et al*., 2001).

In addition to the method of extracting EO as a factor responsible for the difference in composition and antimicrobial potential, the profile of EOs can also be influenced by geographical/environmental location and harvesting seasons of the parent plant (McGimpsey *et al*., 1994; Cosentino *et al*., 1999; Marino *et al*., 1999; Juliano *et al*., 2000). This can partly be understood by the formation of some antibacterial substances from their parent materials in a particular environment. As reported by many studies (Cosentino *et al*., 1999; Jerkovic *et al*., 2001; Ultee *et al*., 2002), the precursors of thymol (5-methyl-2-(1-methylethyl)phenol) and carvacrol (2-methyly-5-(1-methylethyl)-phenol) are γ-terpinene (1-methyl-4-(1-methylethyl)-1,4-cyclohexadiene) and *ρ*-Cymene (1-methyl-4-(1-methyl)-benzene). The specimens obtained from different environmental locations in Greece have been shown to contain the four compounds in equal amounts to the proportion found in Greek oregano plants (Kokkini *et al*., 1997). Similar report has been shown for *Thymus vulgaris* from Italy (Marino *et al*., 1999). This supports the theory that the four compounds are functionally and biologically related (Kokkini *et al*., 1997). In general, the strongest antimicrobial activity has been reported to be exhibited by EOs extracted from herbs which are harvested immediately after flowering or during flowering (McGimpsey *et al*., 1994; Cosentino et al., 1999; Marino *et al*., 1999). Different degree of antimicrobial potency has also been shown by different enantiomers of EOCs (Lis-Balchin *et al*., 1999). The essential oil composition of cilantro, which is obtained from the immature leaves of coriander (*Coriandrum sativum*), has been shown to have a different profile from the EO obtained from the seeds of the same coriander (Delaquis *et al*., 2002). This indicates that there can be wide variation between the compositions of EOs obtained from different parts of the same plant.

Table 1.3 Some types of monoterpenes essential oil compounds and their common examples (Adapted from Bakkali *et al*., 2008).

Type of monoterpenes	Examples
Alcohols	**Monocyclic:** carveol, menthol, α-terpineol, etc.
	Acyclic: linalool, lavandulol, citronellol, nerol, geraniol, etc.
	Bicyclic: fenchol, thuyan-3-ol, borneol, chrysanthenol, etc.
Aldehydes	**Acyclic:** neral, citronellal, geranial, etc.
Carbures	**Monocyclic:** p-cymene, terpinenes, phellandrenes, etc.
	Acyclic: ocimene, myrcene, etc.
	Bicyclic: -3-carene, sabinene, pinenes, camphene, etc.
Ethers	Menthofurane, 1,8-cineole, etc.
phenols	Carvacrol, thymol, etc.
Esters	**Monocyclic:** α-terpinyl acetate or menthyl, etc.
	Acyclic: citronellyl acetate, propionate or linalyl acetate, etc.
	Bicyclic: pinocarvone, fenchone camphor, pinocamphone, thuyone, ombellulone, etc.
Peroxydes	Ascaridole, etc.
Ketones	Monocyclic: carvone, piperitone, menthones, pulegone, etc.

Table 1.4 Some types of sesquiterpenes essential oil compounds and their examples (Adapted from Bakkali *et al.*, 2008).

Types of sesquiterpenes	Examples
Alcohols	β-nerolidol, β-santalol, carotol, patchoulol, farnesol, bisabol, viridiflorol, etc.
Carbures	Elemenes, azulene, zingiberene, β-bisabolene, farnesenes, curcumenes, cadinenes, logifolene, β-caryophyllene, etc.
Epoxide	Humulene epoxides, caryophyllene oxide, etc.
Ketones	Nootkatone, β-vetinone, germacrone, turmerones, cis-longipinan-2,7-dione, etc.

Table 1.5 Some types of phenylpropanoids essential oil compounds and their common examples (Adapted from Bakkali *et al*., 2008).

Types of phenylpropanoids	Examples
Phenols	eugenol, chavicol
Aldehyde	cinnamaldehyde
Methylene dioxy compounds	safrole, apiole, myristicine
Alcohol	cinnamic alcohol
Methoxy derivatives	elemicine, methyleugenols, anethole, estragole

Table 1.6 EOs and their major components which exhibit antimicrobial activities (adopted from Burt, 2004)

Botanical name	Common name	Major compound	Composition (%)	References
Coriandrum sativum (immature leaves)	Cilantro	Linalool,	26%	Delaquis *et al*. (2002)
		E-2-decanal	20%	
Syzygium aromaticum	Clove (bud)	Eugenol,	75-85%	Bauer *et al*. (2001)
		eugenal acetate	8-15%	
Coriandrum sativum (seeds)	Coriander	Linaloo,	70%	Delaquis *et al*. (2002)
		E-2-decanal	-	
Origanum vulgare	Oregano	Carvacrol	Trace-80%	Prudent *et al*. (1995)
		ρ-Cymene	Trace-52%	Marino *et al*.(2001)
		γ-terpinene	2-52%	Russo *et al*. (1998)
		Thymol	Trace-64%	Charai *et al*. (1996)
Thymus vulgare	Thyme	Thymol	10-64%	Lens-Lisbonne *et al*. (1987); McGimpsey *et al*.(1994); Marino *et al*.(2001); Juliano *et al* (2000)
		ρ-Cymene	10-56%	
		γ-terpinene	2-31%	
		Carvacrol	2-11%	
Rosmarinus officinalis	Rosemary	α-pinene	2-25%	Pintore *et al*. (2002)
		1,8-cineole	3-89%	
		Camphor	2-14%	
		Bornyl acetate	0-17%	
Cinnamomum zeylandicum	Cinnamon	Trans-cinnamaldehyde	65%	Lens-Lisbonne *et al*. (1987)
Salvia officinalis L.	Sage	1,8-cineole	6-14%	Marino *et al*.(2001)
		β –pinene	2-10%	
		α-tujone	20-42%	
		Camphor	6-15%	
		α -pinene	4-5%	

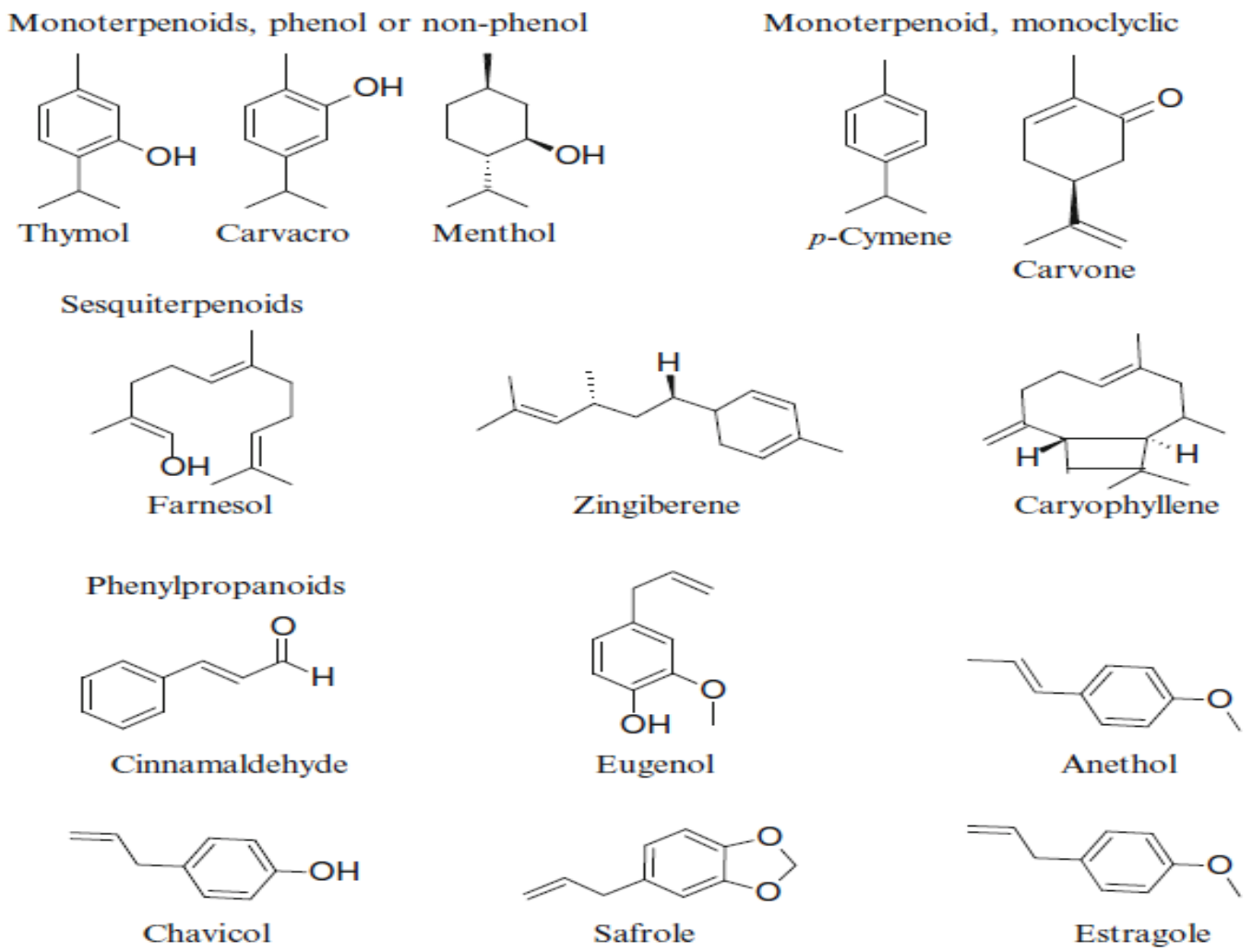


Figure 1.10 Chemical structures of different components of EOs (Adapted from Patra, 2102)

1.7.3. Mechanism of actions of EOs

Considering the large number of EOs and the diverse chemical compounds in them, it is unlikely that there would be one specific mode of antimicrobial action or that there is one specific target site for their actions (Skandamis *et al*., 2001; Carson *et al*., 2002). As indicated in Figure 1.11, a number of sites are thought to be affected by the action of EOCs in different ways. However not all the sites are affected by one action, some sites are affected as the consequence of the action of EOCs in another site (Burt, 2004). In brief, EOCs alter microbial activities through a number of ways: degrading microbial cell wall (Thoroski *et al*., 1989; Helander *et al*., 1998; Figure 1.11), damage to membrane proteins (Ultee *et al*., 1999; Figure 1.11), depletion of the proton motive force (Ultee *et al*., 1999; Ultee and Smid, 2001) and by damaging the cytoplasmic membrane (Ultee *et al*., 2002; Figure 1.11). They also achieve alteration of microbial populations by causing leakage of the cell contents (Cox *et al*., 2000; Lambert *et al*., 2001) and cytoplasmic coagulation (Gustafson *et al*., 1998).

Hydrophobicity of EOs and EOCs which enables them to partition in the phospholipid bilayer of microbial mitochondria and cell membrane, causing damage to the structure and increasing the permeability of the cell contents, is an important characteristic for the success of their mechanisms (Sikkema *et al*., 1994). Because of the increased permeability of microbial cell, leakage of its contents becomes possible (Helander *et al*., 1998; Skandamis *et al*., 2001; Ultee *et al*., 2002). Although death of microbial cell can result from loss of critical cell contents (ions and molecules) or extended loss of cellular materials, leakage of a certain amount of cellular contents may be tolerated with no adverse effect on the viability of the cell (Denyer and Hugo, 1991). This tolerance could be achieved by diverting large amounts of energy into ionic pumps to counterbalance the effect of the lost material, and this consequently slows down bacterial growth (Ultee *et al*., 1999; Cox *et al*., 2001). These mechanisms of actions are less effective against Gram-negative bacteria, where the cell membrane cannot interact directly with the hydrophobic compounds in EOs. This is because the external cell wall around the Gram-negative bacteria is hydrophilic, and therefore, not able to penetrate substances that are lipophilic in nature (Calsamiglia *et al*., 2007). In contrast, because Gram-positive bacteria in which the cell membrane can interact directly with the hydrophobic compounds of EOs, the described mechanism of action is more

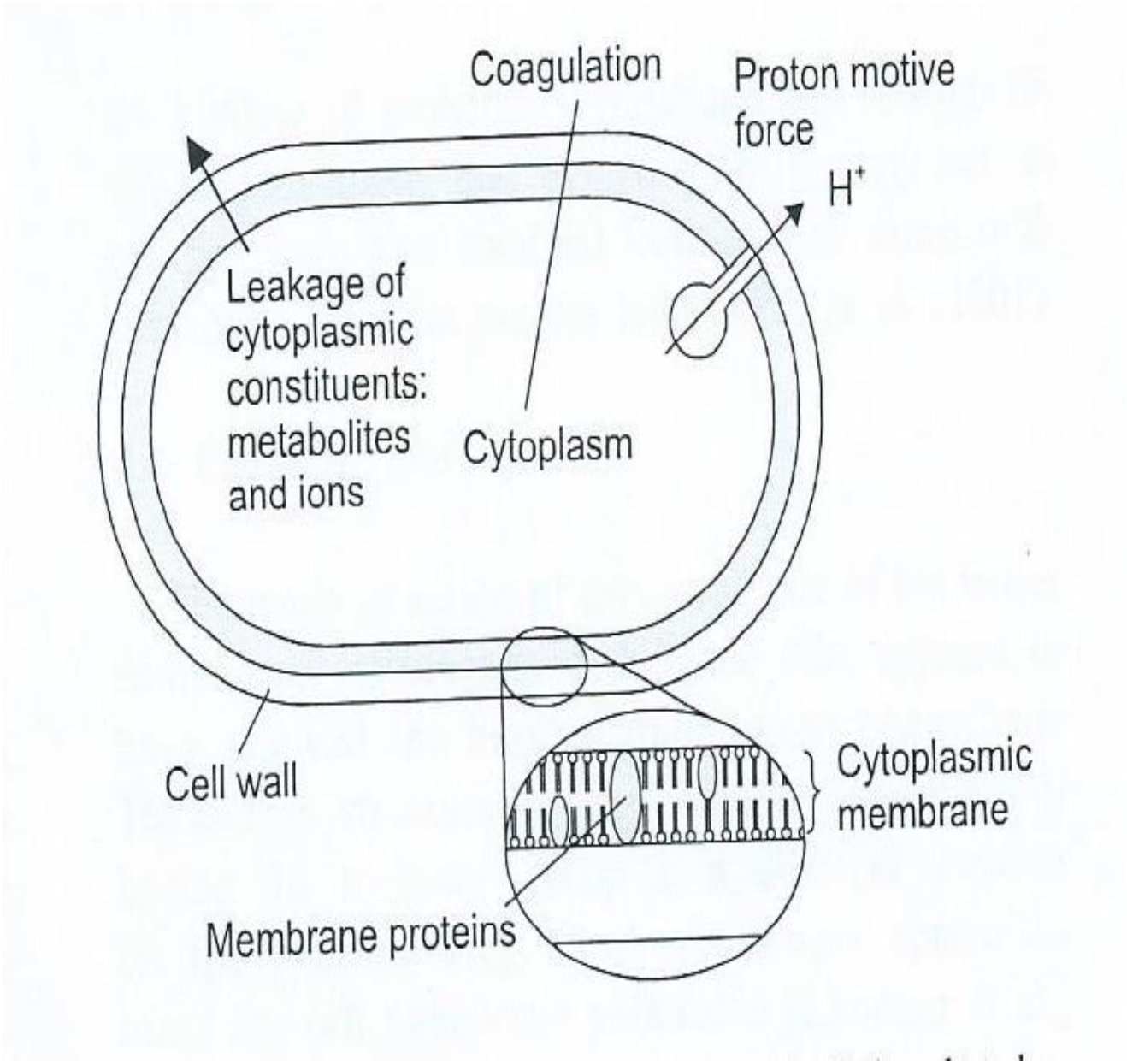


Figure 1.11 Mechanisms and sites in the microbial cell considered to be the locations of actions for EOCs (Adapated from Burt, 2004).

effective against Gram-positive bacteria (Calsamiglia *et al*., 2007). Hence, Gram-positive bacteria are more susceptible to the hydrophobicity of EOs and their compounds than Gram-negative bacteria which have surface layer surrounding the membrane (Chao and Young, 2000; Cimanga *et al*., 2002; Bencharr and Greathead, 2011). This difference is due to the variation in the microbial cell wall composition between the two groups of bacteria. It is this susceptibility of Gram-positive bacteria to the antimicrobial effects of essential oil compounds that makes them potential target. However, because the external membrane of Gram-negative bacteria is not completely impermeable, it can be penetrated by some EOCs that have low molecular weight such as thymol and carvacrol (Cox *et al*., 2001; Calsamiglia *et al*., 2007). This means that the described mechanism of action does not apply to the low-molecular weight molecules. These low-molecular weight compounds can penetrate the outer cell wall and interact with membrane lipid of bacteria by reacting with H_2O through hydrogen bridges, and can diffuse slowly through protein membrane or lipopolysaccharide layer (Griffin *et al*., 1999; Dorman and Deans, 2000; Calsamiglia *et al*., 2007). In addition, destruction of the external cell wall of Gram-negative bacteria leading to an increased penetration of the cytoplasmic membrane and the release of lipopolysaccharides by the ability of some aromatic hydrocarbons found in essential oils have been reported (Helander *et al*., 1998). It can be stated from the above mechanisms of EOCs with low-molecular weight that their selection for use in practice (animal nutrition) is limited due to their practical effectiveness against both the Gram-negative and Gram-positive bacteria. This non-selective effect of the low molecular weight compounds does not have a beneficial implication in animal nutrition as the use of EOs or EOCs is to cause selective inhibition of certain group of bacteria.

EOs containing high proportion of phenolic compounds such as thymol, carvacrol and eugenol (2-methoxy-4-(2-propenyl)phenol as components of their chemical structure exhibit the strongest antimicrobial properties against food borne pathogens (Cosentino *et al*., 1999; Juliano *et al*., 2000; Lambert *et al*., 2001). It is considered that other phenolic compounds are likely to exhibit similar antibacterial characteristics to the above. The mechanisms of action of phenolics are thought to generally include disruption of proton motive force (PMF), coagulation of cell contents, disturbance of cytoplasmic membrane and disrupting active transport and electron flow (Sikkema *et al*., 1995; Davidson, 1997).

The antibacterial activity and specific mode of action of an individual EO is influenced by the chemical structure of EO components (Dormans and Deans, 2000). The presence of hydroxyl group in the chemical structure of phenolics appears to be very vital for their high degree of antibacterial activities. The vital role of the hydroxyl ring in the antimicrobial activity of phenolics was confirmed in previous studies (Dormans and Deans, 2000; Ultee *et al*., 2002). However, the position of the -OH group on phenolic compounds has not been reported to influence the antibacterial activity of EOC to any great extent. For example, the action of carvacrol against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *B. cereus* seems comparable to the mechanisms of thymol against the same set of organisms (Lambert *et al*., 2001; Ultee *et al*., 2002). Nevertheless, Dorman and Deans (2000) reported that thymol and carvacrol behaved differently in their action against Gram-negative and Gram-positive bacteria. The lack of activity of menthol relative to carvacrol in the study by Ultee *et al*. (2002) appears to shed more light on the importance of the phenolic ring itself (destabilized electrons) in determining the degree of antibacterial activity. For the non-phenolic components of EOs, the antimicrobial strength has been associated with the type of alkyl group, with alkenyls such as limonene, where the alkenyl substituents are incorporated into non-phenolic ring, shown to express greater antimicrobial activity than alkyl group such as *p*-cymene (Burt, 2004). This suggests that the stereochemistry and the structure of EOCs have great impact on the antimicrobial characteristics of the compound or its parent oil. This has been confirmed in previous study where limonene (1-methyl-4-(1-methylethenyl)-cyclohexene) was reported to exhibit greater antibacterial potency than ρ-Cymene (Dorman and Deans, 2000). The stereochemistry of EOs had also shown that *trans*-isomers are more active relative to *cis*-isomers; β-isomers like nerol and geraniol are more active compared to α-isomers like α–pinene; the most active compounds are those with methyl-isopropyl cyclohexane rings; and the antibacterial activity is highest in unsaturated cyclohexane such as terpineolene, terpineol or terpinolene (Dorman and Deans, 2000).

The cell proteins found in the cytoplasm of bacterial cell membrane is another site thought to be affected by the mechanisms of action of EOCs (Knobloch *et al*., 1989). Lipid molecules are known to have boundaries with cytoplasmic membrane and the cell membrane is also known to contain enzymes such as ATPases. Direct interaction of the lipophilic compounds with the hydrophobic components of protein and

accumulation of lipophilic hydrocarbons in the lipid bilayer leading to distortion of the lipid-protein interaction have been suggested as two possible ways through which cyclic hydrocarbons could act on this site (Juven *et al*., 1994; Sikkema *et al*., 1995). Components of EOs have also been suggested to influence the synthesis of structural components involved in the enzymes responsible for energy regulation (Conner and Beuchat, 1984). Inhibition of amino acid decarboxylases in *Enterobacter aerogenes* was shown for cinnamon oil and the active components of the oil (Wendakoon and Sakaguchi, 1995).

Effect of EO on stimulation or inhibition of rumen microbes depends on the chemical composition of an essential oil (Dorman and Deans, 2000), and the part of the plant species from which the compound is extracted (Patra and Saxena, 2009). The inhibition of the growth of microbes by different EOCs is mediated by different mode of action due to the variety of EOCs (Calsamiglia *et al*., 2007). As cinnamaldehyde penetrate into the periplasm through porin proteins of the external membrane of microbial cell by its carbonyl group (Nikaido, 1994; Helander *et al*., 1998), carvacrol exchanges its hydroxyl proton with a cation like K^+ by acting as a membrane carrier of monovalent cations (Bodas *et al*., 2012). This mode of action by carvacrol eventually results in bacterial cell death due to the reduction of ATP synthesis (Ultee *et al*., 2002; Busquet *et al*., 2006). The antimicrobial activity of anethol is due to the ether group of the aromatic ring (Davidson and Naidu, 2000). Membrane-active biocide broad spectrum activity is exhibited by some compounds in the tea tree oil (Davidson and Naidu, 2000).

The main effects and mode of action of essential oil compounds tend to be focused on their toxicity to cell membranes (Calsamiglia *et al*., 2007) as described above. However, this is possibly not the only mode of action exhibited by EOCs since other mechanisms have also been reported. Coagulation of certain components of bacterial cell membrane through denaturation of protein is another possible way through which the effect of EOCs can be exerted on microorganisms (Gustafson and Bowen, 1997). Components of essential oil can cause damage to the proteins and lipids (Ultee *et al*., 2002; Burt, 2004) and can cause coagulation of the cytoplasm (Gustafson *et al*., 1998). The leakage of macromolecules and lysis can consequently result from bacterial membrane and cell wall damage (Juven *et al*., 1994; Cox *et al*., 2000; Oussalah *et al*., 2006). These would result ultimately to inactivation of microbial enzymes (Benchaar *et*

al., 2008; Patra and Saxena, 2010). In addition, the potential of some non-phenolic and phenolic EOCs to interact with bioactive molecules like enzymes and to interact with chemical groups of proteins have been reported (Juven *et al*., 1994). Non-phenolic compounds have been suggested to interact with protein by a mode of action such as interaction with the carbonyl group of cinnamaldehyde (Outtara *et al*., 1997). By contrast, the reaction of phenolic compounds with protein is through hydrophobic interaction or reaction with ion and hydrogen bridges (Outtara *et al*., 1997; Prescott *et al*., 2004; Calsamiglia *et al*. (2007). Interaction with proteins and nucleic acids leading to their inactivation through alkylation or crossed bridges could be the mode of actions by other aldehyde compounds (Prescott *et al*., 2004). For instance, it was reported that the enzymatic activity of *Enterobacter aerogenes* could be inhibited through protein binding by the constituents of clove and cinnamon essential oils (Wendakoon and Sakaguchi, 1995).

Compared with most other essential oil compounds, the essential oil of garlic is a special case as most compounds of garlic oil are not found in the entire plant, but only made from thiosulfates when the plant is treated with steam (Pentz and Siegers, 1996). Their activities against a large variety of both gram-negative and gram-positive bacteria, viruses, fungi and parasites have been reported (Reuter *et al*., 1996). Inhibiting the synthesis of proteins of the cell, DNA and RNA are the various modes of actions that have been proposed to substantiate the anti-microbial activity of these compounds (Feldberg *et al*., 1988). The ability to interact with the sulfhydryl groups (-SH) of other active compounds has been suggested as the principal anti-microbial mechanism for the constituents of garlic oil (Reuter *et al*., 1996; O'Gara *et al*., 2000). Several studies (Reuter *et al*., 1996; Ross *et al*., 2001; Busquet *et al*., 2005b) have reported that each additional S atom increases the antimicrobial activity of allyl sulphur compounds of garlic oil. These authors also reported that the antimicrobial action of individual main compounds of garlic oil is less powerful than the activity of garlic oil, indicating that there is synergy among the different constituents of the oil.

1.7.4. Antagonism and synergism between EOCs

The chemical configuration of EOCs, their interactions with one another and their individual proportions are key factors that determine the inherent activity of essential oils (Dorman and Deans, 2000; Marino *et al*., 2001; Delaquis *et al*., 2002). Constituents of EOs could interact in many ways such as synergism, antagonism or additivity. Synergism between EOCs is observed when the effect of an individual component is lesser than the combined effects of the substances (Davidson and Parish, 1989). Antagonism as another form of interaction occurs when the effect of individual substances is greater than the resultant effect of combining one or more compounds. An effect is observed to be additive when the sum of the individual effect is not different from the combined effect of compounds (Burt, 2004). The antibacterial potential of the whole EOs have been reported to be greater than the effects of mixing their major active individual components (Gill *et al*., 2002; Mourey and Canillac, 2002). This suggests that the minor components of the oil are equally involved in determining the antibacterial activity of the oil and may have a synergistic effect with the major components (Burt, 2004).

An additive effect of thymol and carvacrol, the two structurally similar main compounds found in the EO of oregano, was observed in a study with *P. aeruginosa* and *Staphylococcus aureus* (Lambert *et al*., 2001).

Carvacrol and its precursor (ρ-Cymene) have been shown to exhibit synergistic effect when tested against vegetative cells of *B. cereus*. It seems that carvacrol with a weak potential to swell bacterial cell membranes, relies on ρ-Cymene with a weak antibacterial but greater potential to swell microbial cell membranes (Burt, 2004). By this mode of action, the transport of carvacrol into the cell is probably enabled by ρ-Cymene, so that when the two compounds are mixed together synergism is easily achieved (Ultee *et al*., 2000).

A number of effects of interactions such as antagonism, synergism and additivity have been shown when mixing various combinations of coriander, eucalyptus, cilantro and dill EOs, each containing different chemical composition (Delaquis *et al*., 2002). The growth of some microbes such as *Enterobacter sp*., *Micrococcus sp.*, *Staphylococcus sp*. and *Bacillus sp*. was inhibited when 250 ug /mL of cinnamaldehyde

and 500 ug/mL of eugenol were mixed together as opposed to individual application of these compounds (Moleyar and Narasimham, 1992).

1.7.5. Effects of EOs on feed intake, digestion and VFA production

The use of EOs and their constituent components in ruminant nutrition impacts feed intake and digestion in different ways, depending on the dose and type of EO or EOC. Feed intake was not affected by feeding 2 g of juniper berry EO, containing α –pinene (35%) to cows (Yang *et al*., 2007), a mixture of EO (2 g or 0.75 g) to dairy cattle (Benchaar *et al*., 2007a) and 250 mg/day of oregano EO to sheep (Wang *et al*., 2009a). But, feed intake was negatively affected by feeding 500 mg/day of cinnamaldehyde (Busquet *et al*., 2003), and a mixture of eugenol (90 mg/day) plus 180 mg/day of cinnamaldehyde (Cardozo *et al*., 2006). It is possible that problems of diet palatability was associated with the reduction of feed intake, suggesting that encapsulation of these substances might improve feed intake (Calsamiglia *et al*., 2007). By contrast, feed intake and fermentation were increased when 1 g/day of capsicum extract (containing 15% of capsaicin) was added to a concentrate based-diet for beef cattle (Cardozo *et al*., 2006). There was increased (by 13%) ruminal digestibilities of dry matter (DM) when 2 g/cow/day of juniper berry essential oil was added to forage (40%) and barley-based concentrate (60%) in Holstein cows (Yang *et al*., 2007). The digestibilities of different nutrients in dairy goats were not affected by the inclusion of monoterpene blend (0.43 g/kg diet) containing α-pinene (16.0%), β-pinene (2.2%), *p*-cymene (36.7%) and linalool (45.2%) to the diet (Malecky *et al*., 2009). As opposed to reduced digestibility of diet reported by Busquet *et al*. (2005) and Agarwal *et al*. (2009), other studies observed no effect on digestibility of nutrients (Sallam *et al*., 2009; Wang *et al*., 2009a). The differences in the intake and digestibility of DM due to the addition of EOs or EOCs could be attributed to different doses and types of compounds.

Volatile fatty acids such as acetic, butyrate and propionate are the fundamental sources of energy for ruminants. The digestibility of the nutrients in a used feed is reflected by the concentration of these volatile fatty acids in the rumen (Szumacher-Strabel and Cieslak, 2012). Acetic acid is required for the synthesis of fatty acids, whereas other short-chain FA such as valeric, isovaleric and isobutyric acids are necessary to initiate the synthesis of these fatty acids (Wu and Huber, 1994). As reported by several studies, the effects of EOs and EOCs on total volatile fatty acids (TVFA) and molar proportion of acetic

to propionic ratio have mixed results (Busquet *et al*., 2005; Chaves *et al*., 2008; Kamel *et al*., 2008; Patra *et al*., 2010). There was a slight increase in the concentration of total VFA in the rumen when 750 mg/day of a mixture of EOs was added to alfalfa silage (Benchaar *et al*., 2007a). However, the concentration of total VFA in the rumen was reduced when the same mixture and same dose of EOs was added to a feed ration based on corn silage. This suggests that the effects of EOs or EOCs on VFA could depend on the type of diet. The amount of EOs or EOCs has also been demonstrated to influence the concentrations of total and molar proportions of individual volatile fatty acids in the rumen. The addition of increasing doses (0, 3, 30, 300 and 3000 mg/L) of a wide range of EOs such as anise, tea tree and cade oils and EOCs such as anethole, carvacrol and eugenol did not affect the concentrations of total and molar proportions of individual VFA in rumen fluid (Busquet *et al*., 2006). In that study, only the addition of 3000 g/L (the highest dose) of the substances to rumen fluid reduced the concentration of total VFA in the rumen. From the nutritional perspective, a reduction in ruminal concentration of VFA which is due to decreased production of the same fatty acids is an undesirable results because it reflects reduced utilization of dietary energy from structural carbohydrates (Szumacher-Strabel and Cieslak, 2012). Addition of EOs or EOCs is expected to change the molar proportion of VFA towards increasing propionic concentrations and reducing acetic acid without affecting the total VFA. The pattern of VFA production was shifted towards increased proportion of butyrate and reduced concentration of propionate in the rumen when phenolic compounds such as thymol, carvacrol and eugenol were used (Benchaar *et al*., 2007b). Furthermore, Castillejos *et al.* (2006) reported reduced concentration of propionate without affecting the total VFA when 500 mg/L of eugenol was used. In another *in vitro* experiment, supplementation of two EOCs (anethole and *p*-cymene) and three EOs (juniper berry, cinnamon leaf and garlic oils) did not affect the concentration of total VFA but the molar proportion of propionate was reduced by garlic and cinnamon leaf oils (Chaves *et al*., 2008).

The pH of the rumen has also been suggested as another factor which regulates the effects of EOs and EOCs. The pH of the rumen fluid was reported to influence the effects of EOs on the concentration of VFA (Cardozo *et al*., 2005). In that study, cinnamon oil increased the ratio of acetate to propionate at pH 7.0 and decreased the same ratio when the pH was 5.5. In a similar study, Spanghero *et al*. (2008)

reported that at lower pH, a blend of EO changed the end-products of fermentation, particularly, reduced molar proportions of acetate: propionate ratio and proportion of acetate.

In general, from the aforementioned paragraphs, it seems that supplementing diet with EOs and/or EOCs may reduce feed nutrient digestion with concomitant suppression of VFA at high doses or no change at low doses. The pH of the rumen is also a determinant of the effects of EOs or EOCs on VFA production. It has been observed that, in some long-term *in vitro* culture experiments, benefits associated with essential oils and their constituents compounds may decline over time due to possible adaptation of individual microbial species to EOs or EOCs or shifts in microbial population (Benchaar *et al*., 2008; Patra and Saxena, 2009a).

1.7.6. Effects of essential oil compounds on ammonia production

The symbiotic relationship between the ruminal microbial population and the ruminant animal enables ruminants to synthesize biologically high quality protein from non-protein sources of nitrogen (Benchaar *et al*., 2008). Ruminants are able to synthesize proteins for deposition in meat and milk from the microbial protein containing good profile of amino acids (Benchaar *et al*., 2008). Nevertheless, this symbiotic relationship does not provide sufficient microbial protein to synthesize amino acids required by high producing ruminant animals (Benchaar *et al*., 2008; Szumacher-Strabel and Cieslak, 2012). Therefore, extra supplementary protein sources are required to correct the deficits in amino acids requirement. However, due to inefficient utilization of excess protein, ruminant excrete waste materials which are rich in ammonia which causes environmental pollution (Benchaar *et al*., 2008; Szumacher-Strabel and Cieslak, 2012). Therefore, modulation of rumen activities towards improved protein utilization would benefit both the environment and enhance ruminant animal production.

Many *in vitro* studies have been conducted to evaluate the potential influence of essential oils and EOCs to regulate protein utilization by ruminants. EOs and EOCs affect volatile fatty acid (VFA) and ammonia production by altering the activity and population of rumen bacteria, fungi and protozoa (Bodas *et al*., 2012). Therefore, using whole EOs and or their constituent compounds could decrease ruminal NH_3-N production leading to improvement in the efficiency of protein absorption by ruminants (Wallace *et al*.,

2002; Spanghero *et al*., 2008). Reduced production of ammonia results from the decrease in the degradation of feed proteins (Hervas *et al*., 2000; Alexander *et al*., 2008). Some studies suggest that the effects of essential oil compounds on the ruminal production of ammonia are presumably the consequences of a reduction in the population of protozoa (Newbold *et al*., 1997). This is due to the understanding that protozoa are mainly responsible for the degradation of feed protein in the rumen (Jouany, 1996). It has been suggested that added EOs or their individual active components inhibit the break-down of protein to NH_3-N through two mechanisms. These mechanisms are the direct inhibition of hyper ammonia-producing bacteria (HAP) and their deaminase activities, and a reduction in the degradation of protein to peptides (Mclntosh *et al*., 2003; Newbold *et al*., 2004). The reduced production of peptides from the decreased protein degradation suggest reduced peptidolytic activity and explains why some plant extracts like clove bud decrease the concentration of large peptides with no effect on ammonia production (Busquet *et al*., 2005). The HAP bacteria consist only about 1% of the rumen however; they have high potential for deamination (Wallace *et al*., 2004). This suggests that inhibition of the activity of HAP bacteria would impair deamination. There was 9% reduction in the rate of amino acid deamination when casein hydrolysate was incubated (*in vitro* 48 h) with rumen fluid from cows that were offered a silage-based diet with 1 g/day of an added blend of commercial EOCs (Crina ruminants; Akzo Surface Chemistry Ltd., Herfordshire, UK) (Mclntosh *et al*., 2003). The supplemented commercial mixture contained 100-300 g/kg of phenolic compounds such as thymol, eugenol, resorcinol, guaiacol and cresol. Decreased NH_3-N concentrations were reported by Cardozo *et al*. (2005) when oregano (30 and 300 mg/L) and cinnamon oil (0.3 to 300 mg/L) were used. Similarly, cinnamaldehyde (3000 mg/L) reduced the concentration of NH_3-N in the rumen (Busquet *et al*., 2006). Bacterial deaminative activity was reduced (25%) when casein hydrolysate and ruminal fluid from sheep offered diets supplemented with 110 mg of mixture of essential oil compounds were incubated together for 24 h *in vitro* (Newbold *et al*., 2004). It was observed that there was 9% (Mclntosh *et al*., 2003) and 24% (Newbold *et al*., 2004) reduction in deamination activities with no effects on the proteolytic and peptidolytic activities.

Despite the above reports on the potential of EOs and EOCs to cause reduction of ruminal ammonia concentrations, several *in vitro* short term studies with different EOCs report no effect on the

concentrations of NH_3-N in culture (Castillejos *et al*., 2007; Spanghero *et al*., 2008). The use of carvone and carvacrol (300 mg/L) and anethol (3000 mg/L) in *in vitro* studies had no effects on the concentrations of NH_3-N (Busquet *et al*., 2006). Busquet *et al*. (2006) observed that biologically active components of essential oils such as carvacrol, cinnamaldehyde, anethol, eugenol, benzyl salicylate and the whole essential oil themselves (capsicum oil, bud oil, tea tree oil, ginger oil, anise oil, oregano oil) at 3 mg/l showed lack of effect on the concentration of NH_3-N and considerable reduction in NH_3-N concentration at 3000 mg/l. Generally, time, dose and the chemical structure of essential oil have been known as the basic factors determining the effect of the supplemented compound on the concentration of NH_3-N. Variable effects on the ruminal concentration of ammonia N have been observed due to variation in the chemical configuration of essential oil active compound. The potential of the chemical structure of EOCs as a contributing factor to determining their effect on ammonia production has been shown (Castillejos *et al.*, 2006; Benchaar *et al*., 2008). It was observed in those studies that limonene (500 mg), guaiacol (5, 50, 500, 5000 mg/L) and, another phenolic compound eugenol (5, 50 and 500 mg/L) substantially reduced the concentration of NH_3-N in culture. However, the concentration of NH_3-N in culture was not affected by inclusion of vanillic aldehyde (5, 50 and 500 mg/L).

A number of *in vivo* studies where the inclusion of EOs or EOCs did not affect the concentration of NH_3-N have also been reported. Castillejos *et al*. (2005) reported that adding 1.5 mg/l of a mixture of essential oil compounds for 8 days with the pH kept constant did not affect the concentration of NH_3-N. These authors also reported that the degradation of crude protein, concentration of bacteria, dietary nitrogen flow and synthesis of microbial protein were not affected. The lower concentration of the mixture of EOCs (1.5 mg/l) used in that study was suggested as the reason for lack of effect on the metabolism of nitrogen. Nevertheless, when the experiment was repeated for 9 days with the same blend of EOCs at higher doses (5, 50, and 500 mg/l), the effect of the blended mixture of EOCs on nitrogen metabolism (small peptides and amino acid, concentration of NH_3-N and large peptides) was still not observed (Castillejos *et al*., 2007). Mclntosh *et al*. (2003) suggested that the above 35 mg/l is the required concentration of mixture of EOCs in rumen fluid to enhance effective transformation of ruminal protein. There was no change in nitrogen digestibility, retention and NH_3-N concentration when 2 g or 0.75 g of a

mixture of essential oil was supplemented per day in a diet for lactating dairy cows (Benchaar *et al*., 2006b; 2007). This lack of effect on the concentration of NH_3-N in *in vivo* studies due to supplementation of EOCs may suggest a number of possibilities: rapid ruminal metabolism of the active components of essential oil to a less active form, microbial adaptation to EO or shift in the population of individual microbial species (Patra, 2011). The benefits of using EOs or EOcs to modulate protein metabolism include among others, selective inhibition of amino acid degradation due to a selective inhibition of hyper ammonia producing bacteria (HAPB) (Hart *et al*., 2008; Patra and Saxena, 2009a).

1.7.7. Effects of EOs and EOCs on rumen methanogenesis

Methane is considered to be a potent greenhouse gas. Therefore reducing CH_4 emissions from domestic ruminants is receiving worldwide attention because its release into the atmosphere is directly linked with ruminant production (Benchaar *et al*., 2008). Ruminants have the capability (abundant fibre degrading microbes in the rumen, caecum and colon, which help in anaerobic oxidation of feed) to convert non-utilizable feed sources such as lignocellulosic agro-industrial by-products to utilizable form (Kamra *et al*., 2012). During the fermentation of ingested feed, a huge number of microbial populations such as bacteria, protozoa and fungi are involved. These microorganisms act on the food in the rumen to produce VFA, predominantly, acetate, propionate and butyrate, and a number of gases such as CO_2 and H_2 as the end-products of their combined activities (Buddle *et al*., 2011). The VFAs which are absorbed across the rumen wall are used by the animal as the primary source of energy (Kamra *et al*., 2012). Hydrogen (H_2) which results from the fermentation of the feed is used by methanogens as their important source of energy to reduce CO_2 to CH_4 (Hungate, 1967), and may be used by biohydrogenating bacteria to hydrogenate FAs (Chesworth *et al*., 1998). Although formate and methanol are also produced and used by methane producing bacteria, they are not considered as the main sources of energy (Buddle *et al*., 2011). Shown in Figure 1.12 are some of the major sources of methane (H_2 and CO_2, formate, methanol and acetate) synthesis in the rumen. Of these, methane generation from acetate accounts for the highest portion of gas production (Chesworth *et al*., 1998).

The basis for the formation of methane in the rumen is that accumulation of high concentration of H_2 potentially reduces microbial activities and lowers the rate of fermentation, resulting in slow conversion of

feed to VFAs (McAllister and Newbold, 2008). Therefore, ruminal fermentation of feed proceeds more rapidly through the activity of the H_2-consuming methanogens which reduces the concentration of H_2 to about 1 μM of dissolved H_2 (Rees *et al*., 1995; Morvan *et al*., 1996; Buddle *et al*., 2011). This increased fermentation of feed within a given time implies that the animal gains more VFA during that time (Wolin, 1979). However, in this process of methane formation in the rumen, around 5-15% of gross energy consumed in the diet by ruminants is lost (Johnson and Johnson, 1995). Under the prevailing anaerobic conditions of the rumen, CO_2 cannot be oxidized to release energy once it has been reduced to CH_4 (Kamra *et al*., 2012). Therefore, expected benefits from the use of EOs and their constituent compounds, like any other substance, is to save this loss of energy in order to economically produce livestock in a friendly environment.

I. Methane production from acetate

$$CH_3COOH \longrightarrow CH_4 + CO_2$$

II. Methane production from formate

$$4\ H\text{-}COOH \longrightarrow CH_4 + 3CO_2 + 2H_2O$$

III. Methane production from methanol

$$4\ CH_3OH \longrightarrow 3CH_4 + CO_2 + 2H_2O$$

IV. Methane production from Hydrogen and carbon dioxide

$$4H_2 + CO_2 \longrightarrow CH_4 + 2H_2O$$

Figure 1.12 Four separate pathways for the production of methane (Adapted from Chesworth *et al*., 1998).

As reported by Kamra *et al*. (2012), inhibition of methane formation by plant bioactive compounds such as EOs may be achieved through the following actions:

a. Because EOs have antimicrobial activities against a wide range of microbes, they could directly inhibit methanogens.
b. Indirect reduction in the number of methanogens could result from the anti-protozoal activity of EOs. Because of the ecto-symbiotic relationship between methanogens and ciliate protozoa, EOs

might cause the death of ciliate protozoa causing the former to lose its symbiotic relationship and hydrogen supply, leading to decreased production of methane.

c. Since EOs exhibit antimicrobial actions, this might decrease microbial numbers (bacteria and fungi) which results in reduction in the digestibility of feed. Because methane production and feed degradation are directly related to each other, the lower digestibility of feed would directly reduce methanogenic activities.

It was first reported that EO may reduce methane production in rumen cultures when the effect of pinene and limonene on methanogenesis was examined (Crane *et al*., 1957). In that study the inhibitory effect of limonene on methane synthesis was more than that of pinene, suggesting that all terpenes do not have the same toxic effect. After this report, it seems no other information was published on the effect of EO or its compounds until the early 2000s.

The main active compound of *Oreganium* and *Thymus* plants, thymol at 0.4 g/L strongly inhibited *in vitro* methane production (Evans and Martin, 2000). Similarly, 6 mM concentration of *Thymus vulgare* and its principal compounds, thymol, had 99% reduction of methane (Macheboeuf *et al*., 2008). In another report, Eugenol which possesses strong antibacterial effect against both Gram-positive and Gram-negative bacteria was observed to have the potential of influencing rumen fermentation with beneficial effects (Calsamiglia *et al*., 2007). Anethole (86-88%) has been reported as one of the main compounds in mature fennel seeds (Telci *et al*., 2009). In an *in vitro* study, anethole (20 mg/L) reduced methane production (Chaves *et al*., 2008). Although Wang *et al*. (2009) reported that methane production in sheep was inhibited by inclusion of a mixture of EO (0.25 g/day) from oregano plants for 15 days; McGinn (2006) observed no effect of adding 1 g/day of EO to beef cattle diet on methanogenesis when the supplement was used for 21 days. This difference in effect could be due to different dose, time or duration of experiment and type of EO or animal.

About 58% of methane inhibition was reported when eucalyptus oil was used at 1.66 ml/L (Kumar *et al*., 2009), 90.3% at higher (2 ml/L) dose (Sallam *et al*., 2009) and at 0.33 g, α-cyclodextrin-eucalyptus oil complex induced 70% inhibition of methane (Tatsuoka *et al*., 2008). *P-Cymene*, one of the components of

eucalyptus oil (Bhatti *et al*., 2007), at the concentration of 20 mg/L decreased methane synthesis by 29% (Chaves *et al*., 2008). However, there was no effect of α-cyclodextrin cineole (up to 0.33 g/L) on methanogensis (Tatsuoka *et al*., 2008). There was strong inhibition of methane synthesis when oil of peppermint (Tatsuoka *et al*., 2008), and juniper berry and cinnamon oils (Chaves *et al*., 2008) were used. Macheboeuf *et al*. (2008) reported 94% reduction of methanogenesis with 5 mM concentration of cinnamaldehyde, the main component of cinnamon oil. In a batch culture study, Busquet *et al*. (2005) observed 69 and 74% reduction in CH_4 production when 300 mg/L each of diallyl disulphide and garlic oil respectively, were used. It was suggested in that study that direct inhibition of rumen methanogenic archaea was the mode of action through which the compounds reduced methane production. Furthermore, other studies observed that there was 19% reduction in CH_4 production in steers without effect on feed digestibility or protozoal numbers with 20 g/kg DM intake of encapsulated horseradish oil (Mohammed *et al*., 2004). In general, effects of essential oils and their constituent compounds on methanogenesis depend on the dose and the type of substance, with the phenolic compounds or EOs containing them, showing greater antimicrobial potential. In addition, it seems that there is a potential to select EO or EOC to selectively inhibit CH_4 production if the dose is optimized.

1.7.8. Effects of EOs and EOCs on biohydrogenation of PUFA

The *n*-3 PUFA such as EPA and DHA have been reported as important regulators of chronic diseases such as coronary heart disease. As discussed previously, α- linolenic acid (LNA) and linoleic acid (LA), the two metabolic precursors of the long chain *n*-3 fatty acids (20:5n-3 and 22:6n-3), are naturally, the predominant fatty acids in ruminant feedstuff (Morrison, 1977). In the rumen, microbes convert these UFA to different intermediates through the process known as biohydrogenation (BH). Therefore, manipulation of biohydrogenation (BH) is receiving significant attention within the scientific community. During BH, PUFA are converted to saturated fatty acid (SFA) through the following sequence of reactions: hydrolysis, isomerization and reduction before hydrogenation of the free UFA to SFA. Increasing the amounts of the *n*-3 in ruminant food products has been difficult due to the problem of ruminal biohydrogenation of dietary PUFA. Biohydrogenation is carried out by a group of bacteria recognized as cellulolytic bacteria (Kepler and Tove, 1967), particularly, the *Butyrivibrio* group which are

the most active group (Durmic *et al.*, 2008). Although *Clostridium proteoclasticum* as it was formerly called (Kemp *et al.*, 1975; Maia *et al.*, 2007), before it is re-classified as *Butyrivibrio proteoclasticus* (Moon *et al.*, 2008), is the only known bacteria capable of converting VA to 18:0, all bacteria in the *Butyrivibrio* group are capable of producing CLA from linoleic acid. Identification of substances with potential to selectively inhibit the activity of *Butyrivibrio proteoclasticus*, the undesirable microorganisms, would increase both PUFA and BH intermediates (CLA and VA) in ruminant food products (meat and milk).

As mentioned previously, essential oils and EOCs have a wide range of antimicrobial effects against both Gram-positive and Gram-negative bacteria, and ruminal biohydrogenation involves several groups of Gram-positive bacteria (Harfoot and Hazlewood, 1988). Therefore, EOs may potentially modify the process of biohydrogenation through selective inhibition of biohydrogenating bacteria.

There is a scarcity of information on the effects of EOs and EOCs on ruminal biohydrogenation of unsaturated fatty acids. Available data do not show clear effects of EOs and EOCs on biohydrogenation due to the complex chemical composition of EOs. In addition, *in vitro* and *in vivo* data appear not to agree in terms of effects on ruminal biohydrogenation of PUFA.

There was a substantial (58%) inhibition of ruminal biohydrogenation when cinnamaldehyde (500 mg/L) was used in a dual-flow continuous culture fermenter system (Lourenco *et al.*, 2008). However, there was minor (6%) inhibition of biohydrogenation when 250 mg/L of eugenol was supplemented. In the same study, the inclusion of 500 mg/L of cinnamaldehyde caused higher accumulation of biohydrogenation intermediates such as *trans*-11, *cis*-15 C18:2 and *trans*-10 C18:1, *trans*-10, *cis*-12 C18:2 compared with untreated cultures, suggesting that biohydrogenation of linolenic (C18:3) and linoleic (C18:2) acids was affected. However, it is not clear whether the concentrations of the compounds (500 or 250 mg/L) or the type of EOCs (eugenol or cinnamaldehyde) was responsible for this effect. The inclusion of citronella oil (125, 250 and 500 mg/L), Siberian fir needle oil (500 mg/L), rosemary oil (250 and 500 mg/L) and sage oil (500 mg/L) significantly reduced the concentration of stearic acid, the end product of biohydrogenation of C18:2 *n*-6 and C18:3 *n*-3, in a batch culture *in vitro* study (Gunal *et al.*, 2013). In the same study, at the doses mentioned, the concentration of C18:2 *n*-6 was not affected suggesting that

accumulation of C18:0 could be due to inhibition of the last step of biohydrogenation (conversion of C18:1 to C18:0) or that other biohydrogenation intermediates were produced in that study. In another study (Durmic *et al*., 2008), the selective inhibitory potential of selected EO and plant extracts from Australian plants was shown against *Butyrivibrio fibrisolvens* and *Butyrivibrio Proteoclasticus*, the two most recognized groups of bacteria responsible for ruminal biohydrogenation of UFA.

However, the above dramatic inhibition of biohydrogenation reported by Lourenco *et al*. (2008) was not confirmed in a number of *in vivo* studies. The fatty acid composition of milk was not affected by the addition of a monoterpene blend (0.43 g/kg diet) containing α-pinene, β-pinene, p-cymene and linalool to the diet (Malecky *et al*., 2009). Similarly, cinnamaldehyde (1 g/kg) added to the diet of dairy cattle also did not affect the profile of milk fatty acids (Benchaar and Chouinard, 2009).

The fatty acid profile of milk obtained from dairy cows offered 0.75 g/cow/day of a mixture of essential oils and compounds (Crina ruminants; CRINA S.A., Gland, Switzerland) was not affected (Benchaar *et al*., 2007a). The Crina ruminants' mixture contains eugenol, thymol, limonene, guaiacol and vanillin (McIntosh *et al*., 2003; Castillejos *et al*., 2005). It is possible that there was microbial adaptation or shift in microbial populations due to essential oil mixture as the animals were exposed to the experimental treatment for a period of one month (28 days). Nevertheless, the concentration of CLA (*cis*-9, *trans*-11 18:2) in milk fatty acids was increased when the same mixture of essential oil compounds was added at higher concentration (2 g/cow/day) (Benchaar *et al*., 2006a). Chaves *et al*. (2008) reported that supplementing garlic, juniper berry and cinnamaldehyde at 200 mg/kg of dry matter to growing lambs did not modify the fatty acid profile of back and liver fat. In a different study, monoterpenes blend consisting of β-pinene, linalool, α-pinene, *p*-cymene offered at 0.43 g/kg of dry matter intake did not change the fatty acid profile of milk (Malecky *et al*., 2009). Supplementing the diet of dairy cows with 1 g/day of cinnalmaldehyde did not affect the fatty acid composition of milk (Benchaar *et al*., 2007b). Benchaar *et al*. (2006; 2007) observed that supplementing the diet of dairy cows with EO did not alter the FA profile of milk, but the extent of ruminal biohydrogenation was reduced and *cis-9 trans-11* CLA concentration was increased by EO rich in monoterpenes such as carvone and limonene (Lourenco *et al*., 2009). This suggests that these compounds have some effects on biohydrogenating bacteria. This suggests that the

effect of EOCs and whole EOs on the modification of ruminal biohydrogenation of PUFA depends on the type, time or duration of experiment and concentration of essential oil compounds.

As observed by Vasta and Bessa (2012), the lack of agreement between *in vitro* and *in vivo* results on the profile of fatty acids suggests that it is discouraging to use these compounds to modulate ruminal biohydrogenation. However, the great diversity in the composition of these compounds and the huge number of commercially available EOs suggests that more results are required to reach conclusion and conclusions should be drawn with caution.

In addition to altering the fatty acid composition of ruminant food products, there are other potential nutritional and organoleptic benefits associated with using EOs in ruminant feeding. Supplementation of ruminant diets with essential oil compounds could allow the compounds or their derived products to be present in meat or milk. The presence of essential oil compounds or their metabolic products could enhance the value of ruminant products by enriching them with nutritional and organoleptic properties (Chion *et al*., 2010). Carvone and limonene (the main components of caraway essential oil) were reported to be present in the milk when camomile and caraway seeds were fed to goats (Molnar *et al*., 1997). Similarly, a number of reports have shown that different monoterpenes such as sabinene, 3-carene, α-pinene, limonene, β-mircene, camphene and β-pinene were detected in the milk of cows grazing pasture predominated by Alpine (Noni and Battelli, 2008; Chion *et al*., 2010).

1.7.9. Effects of essential oil compounds on rumen microbes

As mentioned earlier, essential oil compounds can cause inhibition of amino acid deamination by inhibiting the growth of hyper ammonia-producing bacteria which are bacteria involved in the production of NH_3-N (Mclntosh *et al*., 2003; Newbold *et al*., 2004; Wallace, 2004; Patra and Saxena, 2009). Mclntosh *et al.* (2003) reported that while some hyper ammonia-producing bacteria such as *Clostridium aminophilus* were less sensitive to the effect of EOCs, others, such as *Peptostreptococcus anaerobius* and *Clostridium sticklandii* were highly sensitive. Although the population of HAP was not affected when high protein diet was offered to sheep, about 77% of the number of hyper ammonia-producing bacteria was decreased when sheep were offered a low protein diet supplemented with 100 mg/day of EOC

(Wallace, 2004). This could suggest that effect of essential oil compounds on HAP may also be diet dependent. At 90 mg/L, the growth of *Selenomonas ruminantium* was selectively inhibited without affecting the activity of *Selenomonas bovis* (Evans and Martin, 2000). However, including 400 mg/L caused complete microbial inhibition. This suggests a selective inhibition of HAP bacteria by essential oil compounds at low doses, and a complete microbial inhibition at higher doses. The peptidolytic activity of ruminal bacteria can also be reduced by essential oil compounds (Busquet *et al*., 2005).

Antiprotozoal activity has also been exhibited by some essential oil compounds (Hristov *et al*., 2003). But there are contrasting reports of these activities. Some studies have reported that essential oil compounds have no effect on the population of protozoa (Mclntosh *et al*., 2003; Newbold *et al*., 2004; Benchaar *et al*., 2007a; b). Ruminal protozoal numbers were not affected when a blend of essential oil compound 750 mg/day (Benchaar *et al*., 2007a) and 110 mg/day (Newbold *et al*., 2004) were fed to dairy cows and sheep respectively. In another study, Patra *et al.* (2010) found lack of effect of fennel extract on the population of protozoa. Similarly, Patra *et al.* (2010) observed that the concentrations of large entodiniomoph in the presence of clove extract were not affected. By contrast, a stimulatory effect on the population of protozoa was observed with EO (Patra and Saxena, 2009). The mode of action by which some studies reported stimulatory effect of EOCs on rumen protozoa is not clear. Other studies (Ando *et al*., 2003; Cardozo *et al*., 2006; Fandino *et al*., 2008) have observed reduction in the concentrations of holotrich, entodiniomorph and total protozoa number due to the antiprotozoal effect of essential oil compounds. Yang *et al*. (2010b) observed that feeding lower concentrations of cinnamaldehyde (0.4-1.6 g/day) has no effect on protozoa, whereas, when 2 g/day of anise containing 100 g/kg of anethol was offered, the numbers of entodiniomorph and holotrich reduced (Cardozo *et al*., 2006). The mechanism of action through which the reported reduction of protozoal population is achieved may be due to the lipophilic nature of EOCs enabling them to penetrate protozoal membrane; however, the actual means of inhibition is not clearly known (Cardozo *et al*., 2006). Generally, there is no conspicuous effect of essential oil compounds on protozoa. There is limited evidence on the effects of essential oil compounds on fungi (Bodas *et al*., 2012). The general effect of EOCs on fungi has been suggested to be the inhibition of their growth (Mclntosh *et al*., 2003).

1.7.10. Microbial adaptation to EOs and compounds

Several factors have been suggested to be responsible for the variation in bacteria's sensitivity to the effects of essential oil compounds, and by extension, the period of time during which the antimicrobial property of EO can be exhibited. Gram-negative bacteria are less sensitive than Gram-positive bacteria which have high susceptibility to the effect of EOCs (Cox *et al.*, 2001). It has been indicated that molecules such as phenolic compounds with hydroxyl and carbonyl groups on their structures are more toxic to bacteria (Griffin *et al.*, 1999), suggesting that the chemical composition of the essential oil compound is another factor that determine the sensitivity of bacteria to them. In addition, as mentioned previously, the concentration of the compound is also a determining factor of the influence of essential oil and their compounds on bacteria. Broudiscou *et al*. (2007) reported that rumen microorganisms in goat degraded β-ocimene, sabinene, α-copaene, α-pinene, myrcene. The same study reported that thymol and camphene were not degraded by the same microbes. This suggests that ruminal microbes can degrade some monoterpenes and some sesquiterpenes to their less active form. Ruminal microbes have the potential to adapt to the effect of essential oil and their compounds over-time (Busquet *et al.*, 2005), suggesting that the actual effect of EOCs may not be shown on results obtained from short-term exposure of ruminal microbial population to EO compounds. Chaves *et al.* (2008) reported that supplementing garlic, juniper berry and cinnamaldehyde at 200 mg/kg of dry matter to growing lambs did not modify the fatty acid profile of back and liver fat. Furthermore, monoterpenes blend consisting of β-pinene, linalool, α-pinene, *p*-cymene at 0.43 g/kg of dry matter intake did not change the fatty acid profile of milk (Malecky *et al.*, 2009). In a separate study, supplementing the diet of dairy cows with 1 g/day of cinnalmaldehyde did not affect the fatty acid composition of milk (Benchaar *et al*., 2007b). These results are compelling evidence that more studies are require to ascertain the potential of EOs and EOCs as potential feed additives in animal nutrition. The unchanged fatty acid profile of animal tissues following the inclusion of essential oil compounds suggest that ruminal microbes can both alter the chemical structure and adapt to some essential oil compounds (Malecky and Broudiscou, 2009).

Specific blend of EO containing limonene, guaiacol and thymol as major constituents was reported to alter ruminal metabolism of nitrogen by inhibiting deamination (Mclntosh *et al*., 2003; Newbold *et al*.,

2004). Contradictory result was reported by Castillejos *et al*. (2005) where the addition of a blend of EO to two different diets (high concentrate with high forage) for a period of eight days in a continuous culture fermentation system did not modify the metabolism of nitrogen but increased the concentration of total VFA. Few changes were made to this last experiment relative to the original study. The dose of the blend of EOCs in the latter experiment was 5 times less and the adaptation time was equally 4 times less than the time in the previous studies (Mclntosh *et al*., 2003; Newbold *et al*., 2004). About twenty eight days of ruminal microbial adaptation was suggested by some of these studies as the minimum period of time required to establish the actual effects of blend of EOs (Mclntosh *et al*., 2003; Newbold *et al*., 2004). Therefore, it becomes clear that the short adaptation time (eight days) and the low dose (1.5 mg/l) in the study by Castillejos *et al*. (2005) were the main factors why there was no change in ruminal nitrogen metabolism. Castillejos *et al*. (2007) observed that addition of a blend of EO at 5 mg/l appears to be the tolerant level for normal ruminal function. An adaptation time of more than 6 days was suggested as minimum amount of time required for the blends of EOs to modify ruminal metabolism of nitrogen. Addition of 400 mg/l of thymol by Evans and Martin (2000) decreased the total VFA, however, increased the acetate to propionate ratio, and indicates that the dose was too toxic to ruminal microbes.

Different mechanisms through which bacteria become insensitive to the effect of supplemented essential oil compounds have been suggested. Spanghero *et al*. (2008) suggested that shift in microbial population is one of the actions that reduce the sensitivity of ruminal microbial population to essential oil compound. The extent of this microbial shift depends on both the concentration and the period of exposure of these compounds to ruminal microbes (Bodas *et al*., 2012). Chizzola *et al*. (2004) suggested that ruminal microbes adapt to essential oil compounds through degrading the active components of these compounds to their less active form. Other authors have proposed that evolution of mechanisms to tolerate the effect of essential oil compounds by ruminal microbes is another mode of action for microbial adaptation to EO (Jouany and Morgavi, 2007).

1.7.11. Legal and safety issues with the use of EOs and EOCs in food

The application of a number of EOs and EOCs as flavourings in foods has been approved and registered by the European commission. The registered components are thought to present zero risk to human health

(Burt, 2004). Some of these flavourings are cinnamaldehyde, limonene, carvacrol, eugenol, menthol, carvone, thymol, citral and ρ-Cymene. About 15 years ago, methyl eugenol and estragole were removed from the list of flavourings approved by the European commission because they were reported to be genotoxic (Commission Decision of 23 January, 2002). New components of EO can only be evaluated for registration and inclusion into the list of flavouring agents after metabolic and toxicological studies have been conducted (Commission Decision of 23 February, 1999; Commission Regulation (EC) No. 1565/2000; Commission Regulation (EC) No.622/2002; Regulation (EC) No.2232/96).

The flavouring agents in the list of European Commission is also part of the list of everything added to Food in the US (EAFUS), which means, the Food and Drug Administration of the United States of America has equally recognized those substances as generally recognized as safe (GRAS) food additives in the US (Burt, 2004).

In other countries of the world, these substances may be recognized as new food additives if their inclusion in food is for any purpose other than for the purpose of adding flavour to the food. As would be expected in those countries, addition to the list of food additives would only be approved after several expensive studies of their potential metabolic and safety issues, and may equally involve prohibitive cost. For those countries, it would be better to consider the use of whole EO or herb or whole spice instead of individual components for economic reasons (Smid and Gorris, 1999).

The Federal Food Drug and Cosmetic Act (FFDCA) recognizes that naturally occurring substances in food should have a different and lower safety standard relative to other ingredients which are intentionally added to food (Smith *et al.*, 2005). According to the Act (21 CFR 172.30), the realistic standard for naturally occurring substances is that the substances must 'not ordinarily render the food injurious to health'. However, for added substances, the Act applies a much higher standard which says 'the food is adulterated if the added substance may render the food injurious to health.

As EOs are considered neither a food nor a direct food additive, no current standard can be easily applied to their safety evaluation (Smith *et al.*, 2005). In the Act standards, EOs occupy an intermediate position

as they are comprised of naturally occurring substances which are intentionally added as individual chemical substances to food (Smith *et al*., 2005).

Essential oils are considered to be safe based on the documented long history of their application in foods and other wide range of human exposures with no known adverse effects (NAS, 1965; 1970; 1975; 1981; 1982; 1987; 21 CFR 172.510). Because of these documented safety history, one may presume with a high degree of confidence that EOs derived from food are likely to be safe (Smith *et al*., 2005).

In terms of actual application in foods, different concentrations (from 0.05% to more than 1% v/w) of whole EOs or their constituent compounds have been reported to present no issues for concern. For instance, treating beef fillets with 0.8% v/w of oregano oil was found to improve the acceptability of the flavour after storage (5^0C) and cooking (Tsigarida *et al*., 2000). Skandamis and Nychas (2001) reported that treating minced beef with 1% v/w of oregano oil enhanced the odour, flavour and colour during storage under modified atmosphere packing and vacuum (5^0C). These authors reported that oregano was almost not detected after cooking. Oragno and thyme oils (0.05%, v/v) have been found to impart an herbal odour when spread on Asian sea bass (Harpaz *et al*., 2003). There were no ill effects on either appearance or flavour of cooked shrimps when thyme oil (0.9%, v/w) was added to a coating compared to considerable reduction in the acceptability of the shrimps when the concentration was increased to 1.8% in the coating (Ouattara *et al*., 2001). Carvacrol and cinnamic acid (1 mM) have been reported to delay spoilage and also maintain the organleptic properties of honeydew melon and kiwifruit (Roller and seedhar, 2002).

Although a great number of EOCs are considered as approved food flavourings in the EU and recognized as GRAS in the US, toxicity and irritation have been reported in some studies (Burt, 2004). For example, in root canal treatment, irritation of mouth tissues has been reported following application of thymol, menthol and eugenol. Manabe *et al*. (1987) observed that the cytotoxicity results of these EOCs indicate that lipid solubility and membrane affinity could be part of the tissue penetration and that irritation of the gum may be caused by surface activity and membrane lysis. Although carvone, cinnamaldehyde and carvacrol appear not to have significant effect in *in vivo* studies, results of *in vitro* studies suggest that at the cellular levels, they exhibit mild to moderate toxicity. At the current level of use, gene-toxicity data

seem not to raise issues for concern (Stammati *et al*., 1999). For people who use EOs and EOCs frequently, allergic contact dermatitis has been observed. However, if these substances are to be used at a very large scale, preventive measures are necessary to ensure that the health condition of workers is not compromised (Carson and Riley, 2001; Bleasel *et al*., 2002). At the above current low levels of intake as flavouring substances, EOs or their compounds have no known or are not expected to pose any significant risk to human health (Smith *et al*., 2005).

1.8. Justification and approach taken in this PhD research

Ruminant derived meat and milk, and their products which constitute a major part of human diets, are characterized by low concentration of long chain *n*-3 PUFA such as C20:5 *n*-3 and C22:6 *n*-3 and high content of SFA. The intakes of these long-chain PUFA are considered to be linked with reduced risk of coronary heart diseases, whilst SFA have been associated with increased risk of cardiovascular diseases. Ruminal biohydrogenation of dietary PUFA results in the production of SFA at the expense of CLA, VA and PUFA. The key to improving the fatty acid profile of milk and meat is dietary manipulation of ruminal biohydrogenation. In order to achieve this dietary modification and optimize the concentration of PUFA in ruminant food products, ruminant nutritionists have been evaluating the suitability of various possible strategies to manipulate the process of biohydrogenation. This manipulation, if it is successfully achieved, would lead to the production of ruminant meats and milk with a high level PUFA, CLA and a lower content of SFA which is of great value for consumer health. Increasing the concentrations in meat or milk, of PUFA such as C20:5*n*-3 and C22:6*n*-3, which have proven human health benefits, would represent an excellent means of increasing their intake by humans. In order to avoid the use of chemicals such as formaldehyde to protect PUFA due to their potential implications for animal product quality and consumer health, and to provide alternative to other protection techniques which are largely inefficient, livestock nutritionists have been evaluating the suitability of plant bioactive compounds such as EOs as modifiers of rumen biohydrogenation of PUFA. The majority of studies with EOs have been focused on their effects on VFA concentration, methane and ammonia production, whilst little attention has been given to their effects on biohydrogenation of PUFA. This modification is carried out by a direct or indirect interaction of these compounds and ruminal microorganisms involved in rumen fermentation. In this PhD study, the potential of EOs and their active compounds (EOCs) as feed additives to reduce the extent of rumen biohydrogenation of *n*-3 PUFA is evaluated. To achieve this objective, a number of experiments were conducted as follows:

Experiment 1

In this study, the effects of fifteen EOCs on the metabolism of *n*-3 PUFA by rumen microorganisms were evaluated *in vitro*.

This study found that some EOCs such as anethole, menthol, 4-allylanisole and *p*-cymene have the potential to reduce the extent of ruminal biohydrogenation of PUFA. However, they also equally caused significant reduction of total VFA. The whole essential oils have a number of attractions compared with the individual EOCs. The whole EOs have been used by man since antiquity in the area of aromatherapy and food industries, hence, are likely to encounter less regulatory hassles compared to individual EOCs. In addition, the synergistic effect between the minor and major components in the whole oils is expected to improve the effectiveness of the whole oils against biohydrogenating bacteria over the individual constituent compounds. These considerations led to the second experiment using the parent whole oils of the ten most effective EOCs in experiment 1.

Experiment 2

In this experiment, the effects of ten whole essential oils on rumen fermentation and biohydrogenation of *n*-3 polyunsaturated fatty acids by rumen microorganisms were examined *in vitro*. This study was conducted to establish whether the parent oils in which some of the individual EOCs (in experiment 1) showing potential are the predominant compounds, are equally as effective at inhibiting the biohydrogenation of PUFA. This study found that some of the whole oils such as anise and cassia oils had the highest potential to reduce the biohydrogenation of *n*-3 PUFA. But, they, like EOCs used in experiment 1, equally caused significant reduction of total VFA in the rumen.

Because the concentration of volatile fatty acids in the rumen reflects the fermentation of nutrients in feed, it was thought that the inclusion of either 300 mg/L of EOCs (experiment 1) or 300 mg/L of whole EOs (experiment 2) reduced the digestibility of feed, hence, decreased ruminal concentration of total VFA. Therefore, to achieve satisfactory inhibition of ruminal biohydrogenation of PUFA without significant suppression of VFA, further *in vitro* study was conducted to establish optimal doses for the two most effective EOCs (experiment 1) and EOs (experiment 2). This led to the next *in vitro* experiment, the third experiment, which screened graded doses of both EOCs and whole EOs.

Experiment 3

This experiment examined the effects of graded doses (0, 100, 200 and 300 mg/L) of two EOCs (4-allylanisole and anethole), and two whole EOs (anise oil and cassia oil) on the fermentation and biohydrogenation of *n*-3 polyunsaturated fatty acids by rumen microorganisms *in vitro*. This study found that at 200 mg/L or less, all substances except cassia oil did not reduce the concentration of total VFA relative to the control treatment.

In the literature, there is disagreement between *in vitro* and *in vivo* effects of EOs or EOCs on metabolism of *n*-3 PUFA. Continuous culture studies and long term *in vivo* studies suggest that the benefits associated with the use of essential oil as feed additive may decline due to two possibilities: (1) adaptation of individual microbial species to the use of EO or, (2) shifts in microbial species composition following long-term use of essential oil (Gladine *et al.*, 2007). Therefore, the last study (semi *in vivo*, experiment 4) was conducted to examine the possibility of microbial adaptation time to anise oil, the most effective EO at 200 mg/L.

Experiment 4

In this experiment, the potential of rumen microbes to adapt to anise oil over 4 weeks on rumen fermentation and metabolism of *n*-3 PUFA was investigated. Six lambs were grouped into two and given similar diet plus anise oil (100 mg/L equals 2.4 g/sheep/day) supplementation for one of the groups for a period of one month. Then, the sheep were slaughtered and ruminal fluids from the two groups of sheep were used in *in vitro* study. The study was a 2 × 2 factorial design experiment (details found in Chapter 6). The study observed that the PUFA content of feeds incubated in rumen fluid extracted from the group of sheep fed anise oil was maintained at higher concentrations compared to the control sheep.

Chapter 2

2.0. General material and methods

In this chapter the general methods, materials and techniques which are similar in all the experiments are discussed. Modifications of the procedures for specific experiments are discussed in the respective chapters.

2.1. Animal management, feeding and collection of rumen fluid

In all the experiments similar breed of sheep (Hartline × Texel cross) were used as rumen fluid donors. The sheep were housed in groups of three or two per pen (depending on whether six or four sheep were used) and straw bedding. Grass hay (*Lolium perenne*) supplied by Patterns Farm, Chelmsford, UK and concentrate (Lamb finisher cubes) from W & H Marriage & Sons LTD, Chelmsford, Essex, were the main feed ingredients. The rumen fluid donor sheep were offered water and hay *ad libitum* and supplemented with additional 400 g/sheep/day of concentrate. The total amount (400 g) of lamb finisher cubes offered per sheep/day was divided into two equal parts (200 g) and fed at 08.00 hours and 16.00 hours. The sheep were placed on the experimental feed for 14 days before slaughter.

Feed was withdrawn from the ewes at 18.00 hours on the eve of the day of slaughter. Sheep were taken to the abattoir (Humphreys and Sons, Chelmsford-Essex) at 07.00 hours in the morning and were slaughtered at 07.30 hours. Whole rumens were then collected and immediately sealed in tough plastic bags to prevent oxygen entry and transported in insulated boxes to maintain rumen temperature to the Lordship Science laboratory. The rumens were incised with a scalpel blade and rumen contents were scooped and the liquor strained through 2 layers of cheesecloth. After straining, the remaining solids were mixed with a volume of buffer (Table 2.1) equal to the rumen liquor removed, and homogenized using a kitchen blender for about 45 seconds to detach rumen microbes attached to solids. The mixture was re-strained with 2 layers of cheesecloth and the filtrate added to the rumen fluid to constitute the buffer rumen fluid mixture as the final inoculum. The mixed fluid was held in a water bath maintained at 39^0C and was flushed with CO_2 to expel oxygen before being dispensed into the *in vitro* incubation flasks.

2.2. Anaerobic buffer

The anaerobic buffer used for all *in vitro* incubations was made by mixing five different solutions (Table 2.1) using the method described by Theodorou *et al*. (1994). The solutions that were mixed to obtain the buffer are macro-mineral solution, buffer solution, micro-mineral solution, reducing solution and anaerobic indicator (resazurin). The solutions were prepared separately then mixed together thoroughly in a 50 litre capacity tank. In order to make a litre of buffer, the individual solutions were measured using a measuring cylinder and mixed together in the proportions shown in Table 2.1. The buffer solution was then sterilized using a Boxer autoclave (LAB3 Ltd, Northampton, UK). The autoclave was pre-set to commence sterilization of buffer at 121^0C for 80 minutes, and then cooled down to 80^0C. The autoclaved buffer was cooled under CO_2 in running cold water.

2.3. The basal diet for *in vitro* incubation

The basal feedstock used throughout the experiments was made from the mixture of a 70:30 rye- grass (*Lolium perenne*, Patterns Farm, Chelmsford, UK) and lamb finisher concentrate (W & H Marriage & Sons LTD, Chelmsford, Essex), respectively. The ingredients, chemical composition and fatty acid content of the basal feedstock used in incubations are shown in Table 2.2. A 70: 30 mixture of the grass hay and concentrate respectively was formulated and milled through 1 mm screen (Glen Creston Ltd, Stammore, England). This diet was supplemented with 32.5 g oil/kg from a mixture on an oil basis, of 60% of fish oil (Sigma-Aldrich Co. Ltd., UK) and 40% of ground linseed (NBTY Europe LTD, Burton-Upon-Trent), as extra sources of *n*-3 PUFA. Linseed was used as a major source of C18:3 *n*-3 and fish oil was included as the main source of C20:5*n* -3 and C22:6*n* -3. In order to make 1 kg of the basal feedstock used in incubations, 700 g of hay, 250 g of concentrate, 30 g of ground linseed and 20 g of fish oil were mixed together as shown in Table 2.2. The composition of the concentrate used according to the supplier (W & H Marriage & Sons LTD, Chelmsford, Essex) was a mixture of wheat (19.6%), wheatfeed (40.1%), molasses (3%), palm kernel extract (12%), sunflower extract (5%), limestone flour (2%), salt (0.8%), mixer oil (0.5%), millspec molasses (6%), spray oil (0.5%), ammonium chloride (0.3%) and malt nuts (10%).

2.4. Incubation, sample collection and storage

Into 125 ml clear glass type 1 serum bottles (R & L Slaughter Ltd, Essex, UK), 1 g of feed substrate, 80 ml anaerobic buffer (Table 2.1), 20 ml inoculum and 300 or less mg/L of either EOs or EOCs (actual amount depends on the objective of the experiment described in each chapter). The bottles were then sealed with rubber cork and incubated at 39^0C using Genlab incubator (Genlab Ltd, Cheshire, UK).

Gas pressure in the bottles during incubation was measured from all the replicates at various times (3, 6, 9, 12, 24, 36 and 48 h) using a pressure transducer (Bailey and Mackey Ltd., Birmingham, UK) which was connected to a digital read-out voltmeter. The pressure was read on the transducer and then the gas was released to return the head-space gas pressure to zero. The bottles were agitated by shaking before returning to the incubator. Fermentation was stopped (at 12, 24 and 48 h) by freezing the contents of incubation bottles at -20^0C for 5 mins. After 5 mins serum bottles were brought to room temperature, then 3 replicates of each treatment were taken to determine ammonia (5 ml) and volatile fatty acids (VFA, 4 ml). The remaining fluid of these replicates was used for determining pH using a pH meter (Hanna instrument Ltd., UK). The aliquots for ammonia were preserved by mixing 5 ml sample with 5 ml of 1M HCL. Volatile fatty acid samples (4 ml) were mixed with 1 ml of a deproteinising solution (2.5.6) and frozen (-20^0C). The remaining 3 replicates of each treatment were mixed with 250 ul of BHT in ethanol (2.5.7) then emptied into plastic tubs and frozen (-20^0C) for subsequent fatty acid analysis.

The individual head-space pressure (psi) was converted to volume of gas (ml) using the following equation:

$V= [(4.899*p)-0.1817]$

Where V is the volume of gas produced (ml) and p is the amount of head-space pressure (psi).

Table 2.1 Anaerobic buffer used for *in vitro* incubation according to Theodorou *et al*. (1994**)**

Compounds	**Proportions/litre**
Macro-mineral solution (g/1000 ml)	
di-sodium hydrogen ortho-phosphate ($Na_2HPO_4.12H_2O$	9.45g
Potassium di-hydrogen ortho-phosphate (KH_2PO_4)	6.20g
Magnesium sulphate 7-hydrate ($MgSO_4.7H_2O$)	0.60g
Buffer solution (g/1000 ml)	
Ammonium hydrogen carbonate (NH_4HCO_3)	4.0g
Sodium hydrogen carbonate ($NaHCO_3$)	35.0g
Micro-mineral solution (g/100 ml)	
Calcium chloride ($CaCl_2.2H_2$)	13.2g
Manganese chloride ($MnCl_2.4H_2O$)	10.0g
Cobalt chloride ($CoCl_2.6H_2O$)	1.00g
Iron chloride ($FeCl_3.6H_2O$	8.00g
Reducing solution (g/100 ml)	
Cysteine $HCl.1H_2O$	0.625g
Anaerobic indicator (g/100 ml)	
Resazurin	1tablet
Proportions of individual solutions for making 1 litre of anaerobic buffer	
Macro-mineral solution	200 ml
Buffer solution	200 ml
Reducing solution	40 ml
Micro-mineral solution	0.1 ml
Anaerobic indicator	1 ml
Deionized water	559 ml

Table 2.2 The ingredients, chemical composition and fatty acid content of the basal feedstock used in incubations

Components	composition
Feed ingredient (g/kg fresh)	
Hay	700.0
[1]Concentrate	250.0
Linseed	30.0
Fish oil	20.0
Chemical composition of basal feedstock (g/kg DM)	
Dry matter	921.0
Crude protein	123.6
Neutral detergent fibre	405.2
Acid detergent fibre	219.5
Ether extract	54.3
Fatty acid composition (g/100 g TFA)	
Linolenic (C18:3 n-3)	21.9
Linoleic (C18:2 n-6)	12.9
Palmitic (C16:0)	12.4
Oleic (C18:1 n-9)	10.0
Eicosapentaenoic (C20:5 n-3)	4.9
Myristic (C14:0)	4.3
Palmitoleic (C16:1)	4.0
Docosahexaenoic (C22:6 n-3)	3.7
Stearic (C18:0)	2.5
Vaccenic (C18:1)	1.6
Arachidonic (C20:4 n-6)	0.1
Remaining fatty acids	21.6
Total fatty acids (mg/g)	59.1

[1]Concentrate= W & H Marriage & Sons LTD, Chelmsford, Essex

2.5. ROUTINE CHEMICAL ANALYSIS

2.5.1. Dry matter

Sub-samples of ground (1 mm screen, Glen Creston Ltd, Stammore, England) mixed feed were weighed into porcelain crucibles (Fisher Scientific, Leicestershire, UK) that were previously pre-heated in the oven at 105°C and cooled. Samples were dried to a constant weight in the oven (105°C). After drying, samples were taken out of the oven into a desiccator to avoid absorption of moisture and were allowed to cool down. After cooling, the weight of the dried feed and the crucible was then taken together. The dry matter content was determined as follows:

$$\text{Dry matter (g/kg)} => \frac{\text{Dry weight (g)}}{\text{Wet wei ght (g)}} \times \frac{1000}{1}$$

2.5.2. Ether Extract

The fat content of the feed was determined using the FOSS Soxtec Extraction system according to the method described by AOAC 920.39 and AACC 30-25.

Approximately 2 g of the basal feedstock was weighed into the extraction thimble of known weight in triplicate. A thin layer of fat-free cotton was placed on the top of the sample and the thimbles were attached to the metal adapters before placing the thimbles and the adapter onto the thimble stand. A previously cleaned and dried aluminium extraction cup was taken from the desiccator and weighed. Using a measuring cylinder, exactly 45 ml of petroleum ether (40-60^{0}C, Sigma-Aldrich Co. Ltd., UK) was added to the aluminium cup and the cup was placed in the cup holder. The thimbles were attached to the magnetic holder in the Soxtec extractor and the extraction cups were inserted before running the extraction programme. The control unit of the Soxtec extractor was preset as follows: boiling time (15 minutes), boiling temperature (90^{0}C), rinsing time (30 minutes) and 10 minutes for the recovery time. The resulting fat residue was dried at 105°C to constant weight and the fat content was then determined gravimetrically using the following formula:

$$\text{Fat (g/kg)} => \frac{\text{weight of extract}}{\text{weight of sample}} \times \frac{1000}{1}$$

2.5.3. Feed total Nitrogen analysis

Total nitrogen of basal feedstock was determined using the Kjeldahl digestion procedure. Approximately 1 g of feed was weighed (in triplicate) into clean Kjeldahl tube (Fisher Scientific, Leicestershire, UK) and digested at 400°C for 2 hours using 20 ml of concentrated H_2SO_4. After cooling to room temperature, the residue was made alkaline through automatic addition of NaOH then the NH_3-N was distilled into a solution of boric acid (4%). This was then titrated with 0.1 M HCL. The sample weight and the volume of hydrochloric acid required to neutralize the ammonia were then used to calculate the nitrogen content of the feed as follows:

1 ml of 0.1 HCl = 0.0014 g of Nitrogen

$$\text{Nitrogen (g/kg)} => \frac{0.0014 \times \text{volume of HCL}}{\text{sample weight (g)}} \times \frac{1000}{1}$$

Crude protein was determined as follows: Nitrogen in feed × 6.25

2.5.4. Ammonia analysis

The concentration of NH_3-N in digesta was determined using the method described by Weatherburn (1967) adapted for use on the plate reader.

2.5.4.1. Reagents

A Standard solution (Ammonium chloride)

The standard solution was made by weighing 0.535 g of dried ammonium chloride and dissolving same in 100 ml distilled water to make 100 mM of ammonium chloride solution. From this solution (100 mM of ammonium chloride), 10 ml was pipetted into a 1000 ml volumetric flask and made up to the mark with distilled water. This solution was then stored in the fridge and used for making dilutions. Range of standard dilutions in 1 ml eppendorf is shown in Table 2.3.

B Phenol and sodium nitroprusside

In order to make this solution, 5 mg of sodium nitroprusside and 1 g of phenol (both from Sigma Aldrich) were separately dissolved in about 30 ml of distilled water. The two solutions were then mixed together and made up to 100 ml with distilled water. Solution was stored in dark bottles at 4°C and used within 4 weeks from the date of constitution.

C Alkaline hypochlorite solution, 14.99%

Sodium hydroxide and sodium hypochlorite were used to make this solution. Half a gram (0.5 g) of NaOH was dissolved in about 30 ml of dH_2O and 0.28 ml of sodium hypochlorite (14.99%) was added. The solution was transferred and made up to 100 ml in volumetric flak. Solution was stored in dark bottles at 4°C and used within 4 weeks from the date of constitution.

2.5.4.2. Analytical procedure

Samples were thoroughly mixed using a vortex mixer for 25 seconds before transferring about 1ml into labelled eppendorf tubes. The tubes were then centrifuged for 15 minutes at 20,000g. The supernatant was then used to make dilutions. Into a new labelled eppendorf tubes 100 uL of sample and 900 uL of dH_2O were added and mixed using vortex mixer for 25 seconds.

After dilutions, 20 uL of standard solution (2.5.4.1A) or sample was added to the 96 wells plate (Table 2.4) in duplicate using 20 uL Gilson pipette (Fisher Scientific, Leicestershire, UK). To each of the wells, 100 uL of reagent B (2.5.4.1B) and 80 uL of reagent C (2.5.4.1C) were sequentially added using a multi-channel pipette before mixing the contents of the wells using an electric shaker. The plate was then incubated at 39°C for 15 minutes to obtain a green to blue-like colour in the wells. In order to obtain a uniform colour in the wells before taking reading, the plate was manually shaken after incubation. Then absorbance reading was taken immediately at 650 nm using the plate reader (Molecular Devices Ltd, Berkshire, UK) with Spetra Max 90 software at room temperature.

Ammonia concentration (mM) was then determined by multiplying the mean result by the dilution factor.

Table 2.3 Range of standard dilutions in 1 ml Eppendorf

Distilled water (ml)	Ammonium chloride (ml)
1	0
1	0
0.800	0.200
0.800	0.200
0.600	0.400
0.600	0.400
0.400	0.600
0.400	0.600
0.200	0.800
0.200	0.800
0	1
0	1

Table 2.4 Sample of 96 well plates

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	0.20	0.20	0.40	0.4	0.6	0.6	0.80	0.8	1.0	1.0
B	Sample 1	Sample 1	Sample 2	Sample 2	Sample 3	Sample 3	Sample 4	Sample 4	Sample 5	Sample 5	Sample 6	Sample 6
C												
D												
E												
F												
G												
H												

NB: Row A1-A12 is standard.

2.5.5. Neutral and acid detergent fiber (NDF and ADF)

The neutral detergent fiber (NDF) and acid detergent fiber (ADF) content of the basal feedstock were determined using the methods of Goering and Van Soest (1970).

Reagents

NDF solution:

Into a 1 litre capacity beaker containing 500 ml of distilled water, 18.61 g of disodium ethylene tetra-acetate dihydrate (EDTA), 6.81 g di-Sodium tetraborate, 30 g of 90% sodium lauryl sulphate and 10 ml triethylene glycol mixed and 4.56 g disodium hydrogen orthophosphate were added. The mixture was then mixed by stirring with a magnetic stirrer then made up to 1L and pH was adjusted to 7.

ADF solution:

Exactly 49.04 ml of 1N H_2SO_4 was added to 1000 ml volumetric flask containing 400 ml of distilled water and this was made up to the mark with distilled water before adding 20 g of cetyl trimethylammonium bromide, CTAB.

Procedure:

Approximately 1 g of dried feed was weighed into preweighed sintered crucibles (Fisher Scientific, Leicestershire, UK) which were previously cleaned, dried (105°C) and cooled in a desiccator. Exactly 0.5 g of sodium sulphite was added to the crucibles containing the feed sample. The feed was then digested for 60 minutes using the Fibertech apparatus (Fibertech 1020 System M-Tecator) with 100 ml of NDF solution and 100 ml of ADF solution. Drops of octanol were added to each column before the heater and the timer were turned on. At the end of 60 minutes, the digest was washed (three times) with about 50 ml of hot water (100°C) and the column drained. Crucibles were then transferred to the cold extraction unit using the clip carrier and were placed in the same manner as with the fibertec. Each crucible was then rinsed with acetone (about 20 ml) and drained. The crucibles with the sample were then oven dried (105°C) overnight and cooled in a desiccator. After cooling, sample and crucible was weighed before ashing at 500°C for 5 hours using Carbolite AAF1100 furnace. The crucible and the ash were then

reweighed after cooling the crucibles in a desiccator. The NDF and ADF content of the feed were calculated as shown below:

$$\text{NDF (g/kg DM)} => \frac{\text{NDF weight (g)}}{\text{Sample weight (g)}} \times 1000$$

$$\text{ADF (g/kg DM)} => \frac{\text{ADF weight (g)}}{\text{Sample weight (g)}} \times 1000$$

2.5.6. Volatile fatty acid analysis

The volatile fatty acids content of rumen fluid were determined using the method of Ottenstein and Bartley (1971).

2.5.6.1. Reagents

A deproteinising solution (DPS) containing 2-ethylbutyric acid as an internal standard was prepared by adding 581 mg of 2-ethylbutyric acid and 50 ml of ortho-phosphoric acid into 250 ml volumetric flask. This solution was then made up to the mark with distilled water and stored at 4°C. *In vitro* fermentation digests (4 ml) were mixed with 1 ml of the DPS and frozen (minus 18^0C) ready for subsequent fatty acid analysis.

2.5.6.2. Sample preparation and analysis

The frozen samples were thawed at 4^0C and transferred to 30 ml Oak Ridge tube (Fisher Scientific, Leicestershire, UK) and centrifuged at 20 000 g for 15 mins at 4^0C. Sub sample of 1.0 ml (the supernatant) was then transferred to a labelled eppendorf tube and frozen at minus 18^0C ready for GC analysis.

Volatile fatty acids sample was removed from the freezer, defrosted and 150 uL was transferred to GC vial and analysed by GC. The operating conditions of the GC (Agilent 6890 with autosampler) were: column (HP-FFAP 10M x 0.53 mm with 1 metre retention gap), injection liner (4mm straight liner with phosphoric acid treated quartz fibre centre packing), carrier gas (Helium), head pressure 35kPa (5psi), column flow (approx. 24 ml/min), temperature (70°C x 1 min. and 70°C x 4 min. for initial and final respectively).

Determination of total fatty acids (mM) was done by simple addition of the individual volatile fatty acids that were identified. Molar proportion of individual volatile fatty acids was calculated as a proportion (mM/mol TVFA) of the total.

2.5.7. Fatty acid analysis

In order to protect fat from oxidation before and during analysis, butylated hydroxyl-toluone (BHT) was added at 0.05% of fat by dissolving 100 mg of BHT in 1 litre of 100% ethanol. Immediately after fermentation was stopped, 250 ul of BHT in ethanol was added to *in vitro* incubation digests before samples were frozen (-20^{0}C).

Direct saponification method (Enser *et al*., 1998) was used to extract fatty acids initially from both feed samples and *in vitro* incubation residues. Then these non-esterified fatty acids (NEFA) were methylated using 1% sulphuric acid in excess methanol (Christie, 2007) and the composition of methyl ester was analysed by gas chromatographic analysis (Folch *et al*., 1957).

2.5.7.1. Reagents

A Butylated hydroxyl-toluone (BHT)

Into a 100 ml volumetric flask, exactly 1 g of BHT was weighed and made up to the mark with chloroform.

B Internal standard (C_{21})

Into a 10 ml volumetric flask approximately 150 mg of Heneicosanoic acid (C_{21}) was weighed and 50 µl of BHT (2.5.7.2A) was added. The mixture was made up to the mark with chloroform. The solution was then thoroughly mixed and stored in the freezer (-20^{0}C).).

C Saponification mixture (5M potassium hydroxide-KOH)

Into a 500 ml measuring cylinder, 280.6 g of KOH was weighed and quickly dissolved in 400 ml of distilled water before capping the cylinder and cooled to room temperature under running cold water. The solution was made up to 500 ml with distilled water after cooling. Quinol (1 g) was weighed into a 200 ml beaker and dissolved in about 50 ml of methanol and subsequently transferred to a 500 ml volumetric flask. This was made up to the mark with methanol. The two solutions were then transferred into IL volumetric flask and thoroughly mixed together to create the saponification mixture.

2.5.7.2. Extraction of fatty acid content of basal feedstock

The FA content of basal diet was extracted by weighing 0.4 g of dried ground samples in duplicate into 30 ml Pyrex tubes. This quantity (0.4 g) was estimated (Wachira *et al.*, 2000) to give around 20-25 mg of fat for an accurate peak in chromatographic analysis. To each sample in the tube was added 0.7 ml of distilled water, 6 ml of 5M saponification mixture (2.5.7.1C), 100 µl of internal standard (2.5.7.1B) and 50 µl BHT (2.5.7.1A; as antioxidant). The tubes were incubated at 60^0C in a water bath for 3 h with shaking at 15 minutes intervals for about 30 seconds to completely hydrolyse and saponify the triglycerides in the sample. After incubation, tubes were taken out of water bath and allowed to cool down before adding 3 ml of 10N sulphuric acid then followed by further 1 h incubation to reform free fatty acids.

After incubation 12 ml of distilled water and 5 ml of petroleum spirit (40-60^0C) were added to the tube and manually shaken vigorously before centrifuging at 1000 g (2300 rpm) for 2 minutes. Absolute ethanol was added drop by drop to clear the gel and the top layer (supernatant) was transferred using a Pasteur pipette into a new soveril tube. This procedure was repeated twice making 3 times. In order to neutralize excess acid and to remove excess water from the fat sample, small amount of sodium hydrogen carbonate was added using small spatula until fizzing subsided then followed by adding some amount of anhydrous sodium sulphate until the power fell through. The tube was then centrifuged at 1000 g for 5 minutes before transferring the supernatant using Pasteur pipette to a clean new 10 ml quick fit tube and stored under oxygen-free nitrogen in the freezer (minus 18^0C) till the samples were methylated.

2.5.7.3. Extraction of fatty acid content of *in vitro* fermentation residues (digesta)

Into a 30 ml Pyrex tube, exactly 0.6 g of well mixed dried digesta was weighed followed by addition of 0.7 ml of distilled water, 6 ml of 5M saponification mixture (2.5.7.1C) and 100 µl of internal standard (2.5.7.1B). The sample was then saponified at 60^0C in a water bath for 2½ h with 15 minutes interval of regular shaking for about 30 seconds to completely hydrolyse the sample. Into each tube was added 12 ml of distilled water and 5 ml of petroleum spirit (40-60^0C) then shaken vigorously before centrifuging for 3 minutes at 1000 g. Absolute ethanol was added in drops to clear the gel before discarding the top layer and the procedure was repeated for 2 more times. Then 3 ml of 10N H_2SO_4 and 5 ml of petroleum spirit

were added followed by vigorous manual shaking of the tubes before centrifuging at 1000 g for 2 minutes. The top layer was transferred into another clean tubes using Pasteur pipette and this process was repeated 2 more times, such that there were three top layers collected into a new tube. In order to neutralize excess acid and to remove excess water from the fat sample, small amount of sodium hydrogen carbonate was added using small spatula until fizzing subsided then followed by addition of some amount of anhydrous sodium sulphate until the power fell through. The tube was then centrifuged at 1000 g for 5 minutes before transferring the supernatant using Pasteur pipette to a clean new quick fit tube and stored under oxygen-free nitrogen in the freezer (minus 18^0C) till the samples were methylated.

2.5.7.5. Methylation of extracted fatty acids samples (feed and digesta)

2.5.7.5.1. Reagents

Reagents used and their preparations for methylation are described below:

A 5% Sodium chloride

Into a 500 ml volumetric flask 25 g of sodium chloride was added and then made up to 500 ml using distilled water to the mark.

B 1% Sulphuric acid/methanol mixtures

Exactly 1 ml of concentrated sulphuric acid was added to a 100 ml volumetric flask containing about 40 ml of methanol and thoroughly mixed. The solution was then made up to the mark using methanol. NB. This mixture was made daily when required.

C 2% Potassium hydrogen carbonate

Into a 500 ml volumetric flask 10 g of potassium hydrogen carbonate was weighed and dissolved in distilled water, then made up to the indicated mark using distilled water.

2.5.7.5.2. Methylation procedures

Frozen samples were taken out of the freezer to defrost in room temperature before drying under Oxygen-free Nitrogen on hot plate maintained at 50^0C. Then 1 ml of hexane was added to the tube to resuspend

the sample followed by brief vortexing. To the sample tube was added 1 ml of the 1% sulphuric acid/methanol mixture (2.5.7.5.1B) before flushing the sample with nitrogen then glass stopper was immediately attached. Tubes were incubated for 1 hour in a water bath maintained at 50^0C. After incubation 2.5 ml of the 5% sodium chloride (2.5.7.5.1A) was immediately added followed by brief vortexing. Then 1 ml of hexane was added and vortexed briefly. Sample tube was allowed to settle in order to form clear layers before transferring the upper section (hexane portion) with a Pasteur pipette to a new clean 10 ml quick fit glass tube and repeating the procedure twice (i.e. addition of 1 ml of hexane plus vortexing). To the tube containing the hexane fraction (supernatants) 1.5 ml of the prepared 2% potassium hydrogen carbonate (2.5.7.5.1C) was added followed by brief vortexing. When two layers were clearly formed, the top layer (hexane fraction) was transferred into clean quick fit tube then dried under nitrogen with temperature maintained at 50^0C. Fatty acid methyl Esters (FAMES) were at last re-suspended by dissolving the dried samples in 500 uL of hexane plus brief vortexing. The re-suspended FAMES was then transferred into vial with insert, capped and then stored at minus 18 till gas chromatogram analysis (GCA).

The GC (HP 6890+, Agilent Technologies, UK Ltd) with a flame ionization detector and fitted with a 100 m fused silica capillary column (Varian CP-7489) of 0.2 µm film thickness and 250 µm diameter was used. Approximately 1 uL of fatty acid samples in hexane were injected at 160^0C (initial temperature of the column) and held at that temperature for 15 minutes. Then the temperature increased at 1.5/minute to 240^0C, and was held at that temperature until the run was completed (at 87 minutes, when the C22:6 *n*-3 was peaked). Helium at a flow rate of 1.2 ml/min was used as the carrier gas. The chromatograms were recorded and processed by a computer installed with Productivity Chemstation Software connected to the GC. Methyl heneicosanoic (C_{21}:$_0$, Sigma-Aldrich Co. Ltd., UK) which was added prior to saponification was used as an internal standard. Fatty acid methyl ester standard mixture (Thames Restek UK) of conjugated linoleic acid (CLA) was used as the standard for identification of peaks. The major peaks were identified by comparing the retention times with the corresponding retention times of known standards.

The concentration of individual fatty acid in sample was determined as follows:

$$\text{Fatty acid (mg)} => \frac{\text{Area of fatty acid} \times (\text{amount of standard/ area of standard})}{\text{sample weight (g)}}$$

$$\text{Fatty acid (g/100 g TFA)} => \frac{\text{fatty acid (mg/g)}}{\text{TFA}} \times \frac{100}{1}$$

Rumen biohydrogenation was calculated as the change in the proportion of individual FA such as 18:3 *n*-3 and 18:3 *n*-2, in the feed relative to the amount left in incubation vessels at a given time as follows:

$$\text{Biohydrogenation (g/100 g)} = \frac{\text{Initial amount of unsaturated FA - amount after incubation}}{\text{Initial amount of unsaturated fatty acid added}} \times 100$$

Calculations of sum of fatty acids are shown as follows:

∑SFA = C14:0+C16:0+C18:0

∑SFA-C18 = C14:0+C16:0

∑MUFAs = C16:1+C18:1 *n*-9+18:1 *trans* 11

∑PUFAs = C18:2 *n*-6+18:2 *cis*-9 *trans* 11 CLA+C18:3 *n*-3+C20:4 *n*-6+C20:5 *n*-3 and C22:6 *n*-3

∑*n*-6/∑*n*-3

P/S = ∑PUFAs/∑SFAs

CHAPTER 3

Effects of Fifteen Compounds from Essential Oil Extracts on the Metabolism of Polyunsaturated Fatty Acids by Rumen Microorganisms *in vitro*

ABSTRACT

The effects of fifteen EOCs on the fermentation activities of rumen microbes and the biohydrogenation (BH) of PUFA were examined *in vitro* using a batch culture system. Rumen fluid was collected from Six Hartline × Texel cross cull ewes offered grass hay (*Lolium perenne*) *ad libitum* and supplemented with additional 400 g/sheep/day of lamb finisher cubes. A basal feedstock comprising of 70:30 grass hay and concentrate was formulated and milled (1 mm screen) then supplemented with 32.5 g oil/kg (40% from ground whole linseed and 60% from fish oil). Serum bottles were incubated at 39^0C; each bottle contained 1 g of feed substrate, 80 ml buffer, 20 ml inoculum, then supplemented with 300 mg/l of EOCs and repeated twice. There were 16 treatments with six replicates per treatment as follows: Control (CON), 3-carene (CAR), 4-Allylanisole (ALA), *trans*-anethole (ANE), (-)-α-bisabolol (BIS), (-)-borneole (BOR), (-)-*trans*-caryophyllene (CPY), *trans*-cinnamaldehyde (CIN), (S)-(-)-β-citronellol (CIT), eucalyptol (EUC), (R)-(+)-limonene (LIM), menthol (MEN), myrtenol (MYT), *P*-cymene (CYM), (-)-α-thujone (THU) and vanillin (VAN). Gas measurement was taken at 3, 6, 9, 12, 24, 36 and 48 h and fermentation was stopped at 12, 24 and 48 h. Samples were collected to analyse NH_3-N, total volatile fatty acids (VFA) and molar proportions of individual VFA; and concentration of individual PUFA including intermediates of BH. Relative to the control, ANE and LIM reduced ($P<0.001$) ammonia concentration by a magnitude of 27%, whilst the other EOCs maintained similar ammonia levels. With the exception of VAN, the addition of all EOCs decreased ($P<0.001$) TVFA compared to the control, with MEN being the most inhibitory compound, inducing an approximately 20% reduction. The concentrations of 18:3 *n*-3 or 18:2 *n*-6 increased significantly in response to EOCs in the sequence: ALA and ANE, MEN and CIT > 3-CAR and BOR, CIN, LIM, MYT, CYM and THU > BIS and EUC > CPY and VAN. The addition of ALA maintained highest the concentrations of C20:5 *n*-3 and C22: 6 *n*-3. These results showed that the phenylpropanoid EOCs (ALA and ANE) and monoterpene alcohols (MEN) had the greatest potential to reduce the disappearance of PUFA and suggest that their use could enhance the concentrations of *n*-3 PUFA in ruminant food products if these effects are confirmed *in vivo*. However, this needs to be balanced against their effects on VFA.

3.1. Introduction

As discussed in section 1.5 of Chapter 1, several technologies such as encapsulation of marine and plant oils in formaldehyde, formation of fatty acids amides and the use of calcium salts of fatty acids have been developed over the years to protect PUFA from ruminal biohydrogenation (BH). However, the majority of these strategies have not satisfactorily protected PUFA from BH. Therefore, there is still an on-going search for a strategy that would satisfactorily protect PUFA from ruminal disappearance. Herbal plants and their extracts have been evaluated as possible alternative since they are natural components of animal feed (Cowan, 1999). The possibility of using whole essential oils and or their bioactive constituent compounds to modify rumen fermentation parameters such as protein break down, VFA and methane production (Calsamiglia *et al*., 2007; Benchaar *et al.*, 2008; Hart *et al*., 2008; Patra, 2011) has been widely studied (see section 1.7). Nevertheless, there is a scarcity of information in the literature on the effects of either EOs or EOCs as potential modifiers of rumen biohydrogenation of PUFA. The antimicrobial effects of EOs have been shown against a wide range of both Gram-positive and Gram-negative bacteria, viruses and fungi (Dean and Ritchie, 1987; Sivropoulou *et al*., 1996; Chao *et al*., 2000) and the possibility that they might be used as feed additives to selectively inhibit rumen microbes responsible for BH of fatty acids needs further investigation. In a recent study, Sgwane *et al*. (2013) evaluated the effects of 20 EOCs (at 300 mg/L) on the metabolism of PUFA *in vitro* and found that some compounds such as linalyl acetate, pinene and pulegone reduced the extent of ruminal BH of PUFA.

It has been established that effects of EOCs depends on the chemical type or structural configuration of the compound (Chapter 1.7). The diverse nature and number of commercially available EOCs warrants further screening. Hence, the aim of this study was to widen the range of EOCs screened in the study of Sgwane *et al*. (2013) at the same dose (300 mg/L). As ruminal biohydrogenation of PUFA involves several groups of Gram-positive bacteria (Harfoot and Hazlewood 1988), some EOCs used in this study were selected based on either their previous effects or the effects of their parent whole oils on Gram-positive bacteria (Table 3.1). In addition, as both biohydrogenation and methanogenesis depend on the availability of hydrogen (H_2) from feed digestion; other EOCs used were selected based on their previous effects on methanogenesis (Table 3.1). A number of other compounds were also chosen because they belong to the same chemical group as others which were previously reported to be effective (Table 3.1).

Table 3.1 Selected essential oil compounds used in this study and examples of microbes or process inhibited in previous studies

EOC	Microbes/process inhibited	Reference
P-cymene	*Bacillus subtilis, Salmonella, typhi, Staphylococcus aureus, Pseudomonas aeruginosa etc*	Roengsumran *et al*. (1997);
	Methanogenesis	Chaves *et al*., 2008
Cinnamaldehyde	*Staphylococcus aureus, E. coli and monocytogenes*	(Oussalah *et al*., 2007)
	Methanogenesis	Chaves *et al*., 2008; Macheboeuf *et al*. (2008
Menthol	*Camphylobacter jejuni, Listeria monocytogenes and Staphylococcus aureus*	Smith-Palmer *et al*. (1998);
	oil of peppermint rich in menthol inhibited methanogenesis	Tatsuoka *et al*., 2008
borneol	*Pseudomonas aeruginosa*, *Bacillus subtilis, Klebsiella pneumoniae* and *Proteus vulgaris,*	Prabuseenivasan *et al.* (2006)
4-allylanisole	*Staphylococcus epidermidis,* S. *aureus*	Singh *et al*., 2002
Limonene	Gram-positive and Gram- negative bacteria	Espina *et al*., (2011)
β-caryophyllene, 1,8-cineole	*Listeria monocytogenes*, *Staphylococcus aureus*, *Camphylobacter jejuni*, *E. coli*	Kalemba *et al*., 2012; Smith-Palmer *et al*. (1998)
Anethole	Methanogenesis	Telci *et al*., 2009
Eucalyptol	Eucalyptus oil rich in eucalyptol inhibited methanogenesis	Kumar *et al*., 2009
Thujone, myrtenol, citronellol	Same chemical class as *P*-cymene (Table 3.3)	Table 3.3
Bisabolol	Same chemical class as caryophyllene (Table 3.3)	Table 3.3
3-carene	Same chemical class as borneol and pinene (Table 3.3)	Sgwane *et al*. (2013); Table 3.3

3.2. Materials and methods

3.2.1. Animal management and collection of rumen fluid/sampling

In this experiment, six Hartline × Texel cross cull ewes (mean weight 50 ± 9.9 kg) were used as rumen fluid donors. Details of animal housing, experimental diet, feeding, duration of adaptation and collection of rumen fluid are as described in the general materials and methods (see section 2.1).

3.2.2. Basal feedstock, treatments and *in vitro* incubation

The basal feedstock was a mixture of good quality rye-grass hays (*Lolium perenne*), lamb finisher concentrate, whole ground linseed and fish oil. See section 2.3 for a detail description of the basal feedstock used in this *in vitro* incubation. The ingredients, chemical and fatty acid composition of the diet are shown in Table 2.2.

The effects of 15 essential oil constituent compounds were evaluated using the *in vitro* gas production batch culture method described by Theodorou *et al*. (1994). All EOCs used were purchased from Sigma-Aldrich Co. Ltd., UK and were stored at the required temperatures as specified on delivery notes prior to use. The description of the EOCs as purchased from Sigma-Aldrich is shown in Table 3.2. All the EOCs purchased and used had purity from 95% and above (Table 3.2). The typical parent whole oils from which the EOCs can be extracted are shown in Table 3.3.

There were 16 treatments and six replicates as follows: 3-carene (CAR), 4-Allylanisole (ALA), *trans*-anethole (ANE), (-)-α-bisabolol (BIS), (-)-borneole (BOR), (-)-*trans*-caryophyllene (CPY), *trans*-cinnamaldehyde (CIN), (S)-(-)-β-citronellol (CIT), eucalyptol (EUC), (R)-(+)-limonene (LIM), menthol (MEN), myrtenol (MYT), *P*-cymene (CYM), (-)-α-thujone (THU) and vanillin (VAN).

Incubation was done in 125 ml clear glass type 1 serum bottles (R & L Slaughter Ltd, Essex, UK) for 48 h in each run and repeated twice. In each run 432 serum bottles were incubated, each bottle contained 1 g of feed substrate, 300 mg/l of EOC, 80 ml anaerobic buffer (see Table 2.1) and 20 ml inoculum and the bottle sealed with rubber cork before incubation.

Table 3.2 Description of the essential oil compounds (EOCs) used in this experiment

Name of EOCs	Abbreviation	Catalog number	Purity	Density	Form of EOC
3-carene	CAR	**94415**	≥98.5%	0.864 g/mL	Liquid
4-Allylanisole	ALA	A29208	≥98%	0.961 g/mL	Liquid
Trans-anethole	ANE	10368	99.50%	0.988 g/mL	Liquid
(-)-α-bisabolol	BIS	14462	95%	0.92 g/mL	Liquid
(-)-borneole	BOR	15598	99%	1.011 g/mL	Powder
(-)-*trans*-caryophyllene	CPY	22075	98.5%	0.902 g/mL	Liquid
trans-cinnamaldehyde	CIN	239968	95%	1.05 g/mL	Liquid
(S)-(-)-β-citronellol	CIT	W509205	99%	0.856 g/mL	Liquid
Eucalyptol	EUC	C80601	99%	0.921 g/mL	Liquid
(R)-(+)-limonene	LIM	183164	99%	0.842 g/mL	Liquid
Menthol	MEN	M2772	99%	0.89 g/mL	Solid
Myrtenol	MYT	W343900	95+	0.954 g/mL	Solid
P-cymene	CYM	30039	≥99.5%	0.86 g/mL	Liquid
(-)-α-thujone	THU	89231	96%	0.914 g/mL	Liquid
Vanillin	VAN	V1104	99%	1.06 g/mL	Solid

Supplier of EOCs: Sigma-Aldrich Co. Ltd., UK

Table 3.3 Typical parent whole oils from which the EOCs used in this study can be extracted from

Common name	Source of EOC	Typical content in EO	Chemical class[1]	References
3-carene	Turpentine oil	70%	Bicyclic monoterpene	Mahboubi and Kazempour (2012)
4-Allylanisole	Basil oil	58.3%	phenylpropanoids	Chalchat and Ozcan (2008)
Trans-anethole	Anise oil	82.7%	phenylpropanoids	Soher *et al*. (2014)
(-)-α-bisabolol	*Artemisia ordosica*	27%	Sesquiterpene	Yang *et al*. (2012)
(-)-borneole	*Cymbopogon olivieri*	26%	Bicyclic monoterpene	Mahboubi and Kazempour (2012)
(-)-*trans*-caryophyllene	Clove	14%	Bicyclic sesquiterpene	Kalemba *et al*. (2012); EP 5
trans-cinnamaldehyde	Cassia oil	70-90	phenylpropanoids	EP 5; Kalemba *et al*. (2012)
(S)-(-)-β-citronellol	Citronella oil	31.5%	Monoterpene	Pandu *et al*. (2014)
Eucalyptol	Eucalyptus oil	68%	Monoterpene (ethers)	Elaissi *et al*. (2011)
(R)-(+)-limonene	Mandarin oil	75%	Monoterpene	Yu *et al*. (2007), EP 5
Menthol	Cornmint oil	30-55%	Monoterpene alcohol	EP 5; Patra (2012)
Myrtenol	*Astartea*	26%	Monoterpene	EP 5, Lowe *et al*. (2004)
P-cymene	Thyme oil	56%	Monoterpenoid	Juliano *et al*. (2000), EP 5; Kalemba *et al*., 2012
(-)-α-thujone	Sage oil	42%	Monoterpene	Marino *et al*. (2001)
vanillin	Vanilla oil	Main	Phenolic aldehyde	López-Malo *et al*. (2000)

[1=] Source of chemical class= Bakkali *et al*. (2008); Table 1.5.

3.2.3. Sample collection and preservation

The procedures for gas pressure measurement, fermentation stopping times, collection and storage of samples are described in the general material and methods (see section 2.4).

3.2.4 Chemical analysis

The concentration of NH_3-N in digesta was analysed using the phenol-hypochlorite method (Weatherburn, 1967; Broderick and Kang, 1980) adapted for use on the plate reader as described in general materials and methods (see section 2.5.4).

The concentration of volatile fatty acid (VFA) was determined by gas chromatography (GC) as described by Ottenstein and Bartley (1971). Details of this method are found in the general materials and methods (see section 2.5.6).

The concentrations of fatty acids in feed and in freeze dried samples of effluent of in vitro digests were extracted by direct saponification method described by Enser *et al*. (1998). Details of this method are outlined in the general materials and methods (see section 2.5.6).

3.2.5. Experimental design and statistical analysis

The objective was to examine the effects of fifteen EOCs on the extent of rumen biohydrogenation of *n*-3 PUFA *in vitro*, including their effects on fermentation parameters (gas production, NH_3-N concentration and on concentrations of TVFA and molar proportions of individual VFA. The null hypothesis was that inclusion of 300 mg/L of EOCs would have no effect on fermentation and biohydrogenation data. The alternative hypothesis was that the EOCs would affect (decrease or increase) fermentation activities.

This study was a completely randomized design (CRD) experiment with 16 treatments randomly allocated to fermentation flask as outlined in section 3.2.2. Data were analysed by analysis of variance (ANOVA) using GenStat 15th edition (VSN international Ltd, Registered to: Writtle College) with experimental runs as a blocking factor. Differences between treatments were declared by least

significance difference (LSD) and significance was declared at P< 0.05. Data were analysed separately for each time point (12, 24 and 48 h).

3.3. RESULTS

3.3.1. *In vitro* fermentation parameters

The effects of EOCs on cumulative gas production (ml/g OM), NH_3-N concentration and pH of fermentation vessels are shown in Table 3.4.

Except for VAN and LIM which maintained total gas production levels similar to the control (118.6 ml/g OM), the addition of most EOCs significantly (P<0.001) reduced total gas production at 12 h, with CIN (65.5 ml/g OM) being the most inhibitory EOC, inducing approximately 45% reduction of total gas. At 24 and 48 h, VAN had similar effects to the control (175.1 and 220 ml/g OM for 24 and 48 h respectively), whilst the rest of the treatments reduced gas production relative to the control, with the lowest reductions in gas production found in vessels with MEN and MYT (means 120.4 and 146.8 for 24 and 48 h, respectively).

The concentration of NH_3-N in cultures increased as the time of incubation progressed (lowest at 12 and highest at 48 h). There were no effects of treatments on the concentration of ammonia N in cultures at 12 h. After 24 h ANE and LIM caused a 27% mean reduction of NH_3-N concentration relative to the control. At 48 h, only ANE and CIN significantly decreased (P<0.001) NH_3-N concentration (mean decrease 5.5 mM) compared with the control, other treatments maintained NH_3-N levels similar to the control (6.9 mM).

The pH of cultures at 12 h was not affected by the addition of all EOCs except in cultures with added BOR which increased it (7.0) relative to the control (6.7). Relative to the control, the pH of cultures was significantly (P<0.001) increased with the addition of all EOCs at both 24 and 48 h (average 6.8 for both 24 and 48) except VAN (both 24 and 48) and CPY (24 h only) which had similar pH to the control (6.7 for both 24 and 48 h).

The concentration of total TVFA (mM) and molar proportions of individual VFA (mM/mol TVFA) are presented in Table 3.5. The average concentration of TVFA was highest in the control and VAN (63.8

and 71.0 mM for 24 and 48 h, respectively) and low ($P<0.001$) in the remaining treatments, with the lowest level observed in MEN (53.7 and 54.1 mM for 24 and 48 h, respectively). At 24 h the average molar proportion of acetate was lowest in vessels supplemented with MEN (67.2 mM/mol TVFA) and was highest in cultures with CIT (71.4 mM/mol TVFA), relative to the control (68.1 mM/mol TVFA). After 48 h the cultures with the lowest molar proportion of acetate was CIN (63.9 mM/mol TVFA), an approximately 2% reduction in acetate compared to the control, whilst CIT (69.0) had the highest molar proportion of acetate. Propionate was not affected by VAN (24 h), but was increased by EUC and VAN at 48 h (average increase of 10%), and was reduced by other compounds with CIT inducing the greatest reduction (20 % and 21 % for 24 and 48 h, respectively). Whilst VAN, EUC, CAR, CPY and BIS (48 h) had no effect on the proportion of butyrate, other compounds reduced the proportion of butyrate with MEN expressing the greatest reduction (21 and 34 % reduction at 24 and 48 h respectively). Although CIN, EUC and VAN had no effect on the acetate: propionate ratio, other EOCs increased ($P<0.001$) it with the highest increase recorded in vessels with CIT (about 35 % average increase at 24 and 48 h).

Table 3.4 Effects of EOCs on cumulative gas production (ml/g OM), pH and ammonia concentration (mM) in cultures during 48 h *in vitro* incubation

Variables	Time (h)	EOCs																	
		CON	3-CAR	ALA	ANE	BIS	BOR	CPY	CIN	CIT	EUC	LIM	MEN	MYT	CYM	THU	VAN	sed	P-values
Gas	12	118.6^{a}	97.1^{bc}	95.5^{b}	100.7^{bc}	106.7^{c}	96.9^{b}	96.5^{b}	65.5^{d}	84.2^{e}	102.9^{bc}	113.4^{a}	81.8^{e}	82.6^{e}	105.2^{c}	91.4^{b}	115.2^{a}	5.14	0.001
	24	175.1^{a}	130.4^{bd}	132.5^{bd}	137.9^{bd}	148.6^{cde}	137.7^{bd}	138.2^{bd}	126.9^{b}	128.1^{b}	148.3^{cde}	151.5^{c}	119.1^{b}	121.7^{b}	140.7^{cde}	130.1^{b}	172.0^{a}	6.72	0.001
	48	220.0^{a}	153.4^{bc}	154.5^{bc}	158.0^{b}	174.7^{b}	163.4^{b}	171.3^{b}	168.9^{b}	167.9^{b}	182.0^{b}	177.4^{bd}	141.4^{c}	152.2^{bc}	165.8^{b}	152.4^{bc}	215.7^{a}	7.80	0.001
NH_3-N	12	4.3	4.3	4.7	4.1	4.0	4.7	3.3	5.5	3.4	3.7	4.7	4.9	5.3	4.0	4.8	3.8	0.72	NS
	24	5.7^{a}	5.2^{ab}	4.9^{ab}	4.2^{b}	5.6^{a}	5.1^{ab}	7.5^{c}	4.53^{ab}	5.3^{ab}	5.2^{ab}	4.1^{b}	5.6^{a}	5.3^{ab}	4.5^{ab}	5.4^{ab}	4.8^{ab}	0.67	0.002
	48	6.9^{ace}	6.1^{abd}	6.0^{abd}	5.5^{b}	8.0^{e}	5.8^{bd}	6.4^{abc}	5.4^{b}	5.9^{bd}	6.5^{abd}	5.9^{bd}	6.8^{adc}	6.9^{ace}	5.9^{bd}	7.3^{ce}	6.3^{abc}	0.57	0.001
pH	12	6.7^{a}	6.8^{a}	6.8^{a}	6.8^{a}	6.8^{a}	7.0^{b}	6.8^{a}	6.9^{ab}	6.9^{ab}	6.8^{a}	6.8^{a}	6.9^{ab}	6.8^{a}	6.8^{a}	6.8^{a}	6.7^{a}	0.06	0.001
	24	6.7^{a}	6.8^{b}	6.8^{b}	6.8^{b}	6.8^{b}	6.8^{b}	6.7^{a}	6.8^{b}	6.8^{b}	6.8^{b}	6.8^{b}	6.8^{b}	6.8^{b}	6.8^{b}	6.8^{b}	6.7^{a}	0.03	0.001
	48	6.7^{a}	6.8^{b}	6.9^{c}	6.9^{c}	6.8^{b}	6.8^{b}	6.8^{b}	6.8^{b}	6.8^{b}	6.8^{b}	6.8^{b}	6.9^{c}	6.8^{b}	6.8^{b}	6.9^{c}	6.7^{a}	0.05	0.001

Means within row with different superscripts letters are different (P<0.05); CON, control; CAR, 3-carene; ALA, 4-Allylanisole; ANE, *trans*-anethole; BIS, (-)-α-bisabolol; BOR, (-)-borneole; CPY, (-)-*trans*-caryophyllene; CIN, *trans*-cinnamaldehyde; CIT, (S)-(-)-β-citronellol*;* EUC, eucalyptol*;* LIM, (R)-(+)-limonene*;* MEN, menthol*;* MYT, myrtenol*;* CYM, *P*-cymene*;* VAN, (-)-α-thujone*;* vanillin; EOCs= essential oils compounds.

Table 3.5 Effects of EOCs on concentration of total (mM) and molar proportions of individual volatile fatty acids (mM/mol TVFA) in cultures during 48 h *in vitro* incubation

		EOCs																	
FA	**Time (h)**	**CON**	**3-CAR**	**ALA**	**ANE**	**BIS**	**BOR**	**CPY**	**CIN**	**CIT**	**EUC**	**LIM**	**MEN**	**MYT**	**CYM**	**THU**	**VAN**	**sed**	**P-values**
TVFA	24	64.6^{a}	55.6^{bd}	54.4^{bd}	54.7^{bd}	58.7^{ce}	55.9^{b}	61.1^{ef}	54.5^{bd}	55.0^{bd}	60.5^{c}	55.9^{b}	53.7^{d}	57.5^{e}	54.5^{bd}	57.0^{e}	63.0^{af}	0.97	0.001
	48	71.7^{a}	59.3^{bf}	56.6^{bd}	57.4^{bdf}	63.5^{cef}	58.8^{bf}	71.8^{a}	60.6^{bcf}	66.4^{e}	67.5^{e}	60.8^{bef}	54.1^{d}	61.0^{f}	58.8^{bf}	58.5^{bf}	70.3^{ae}	2.10	0.001
Acetate	24	68.1^{ad}	69.6^{b}	69.2^{b}	69.1^{b}	68.5^{a}	67.9^{ac}	68.5^{a}	68.2^{a}	71.4^{e}	67.5^{cd}	69.6^{b}	67.2^{d}	68.5^{a}	69.8^{b}	67.9^{ac}	68.2^{a}	0.34	0.001
	48	65.1^{a}	67.8^{b}	67.3^{b}	68.0^{bd}	66.9^{b}	65.6^{a}	67.3^{b}	63.9^{c}	69.0^{d}	64.3^{a}	67.5^{b}	65.5^{a}	66.7^{b}	67.9^{b}	65.7^{a}	65.0^{a}	0.54	0.001
Propionate	24	20.2^{a}	18.3^{c}	17.6^{d}	17.6^{d}	18.5^{c}	18.6^{cd}	18.5^{c}	19.4^{b}	16.1^{e}	19.5^{b}	18.1^{cd}	17.9^{d}	17.8^{d}	17.5^{d}	18.2^{cd}	20.0^{a}	0.29	0.001
	48	21.6^{a}	18.6^{b}	18.0^{bc}	17.8^{c}	19.2^{b}	19.9^{d}	19.9^{d}	20.7^{e}	17.1^{f}	22.3^{g}	18.4^{b}	18.3^{b}	18.3^{b}	17.8^{c}	19.4^{b}	22.0^{g}	0.34	0.001
Butyrate	24	9.3^{a}	9.5^{ac}	10.4^{de}	10.1^{ef}	9.7^{bc}	10.6^{d}	9.9^{bf}	10.0^{be}	9.7^{bc}	9.6^{ac}	9.7^{bc}	11.2^{f}	10.2^{ef}	10.0^{be}	10.4^{de}	9.3^{a}	0.17	0.001
	48	9.3^{a}	9.9^{a}	11.0^{bc}	10.7^{b}	9.8^{a}	10.7^{b}	9.0^{a}	11.8^{c}	10.3^{b}	9.6^{a}	10.0^{ab}	12.5^{c}	10.8^{b}	10.5^{b}	10.9^{b}	9.2^{a}	0.42	0.001
A:P	24	3.4^{a}	3.8^{bcd}	4.0^{b}	4.0^{b}	3.7^{cd}	3.7^{be}	3.8^{d}	3.5^{ae}	4.5^{f}	3.5^{ae}	3.9^{d}	3.8^{d}	3.9^{bdg}	4.0^{bg}	3.8^{d}	3.4^{a}	0.09	0.001
	48	3.0^{ag}	3.7^{b}	3.8^{bc}	3.9^{c}	3.5^{be}	3.3^{d}	3.4^{de}	3.1^{a}	4.1^{f}	2.9^{a}	3.7^{b}	3.7^{b}	3.7^{b}	3.9^{c}	3.4^{de}	3.0^{a}	0.09	0.001

Means within row with different superscripts are different (P<0.05); TVFA, total volatile fatty acid; A/P, acetate to propionate ratio. CAR, 3-carene; ALA, 4-Allylanisole; ANE, *trans*-anethole; BIS, (-)-α-bisabolol; BOR, (-)-borneole; CPY, (-)-*trans*-caryophyllene; CIN, *trans*-cinnamaldehyde; CIT, (S)-(-)-β-citronellol*;* EUC, eucalyptol*;* LIM, (R)-(+)-limonene*;* MEN, menthol*;* MYT, myrtenol*;* CYM, *P*-cymene*;* VAN, (-)-α-thujone*;* vanillin; fatty acid; EOCs= essential oils compounds

3.3.2. Effects of EOCs on fatty acid metabolism

The effects of EOC treatments on concentrations of selected C14 and C16 fatty acids are presented in Table 3.6. The mean concentrations of C14:0 at 12 h was 5.0 g/100 g TFA and was similar for all EOCs except BIS and CYM which decreased the level ($P<0.001$; average 4.0 g/100 g TFA). Relative to the control, the fermentation vessel concentrations of C14:0 were not affected by the addition of 3-CAR, BIS, CPY, CIN, LIM and VAN at 24 h, but it was significantly higher ($P<0.001$) in cultures in which the rest of the treatments were added (mean values 5.0 g/100 g TFA). After 48 h the addition of all EOCs did not change the levels of C14:0 in the cultures except with ALA, CPY, EUC, MEN, THU and VAN which increased it (average 5.0 g/100 g TFA) relative to the control (4.0 g/100 g TFA).

After 12 h, the concentration of C16:0 was lowest ($P<0.001$) in vessels supplemented with BIS and CIT (mean values 14.0 g/100 g TFA) and highest in cultures in which the control, VAN, CPY and LIM were added (mean values 16.0 g/100 g TFA). Although the levels of C16:0 were not affected by treatments at 24 h, after 48 h it was lowest ($P<0.001$) in the cultures where BIS and MEN were added (mean values 14.1 g/100 g TFA). This level was approximately 12% and 7% lower than the concentrations found in the control (16.0 g/100 g TFA), and in ALA and ANE (mean values 15.2), respectively.

At 12 h the levels of C16:1 were highest ($P<0.001$) in cultures supplemented with BOR, CIT and MYT (average 2.7 g/100 g TFA), this was followed by ALA and THU (mean values 2.5 g/100 g TFA), and lowest in vessels with the control, BIS and VAN (average 2.1 g/100 g TFA). However, after 24 h, CPY had the lowest concentration of C16:1 (1.7 g/100 g TFA), followed by the control, 3-CAR, ANE, CIN, LIM, CYM and VAN (mean values 1.9 g/100 g TFA), whilst the highest levels of C16:1 was observed in vessels with added BOR and MYT (average 2.6 g/100 g TFA). At 48 h, BOR and MYT maintained the highest levels of C16:1 (means 2.7 g/100 g TFA) relative to the control (1.7 g/100 g TFA), which had similar concentration of C16:1 to 3-CAR, LIM, CYM and VAN.

The concentrations of selected C18 fatty acids in response to supplementation with EOCs are shown in Table 3.7. Relative to the control, CIT elicited an approximately 14% reduction in the concentration of C18:0 at 12 h, whilst the remaining EOCs maintained C18:0 at level similar to the control (14.0 g/100 g

TFA). The mean concentration of 18:0 was increased (P<0.001) by the addition of CYM, LIM and CIT to fermentation cultures at 24 h (average 14.6 g/100 g TFA), after 48 h, C18:0 was reduced (P<0.001) in the vessels where BOR, BIS, CIT, EUC, MEN, MYT and THU were added (11.4 g/100 g TFA) compared to the control (12.9 and 13.6 g/100 g TFA for 24 and 48 h respectively). The remaining treatments maintained similar levels of 18:0 to the control at both 24 and 48 h.

At 12 h, the average concentrations of C18:1 *n*-9 in cultures with added CIN, LIM, CPY and MEN was 7.0 g/100 g TFA, which exceeded (P=0.015) the mean values recorded in vessels with the control, BIS, BOR, CIT, EUC and VAN (6.3 g/100 g TFA). After 24 h, CIT caused about a twofold increase in the concentration of C18:1 *n*-9 relative to the control (P=0.015), whilst the levels were intermediate in the rest of the other EOCs. Supplementation with ALA, BIS, CIT, MEN and THU doubled the vessel content of C18:1 *n*-9 (mean values 5.3 g/100 TFA) relative to either the control or VAN (average 2.7 g/100 TFA) after 48 h.

No dietary treatment effect on the vessel content of C18:1 *trans* 11 was observed after 12 h. However, C18:1 *trans* 11 was increased by the addition of all EOCs at both 24 and 48 h with the exception of VAN. The greatest increases were found in cultures with MEN and BOR (mean values 1.2 and 1.3 g/100 g TFA for 24 and 48 h, respectively) compared to the control (average 1.0 for both 24 and 48 h).

The use of all EOCs did not affect the levels of 18:2 *cis*-9 *trans*-11 CLA at all incubation time points except for 48 h. After 48 h MEN, BOR and BIS caused marginal but significant (P=0.002) increases on the level of CLA (mean values 0.14, 0.11, 0.11 and 0.08 g/100 g TFA for MEN, BOR, BIS and control respectively).

The content of 18:2 *n*-6 decreased with time, being highest at 12 h and lowest at 48 h, in both the control and across all cultures supplemented with EOCs. At 12 h the vessel content of 18:2 *n*-6 increased significantly in response to EOCs in the sequence: CIT (4.7 g/100 g TFA) > ALA, ANE, CIN, MEN, MYT (average 4.2 g/100 g TFA) > 3-CAR, BOR, CPY, LIM, CYM and THU (mean values 3.4 g/100 g TFA) > VAN and the control (means 2.5 g/100 g TFA). The concentration of 18:2 *n*-6 at 24 h decreased significantly in response to EOCs in the order: control, CPY and VAN (1.4 g/100 g TFA) < BIS and EUC

(1.7 g/100 g TFA) < 3-CAR, BOR, CIN, LIM, MYT, CYM and THU (2.2 g/100 g TFA) < ALA, ANE, CIT and MEN (2.7 g/100 g TFA). After 48 h, the average level of 18:2 *n*-6 in cultures with the most effective EOCs (ANE and ALA) was more than doubled relative to the control, whilst the concentration was intermediate with the addition of the rest of the treatments.

The pattern of effects of treatments on the vessel content of 18:3 *n*-3 at all incubation times (12, 24 and 48 h) were similar to the reported effects of treatments on the levels of 18:2 *n*-6 above. After 12 h the vessel content of 18:3 *n*-3 increased significantly in response to EOCs in the sequence: CIT (7.0 g/100 g TFA) > ALA, ANE, CIN, MEN, MYT (average 6.2 g/100 g TFA) and lowest in cultures supplemented with VAN and the control (means 3.1g/100 g TFA). The values of 18:3 *n*-3 at 24 h decreased significantly in response to EOCs in the order: control and VAN (1.7 g/100 g TFA) < BIS and EUC (2.2 g/100 g TFA) < 3-CAR, BOR, CIN, LIM, MYT, CYM and THU (3.0 g/100 g TFA) < ALA, ANE and MEN (3.9 g/100 g TFA). After 48 h, the average level of 18:3 *n*-3 in cultures with the most effective EOCs (ANE and ALA) tripled the concentration observed in the control, whilst the concentration was intermediate with the addition of the rest of the treatments.

The effects of treatments on the concentration of selected C20 fatty acids are presented in Table 3.8. No effect of treatment was found on the concentration of C20:4 *n*-6 in cultures at all times of incubation.

The levels of C20:5 *n*-3 decreased with time, being highest at 12 h and lowest at 48 h, in both the control and in cultures with added EOCs. The effect of EOCs on the concentration of C20:5 *n*-3 at 12 h can be ranked in ascending order as follows: control and VAN (2.1) < 3-CAR, BIS, CPY, LIM and CYM (2.3 g/100 g TFA) < ALA, ANE, CIN and EUC (2.6 g/100 g TFA) < BOR, CIT, MEN, MYT and THU (2.7 g/100 g TFA). After 24 h, the effects of EOCs on the concentration of C20:5 *n*-3 in a descending order can be ranked as follows: ALA, ANE, BOR, EUC, MEN, MYT and THU (2.3 g/100 g TFA) > 3-CAR, BIS, CPY, CIN, CIT, LIM and CYM (1.8 g/100 g TFA) > the control and VAN (1.7 g/100 g TFA). Nevertheless, at 48 h period, the content of C20:5 *n*-3 in cultures with BOR (2.3 g/100 g TFA), followed by ALA, ANE, EUC, MEN, MYT and THU (average 2.0 g/100 g TFA) were more than doubled and doubled, respectively, relative to the control (1.0 g/100 g TFA).

The pattern of effects of treatments on the levels of C22:6 *n*-3 in vessels at all incubation times (12, 24 and 48 h) were similar to the reported effects of treatments on the levels of C20:5 *n*-3 above, with ALA, ANE and BOR among the most effective compounds to maintain the highest levels of C22:6 *n*-3.

The concentrations of the sums of fatty acids (g/100 g TFA) and content of total fatty acid (mg/g) are presented in Table 3.9. No effect of treatments was observed on the concentrations of remaining fatty acids after 12 h except in cultures with added BIS and EUC, which increased it relative to the control. At 24 and 48 however, ALA, ANE, CIT, LIM, MEN and THU reduced the levels of RFA in cultures compared to the control.

At 12 h the ∑SFA was significantly reduced by supplementation with CIT, BIS, BOR, CIN EUC, MEN, MYT and THU (average 32.4 g/100 g TFA) relative to the control (35.0 g/100 g TFA). No effect of treatment on ∑SFA was observed after 24 h. However, there was about 11% reduction of ∑SFA in cultures with added BOR, CIT, MEN, MYT and THU compared to the control after 48 h. The effects of EOCs on ∑SFA-18:0 was similar to the reported effects on ∑SFA; however, CYM which did not affect ∑SFA at 48 h caused 5% reduction in the content of ∑SFA-18:0 after 48 h relative to the control.

The content of ∑MUFA in cultures was reduced ($P<0.001$) with the addition of all EOCs at 12 except in cultures with CPY and VAN, being lowest in cultures supplemented with CIT (10.7 g/100 g TFA) relative to the control vessels (13.5). At 24 h, ∑MUFA concentration was highest in cultures with added CIT (10.8 g/100 g TFA) compared to the control (6.6 g/100 g TFA), which was not different from VAN. Except in vessels supplemented with CIN and VAN which had levels of ∑MUFA similar to the control, the rest of the treatments increased ∑MUFA after 48 h.

The content of ∑PUFA decreased as incubation time increased (i.e. highest at 12 h and lowest at 48 h). At 24 h, the content of ∑PUFA in cultures were in the following decreasing order of significant magnitude: ALA, ANE, CIT and MEN (average 10.9 g/100 g TFA) > 3-CAR, LIM, MYT, CYM and THU (9.5 g/100 g TFA) > BOR, BIS, CPY and EUC (8.0 g/100 g TFA) > VAN and the control (6.7 g/100 g TFA). After 48 h, the levels of ∑PUFA observed in cultures with ALA, ANE and MEN (9.1 g/100 g TFA), which

were the most effective EOCs, was more than double the concentration found in cultures with the control (4.5 g/100 g TFA).

No effect of inclusion of VAN and CIN was found on TFA at 12, 24 and 48 h. However, all other EOCs reduced (P<0.001) TFA relative to the control, with CPY, MEN and CYM (12 h), CIT, EUC and MYT (24 h), and ALA and ANE (48 h), being the EOCs that elicited the greatest reduction of TFA compared to the control (Table 3.9).

As indicated in Table 3.10, although no effect of treatments on ∑*n*-6/∑*n*-3 was observed at 12 h, it was consistently reduced (P<0.001) in cultures with BIS, BOR, EUC, MYT, THU and VAN (mean values 0.3) at both 24 and 48 h relative to the control (average 0.4).

The ratio of PUFA to SFA (P: S) in cultures decreased with time of incubation. At both 12 and 24 h, the P/S was increased (P<0.001) by all EOCs except EUC and VAN relative to the control (Table 3.10). At 48 h of incubation, the ratio of P/S in cultures with ALA, ANE, BOR, MEN, MYT and THU (EOCs with the highest levels of P/S) tripled (0.3) the content of P/S in the control (0.1).

The biohydrogenation (g/100 g) of 18:2 *n*-6 and *n*-3 PUFA are presented in Table 3.11. The extent of biohydrogenation of linoleic acid in the fermentation vessels increased as the time of incubation progressed; being lowest at 12 h and highest at 48 h. At 12 h the disappearance of 18:2 *n*-6 from vessels supplemented with EOCs can be ranked in the following increasing order: ALA, CIN, MYT and CIT (52.1 g/100 g) > ANE, BOR, MEN, CYM and THU (61.3%) > LIM, EUC, CPY, BIS, 3-CAR, VAN and the control (70.4). At 24 h, the biohydrogenation of 18:2 *n*-6 increased significantly in response to EOCs (P<0.001) in the sequence: ALA (70.2 g/100 g) >ANE and CYM (73.1 g/100 g) > THU, MYT, MEN (75.8 g/100 g) > 3-CAR, BOR, CIN and LIM (78.1 g/100 g) > CPY, EUC, VAN and the control (84.7 g/100 g). At 48 h, the protection of 18:2 *n*-6 from ruminal disappearance was least in the control, VAN and CPY (average 89.3 g/100 g), highest in cultures with ALA (74.4 g/100 g) followed by ANE and CYM (78.6), whilst rates were intermediate in the remaining EOCs.

The pattern of effects of EOCs on biohydrogenation of 18:3 *n*-3 was similar to their reported effects on the disappearance of vessel content of 18:2 *n*-6 at all incubation times. After 12 h the biohydrogenation of

18:3 *n*-3 from vessels supplemented with EOCs can be ranked in the following increasing order: ALA, CIN, and CIT (57.3 g/100 g) > ANE, BOR, MEN, CYM, MYT and THU (67.0%) and highest in the rest of the treatments such as LIM, EUC, CPY, BIS, 3-CAR, VAN and the control (70.4). The biohydrogenation values for 24 h in increasing response were: ALA (73.6 g/100 g) >ANE and CYM (77.1 g/100 g) > THU, MYT, MEN (80.1 g/100 g) and levels were intermediate in the rest of the other EOCs but highest in CPY, EUC, VAN and the control (84.7 g/100 g). At 48 h, the protection of 18:3 *n*-3 from ruminal disappearance was least in the control, VAN and CPY (average 94.3 g/100 g), highest in cultures supplemented with ALA (79.0 g/100 g) followed by ANE and CYM (82.2 g/100 g), whilst it was intermediate in the remaining EOCs.

The extent of disappearance of 20:5 *n*-3 in vessels increased as the time of incubation advanced, being lowest at 12 h and highest at 48 h (Table 3.11). Biohydrogenation of C20:5 *n*-3 at 12 h of incubation in increasing magnitude was as follows: ALA, MYT, CIT, CIN, BOR and THU (27.5 g/100 g) < ANE, BIS, MEN and CYM (37.2 g/100 g) < 3-CAR, CPY, LIM, VAN and the control (46.5 g/100 g). After 24 h, biohydrogenation increased significantly in response to EOCs ($P<0.001$) in the progression: control, 3-CAR, CPY, CIN, LIM and VAN (46 g/100 g) and was highest in ALA, BOR, MYT and THU (62.9 g/100 g), with potential being intermediate in the other EOCs. After 48 h, biohydrogenation of C20:5 *n*-3 was lowest in vessels with added ALA and THU (42 g/100 g) relative to the control (71.4 g/100 g).

The trend of biohydrogenation in the vessel content of C22:6 *n*-3 was similar to the reported trend in the biohydrogenation of C20:5 *n*-3, with ALA, BOR and THU showing the highest ($P<0.001$) potential to reduce the disappearance of C22:6 *n*-3 after 24 and 48 h, whilst 3-CAR, CPY and LIM, had the lowest degree of protection of C22:6 *n*-3 from biohydrogenation relative to the control.

Table 3.6 Effects of EOCs on selected C14 and C16 fatty acids concentration (g/100 g total fatty acids) in cultures at 12, 24 and 48 h *in vitro* incubation

		EOCs																	
FA	**Time (h)**	**CON**	**3-CAR**	**ALA**	**ANE**	**BIS**	**BOR**	**CPY**	**CIN**	**CIT**	**EUC**	**LIM**	**MEN**	**MYT**	**CYM**	**THU**	**VAN**	**sed**	**P-values**
C14:0	12	5.01^{a}	5.00^{a}	5.02^{a}	5.01^{a}	4.01^{b}	5.00^{a}	5.02^{a}	5.00^{a}	5.01^{a}	4.99^{a}	5.01^{a}	5.01^{a}	5.00^{a}	4.03^{b}	5.01^{a}	5.02^{a}	0.20	0.001
	24	4.02^{a}	4.03^{a}	5.03^{b}	5.02^{b}	4.03^{a}	5.01^{b}	4.01^{a}	4.03^{a}	5.00^{b}	5.02^{b}	4.02^{a}	4.98^{b}	5.01^{b}	5.01^{b}	5.02^{b}	4.01^{a}	0.20	0.001
	48	4.01^{a}	4.02^{a}	5.00^{b}	4.01^{a}	4.00^{a}	4.01^{a}	5.01^{b}	4.02^{a}	4.00^{a}	5.01^{b}	4.01^{a}	4.99^{b}	4.01^{a}	4.00^{a}	5.01^{b}	5.01^{b}	0.10	0.001
C16:0	12	16:0^{a}	15.0^{b}	15.0^{b}	15.0^{b}	14.0^{c}	15.0^{b}	16.0^{a}	15.0^{b}	14.0^{c}	15.0^{b}	16.0^{a}	15.0^{b}	15.0^{b}	15.0^{b}	15.0^{b}	16.0^{a}	0.40	0.001
	24	15.2	14.5	14.6	14.6	14.4	14.7	13.6	14.3	15.5	15.5	15.0	14.8	14.7	15.3	14.9	14.9	0.62	NS
	48	16.0^{a}	15.5^{a}	15.2^{b}	15.2^{b}	14.3^{c}	14.9^{bc}	15.5^{b}	15.2^{b}	13.8^{c}	15.5^{b}	15.6^{b}	13.9^{c}	14.8^{bc}	15.0^{b}	14.8^{bc}	16.1^{a}	0.39	0.001
C16:1	12	2.00^{a}	2.10^{ad}	2.40^{be}	2.30^{b}	2.10^{a}	2.70^{c}	2.10^{a}	2.20^{d}	2.70^{c}	2.30^{b}	2.20^{d}	2.60^{ce}	2.70^{c}	2.00^{a}	2.50^{e}	2.10^{a}	0.100	0.001
	24	1.90^{a}	1.80^{ad}	2.20^{b}	2.00^{a}	2.10^{b}	2.60^{c}	1.70^{d}	1.80^{ad}	2.30^{b}	2.30^{b}	1.90^{a}	2.30^{b}	2.50^{c}	1.90^{a}	2.30^{b}	1.90^{a}	0.100	0.001
	48	1.70^{ad}	1.70^{ad}	2.20^{b}	2.00^{b}	2.10^{b}	2.60^{c}	1.90^{a}	1.60^{d}	2.00^{b}	2.20^{b}	1.70^{ad}	2.20^{b}	2.70^{c}	1.70^{ad}	2.20^{b}	1.80^{ad}	0.110	0.001

Means within row with different superscripts letters are different (P<0.05); CON, control; CAR, 3-carene; ALA, 4-Allylanisole; ANE, *trans*-anethole; BIS, (-)-α-bisabolol; BOR, (-)-borneole; CPY, (-)-*trans*-caryophyllene; CIN, *trans*-cinnamaldehyde; CIT, (S)-(-)-β-citronellol*;* EUC, eucalyptol*;* LIM, (R)-(+)-limonene*;* MEN, menthol*;* MYT, myrtenol*;* CYM, *P*-cymene*;* VAN, (-)-α-thujone*;* vanillin; FA= fatty acids. Values with extreme similarity are given in two decimal places

Table 3.7 Effects of EOCs on selected C18 fatty acids composition (g/100 g total fatty acids) in cultures at 12, 24 and 48 h *in vitro* incubation

		EOCs																	
FA	**Time (h)**	**CON**	**3-CAR**	**ALA**	**ANE**	**BIS**	**BOR**	**CPY**	**CIN**	**CIT**	**EUC**	**LIM**	**MEN**	**MYT**	**CYM**	**THU**	**VAN**	**sed**	**P-values**
C18:0	12	14.0^{ab}	15.0^{a}	14.0^{ab}	15.0^{a}	13.0^{bc}	14.0^{ab}	14.0^{ab}	14.0^{ab}	12.0^{c}	13.0^{bc}	15.0^{a}	13.0^{bc}	13.0^{bc}	15.0^{a}	13.0^{bc}	14.0^{ab}	0.60	0.001
	24	12.9^{a}	13.4^{a}	13.0^{a}	13.8^{ab}	13.1^{a}	12.6^{a}	12.4^{a}	13.3^{a}	15.0^{b}	13.2^{a}	14.0^{b}	13.4^{a}	12.8^{a}	14.7^{b}	13.0^{a}	12.7^{a}	0.65	0.006
	48	13.6^{ac}	14.1^{a}	12.3^{bc}	13.2^{ac}	11.0^{b}	11.4^{b}	12.1^{bc}	14.1^{a}	11.7^{b}	11.6^{b}	14.6^{a}	11.5^{b}	11.4^{b}	13.3^{ac}	11.4^{b}	13.0^{ac}	0.78	0.001
C18:1 *n*-9	12	6.3^{a}	6.8^{ab}	6.7^{ab}	6.6^{ab}	6.3^{a}	6.3^{a}	6.9^{b}	7.0^{b}	6.3^{a}	6.2^{a}	7.0^{b}	6.9^{b}	6.6^{ab}	6.6^{ab}	6.6^{ab}	6.3^{a}	0.27	0.015
	24	3.7^{a}	6.0^{bf}	5.7^{bc}	5.7^{bc}	5.5^{bc}	5.2^{c}	5.3^{cd}	5.9^{bd}	7.2^{e}	5.4^{bc}	6.4^{f}	6.1^{bf}	5.7^{bc}	6.2^{bf}	5.8^{bd}	4.0^{a}	0.35	0.001
	48	2.5^{a}	4.3^{bd}	5.3^{c}	4.2^{bd}	5.3^{c}	4.8^{b}	3.9^{d}	3.1^{a}	5.3^{c}	4.3^{bd}	4.6^{b}	5.1^{c}	5.0^{c}	4.0^{bd}	5.3^{c}	2.8^{a}	0.42	0.001
C18:1 tr 11	12	1.1	1.2	1.2	1.2	1.1	1.4	1.2	1.3	1.3	1.2	1.2	1.4	1.5	1.1	1.4	1.1	0.60	NS
	24	1.0^{a}	1.1^{c}	1.1^{c}	1.0^{a}	1.1^{c}	1.2^{d}	1.0^{a}	0.9^{b}	1.2^{d}	1.1^{c}	1.1^{c}	1.2^{d}	1.2^{d}	1.1^{c}	1.1^{c}	1.0^{a}	0.05	0.001
	48	1.0^{a}	1.1^{c}	1.1^{c}	1.1^{c}	1.1^{c}	1.2^{d}	1.1^{c}	0.9^{b}	1.1^{c}	1.1^{c}	1.1^{c}	1.3^{e}	1.2^{d}	1.0^{a}	1.1^{c}	1.0^{a}	0.05	0.001
C18:2 CLA	12	0.20	0.21	0.22	0.25	0.22	0.18	0.20	0.21	0.23	0.20	0.20	0.23	0.23	0.22	0.24	0.20	0.030	NS
	24	0.14	0.16	0.18	0.19	0.14	0.15	0.22	0.14	0.19	0.15	0.15	0.15	0.16	0.14	0.15	0.17	0.027	NS
	48	0.08	0.07	0.1	0.1	0.11^{a}	0.11^{a}	0.08	0.08	0.08	0.1	0.07	0.14^{a}	0.1	0.08	0.1	0.09	0.016	0.002
C18:2 *n*-6	12	2.4^{a}	3.3^{b}	4.1^{cd}	4.0^{c}	2.8^{a}	3.3^{b}	3.1^{b}	4.6^{cd}	4.7^{d}	2.8^{a}	3.5^{b}	4.2^{cd}	4.1^{cd}	3.5^{b}	3.5^{b}	2.5^{a}	0.33	0.001
	24	1.3^{a}	2.2^{b}	2.7^{c}	2.7^{c}	1.6^{d}	2.0^{b}	1.5^{a}	2.0^{b}	2.8^{c}	1.7^{d}	2.2^{b}	2.6^{c}	2.2^{b}	2.4^{b}	2.2^{b}	1.4^{a}	0.14	0.001
	48	0.9^{a}	1.7^{cg}	2.3^{d}	2.1^{de}	1.3^{bf}	1.6^{c}	1.2^{b}	1.3^{bf}	1.4^{bc}	1.2^{b}	1.6^{c}	2.0^{e}	1.5^{cf}	1.9^{eg}	1.6^{c}	1.0^{a}	0.11	0.001
C18:3 *n*-3	12	3.0^{a}	4.5^{bf}	5.8^{c}	5.7^{cf}	3.7^{a}	4.6^{bf}	4.0^{b}	6.8^{de}	7.0^{d}	3.7^{a}	4.7^{bf}	6.1^{e}	5.7^{cf}	4.7^{bf}	4.9^{f}	3.2^{a}	0.45	0.001
	24	1.6^{a}	3.1^{c}	4.0^{d}	3.9^{d}	2.3^{e}	2.8^{b}	2.0^{e}	2.7^{b}	3.6^{dg}	2.1^{f}	3.1^{c}	3.9^{d}	2.9^{bc}	3.4^{c}	3.0^{bc}	1.8^{af}	0.19	0.001
	48	1.1^{a}	2.2^{c}	3.2^{d}	3.0^{de}	1.7^{bf}	2.1^{c}	1.5^{f}	1.6^{bf}	2	1.4^{abf}	2.2^{c}	2.7^{e}	1.9^{bc}	2.7^{e}	2.2^{c}	1.2^{af}	0.16	0.001

Means within row with different superscripts letters are different (P<0.05); FA= fatty acids; CON, control; CAR, 3-carene; ALA, 4-Allylanisole; ANE, *trans*-anethole; BIS, (-)-α-bisabolol; BOR, (-)-borneole; CPY, (-)-*trans*-caryophyllene; CIN, *trans*-cinnamaldehyde; CIT, (S)-(-)-β-citronellol*;* EUC, eucalyptol*;* LIM, (R)-(+)-limonene*;* MEN, menthol*;* MYT, myrtenol*;* CYM, *P*-cymene*;* VAN, (-)-α-thujone*;* vanillin; FA= fatty acid

Table 3.8 Effects of EOCs on selected long chain fatty acids composition (g/100 g total fatty acids) in cultures at 12, 24 and 48 h *in vitro* incubation

		EOCs																	
FA	**Time (h)**	**CON**	**3-CAR**	**ALA**	**ANE**	**BIS**	**BOR**	**CPY**	**CIN**	**CIT**	**EUC**	**LIM**	**MEN**	**MYT**	**CYM**	**THU**	**VAN**	**sed**	**P-values**
	12	0.30	0.32	0.20	0.21	0.31	0.20	0.22	0.20	0.22	0.20	0.21	0.22	0.21	0.30	0.30	0.31	0.02	NS
C20:4 *n*-6	24	0.31	0.33	0.31	0.30	0.33	0.31	0.43	0.30	0.32	0.30	0.31	0.32	0.30	0.30	0.31	0.31	0.03	NS
	48	0.31	0.30	0.29	0.31	0.30	0.28	0.31	0.31	0.30	0.32	0.29	0.29	0.20	0.31	0.30	0.28	0.02	NS
	12	2.0^{a}	2.2^{b}	2.6^{cd}	2.6^{cd}	2.3^{b}	2.7^{ce}	2.2^{b}	2.5^{d}	2.7^{ce}	2.5^{d}	2.3^{b}	2.7^{ce}	2.8^{e}	2.3^{b}	2.7^{ce}	2.1^{a}	0.08	0.001
C20:5 *n*-3	24	1.6^{a}	1.8^{bc}	2.2^{e}	2.2^{e}	1.9^{c}	2.4^{d}	1.8^{bc}	1.8^{bc}	1.9^{c}	2.2^{e}	1.9^{c}	2.3^{de}	2.3^{de}	1.8^{bc}	2.3^{de}	1.7^{ab}	0.07	0.001
	48	1.0^{a}	1.4^{b}	2.0^{dg}	1.9^{de}	1.8^{e}	2.3^{c}	1.4^{b}	1.3^{b}	1.3^{b}	2.0^{dg}	1.4^{b}	2.0^{dg}	2.1^{g}	1.6^{f}	2.1^{g}	1.3^{b}	0.1	0.001
	12	1.6^{a}	1.7^{ac}	1.9^{bd}	1.9^{bd}	1.8^{bc}	2.0^{d}	1.6^{a}	1.8^{bc}	1.8^{bc}	1.9^{bd}	1.8^{bc}	2.0^{d}	2.1^{d}	1.8^{bc}	2.1^{d}	1.7^{a}	0.08	0.001
C22:6 *n*-3	24	1.5^{ab}	1.6^{b}	1.8^{de}	1.8^{de}	1.7^{d}	2.0^{c}	1.7^{e}	1.4^{a}	1.5^{ab}	1.8^{de}	1.6^{b}	1.8^{de}	1.9^{d}	1.5^{ab}	1.9^{d}	1.6^{b}	0.08	0.001
	48	1.0^{ag}	1.1^{a}	1.7^{de}	1.6^{d}	1.8^{ef}	2.0^{e}	1.3^{b}	1.0^{ag}	0.9^{g}	1.8^{ef}	1.1^{a}	1.7^{de}	1.9^{ef}	1.2^{b}	1.9^{ef}	1.2^{b}	0.08	0.001

Means within row with different superscripts letters are different (P<0.05); CON, control; CAR, 3-carene; ALA, 4-Allylanisole; ANE, *trans*-anethole; BIS, (-)-α-bisabolol; BOR, (-)-borneole; CPY, (-)-*trans*-caryophyllene; CIN, *trans*-cinnamaldehyde; CIT, (S)-(-)-β-citronellol*;* EUC, eucalyptol*;* LIM, (R)-(+)-limonene*;* MEN, menthol*;* MYT, myrtenol*;* CYM, *P*-cymene*;* VAN, (-)-α-thujone*;* vanillin; FA= fatty acids. Values with extreme similarity are given in two decimal places.

Table 3.9 Effects of EOCs on the concentration of sums of fatty acids (g/100 g TFA) and content of total fatty acids (mg/g) in cultures at 12, 24 and 48 h *in vitro* incubation

FA	Time (h)	EOCs																sed	P-value
		CON	3-CAR	ALA	ANE	BIS	BOR	CPY	CIN	CIT	EUC	LIM	MEN	MYT	CYM	THU	VAN		
RFAs	12	35.5^{a}	33.4^{a}	32.2^{a}	32.9^{a}	39.0^{b}	34.6^{a}	33.3^{a}	32.3^{a}	35.0^{a}	37.0^{b}	31.8^{c}	33.2^{ac}	33.8^{ac}	34.9^{a}	35.4^{a}	36.1^{a}	1.73	0.005
	24	43.0^{a}	39.1^{a}	38.2^{b}	37.9^{b}	40.4^{ab}	39.8^{ab}	43.9^{a}	41.2^{ab}	32.4^{c}	38.7^{ab}	37.3^{b}	35.9^{c}	38.8^{ab}	36.1^{cb}	38.0^{b}	43.0^{a}	2.37	0.001
	48	43.2^{ac}	38.8^{b}	37.8^{b}	39.3^{bc}	41.9^{cd}	41.1^{cd}	41.3^{c}	43.9^{ac}	43.2^{a}	41.8^{ab}	38.3^{b}	40.8^{d}	41.1^{cd}	40.1^{d}	40.1^{d}	43.0^{ac}	1.16	0.001
∑SFA	12	35.0^{a}	35.2^{a}	34.6^{a}	34.8^{a}	31.5^{b}	33.7^{c}	35.0^{a}	33.6^{c}	31.4^{b}	32.9^{b}	36.0^{a}	32.2^{b}	32.2^{b}	34.1^{a}	32.0^{b}	34.6^{a}	0.97	0.001
	24	32.3	32.1	32.2	33.1	31.8	32.0	29.9	31.9	35.3	33.4	33.3	32.9	32.1	34.5	32.7	32.1	1.48	NS
	48	34.0^{ad}	34.1^{ad}	32.1^{acd}	32.9^{acd}	29.6^{be}	30.7^{ce}	32.3^{acde}	33.4^{ad}	29.8^{be}	31.8^{ae}	34.6^{d}	30.0^{e}	30.7^{e}	32.8^{acd}	30.8^{e}	33.6^{ad}	1.2	0.001
∑SFA-18:0	12	20.7^{a}	20.3^{ac}	20.3^{ac}	20.2^{ac}	18.4^{b}	20.1^{ac}	20.9^{a}	19.8^{c}	19.6^{c}	19.8^{c}	20.5^{ac}	19.6^{c}	19.6^{c}	19.3^{c}	19.4^{c}	20.6^{a}	0.52	0.001
	24	19.5	18.7	19.2	19.3	18.8	19.4	17.5	18.6	20.3	20.3	19.3	19.5	19.3	19.9	19.7	19.4	0.78	NS
	48	20.5^{a}	20.0^{a}	19.8^{ac}	19.7^{ac}	18.6^{b}	19.3^{c}	20.2^{a}	19.2^{c}	18.1^{b}	20.2^{a}	20.0^{a}	18.5^{b}	19.3^{c}	19.5^{c}	19.4^{c}	20.6^{a}	0.46	0.001
∑MUFA	12	13.5^{a}	12.5^{bc}	11.7^{cd}	11.1^{de}	12.2^{bc}	12.5^{bc}	13.5^{a}	11.0^{de}	10.7^{e}	12.6^{bc}	12.5^{bc}	12.3^{bc}	12.1^{bc}	11.7^{cd}	12.3^{bc}	13.1^{a}	0.46	0.001
	24	6.6^{a}	8.9^{b}	9.0^{b}	8.8^{b}	8.8^{b}	9.1^{b}	8.0^{b}	8.6^{b}	10.8^{c}	8.8^{b}	9.3^{b}	9.6^{b}	9.4^{b}	9.1^{b}	9.3^{b}	6.9^{a}	0.47	0.001
	48	5.2^{a}	7.1^{b}	8.6^{c}	7.2^{b}	8.6^{c}	8.6^{c}	6.8^{d}	5.6^{a}	8.4^{c}	7.6^{b}	7.3^{b}	8.6^{c}	8.9^{c}	6.8^{d}	8.7^{c}	5.6^{a}	0.52	0.001
∑PUFA	12	9.5^{a}	12.2^{b}	14.8^{c}	14.6^{c}	11.1^{b}	13.0^{d}	11.2^{b}	16.2^{e}	16.5^{e}	11.3^{b}	12.7^{b}	15.5^{e}	15.2^{e}	12.7^{b}	13.7^{d}	9.9^{a}	0.83	0.001
	24	6.4^{a}	9.2^{b}	11.1^{c}	11.0^{c}	8.0^{d}	9.5^{b}	7.6^{d}	8.3^{d}	10.3^{c}	8.2^{d}	9.1^{b}	11.0^{c}	9.7^{b}	9.5^{b}	9.7^{b}	7.0^{a}	0.6	0.001
	48	4.5^{a}	6.8^{b}	9.5^{c}	9.0^{c}	7.0^{d}	8.4^{d}	5.7^{e}	5.5^{e}	5.9^{e}	6.7^{e}	6.7^{e}	8.9^{c}	7.6^{d}	7.8^{d}	8.2^{d}	5.1^{a}	0.45	0.001
TFA	12	47.8^{a}	44.6^{a}	46.9^{a}	47.7^{a}	44.3^{a}	47.1^{a}	38.0^{b}	46.8^{a}	46.9^{a}	40.7^{b}	44.3^{ab}	39.5^{b}	39.5^{b}	39.5^{b}	40.4^{ab}	47.6^{a}	2.63	0.001
	24	62.3^{a}	56.3^{a}	57.7^{a}	56.8^{a}	53.8^{b}	56.6^{a}	52.3^{b}	57.1^{a}	40.5^{c}	47.0^{d}	54.7^{b}	48.0^{d}	46.8^{d}	48.5^{d}	47.3^{d}	57.5^{a}	3.28	0.001
	48	58.1^{ac}	50.3^{b}	49.6^{b}	49.8^{b}	61.5^{a}	51.4^{b}	52.4^{ab}	52.1^{ab}	53.4^{bc}	56.9^{c}	50.5^{b}	51.4^{b}	54.2^{bc}	52.8^{ab}	55.2^{c}	53.6^{ab}	3.17	0.017

∑SFA = sum of saturated fatty acids, MUFA= monounsaturated fatty acids, PUFA= polyunsaturated fatty acids, TFA= total fatty acids; FA= fatty acids; Means within row with different superscripts letters are different (P<0.05); CON, control; CAR, 3-carene; ALA, 4-Allylanisole; ANE, *trans*-anethole; BIS, (-)-α-bisabolol; BOR, (-)-borneole; CPY, (-)-*trans*-caryophyllene; CIN, *trans*-cinnamaldehyde; CIT, (S)-(-)-β-citronellol*;* EUC, eucalyptol*;* LIM, (R)-(+)-limonene*;* MEN, menthol*;* MYT, myrtenol*;* CYM, *P*-cymene*;* VAN, (-)-α-thujone*;* vanillin

Table 3.10 Effects of EOCs on fatty acid ratios *n*-6/*n*-3 and P:S in cultures at 12, 24 and 48 h *in vitro* incubation

		EOCs																	
FA ratio	**Time (h)**	**CON**	**3-CAR**	**ALA**	**ANE**	**BIS**	**BOR**	**CPY**	**CIN**	**CIT**	**EUC**	**LIM**	**MEN**	**MYT**	**CYM**	**THU**	**VAN**	**sed**	**P-values**
***n*-6/*n*-3**	12	0.401	0.400	0.402	0.401	0.403	0.402	0.401	0.400	0.403	0.400	0.403	0.401	0.401	0.403	0.402	0.401	0.020	NS
	24	0.401^{a}	0.400^{a}	0.400^{a}	0.401^{a}	0.301^{b}	0.302^{b}	0.301^{b}	0.400^{a}	0.401^{a}	0.300^{b}	0.401^{a}	0.401^{a}	0.301^{b}	0.400^{a}	0.302^{b}	0.301^{b}	0.010	0.001
	48	0.402^{a}	0.401^{a}	0.402^{a}	0.402^{a}	0.300^{b}	0.301^{b}	0.400^{a}	0.402^{a}	0.401^{a}	0.301^{b}	0.401^{a}	0.302^{b}	0.301^{b}	0.403^{a}	0.301^{b}	0.302^{b}	0.020	0.001
P:S	12	0.302^{a}	0.301^{a}	0.402^{b}	0.400^{b}	0.401^{b}	0.402^{b}	0.303^{a}	0.501^{c}	0.503^{c}	0.300^{a}	0.403^{b}	0.502^{c}	0.501^{c}	0.401^{b}	0.400^{b}	0.302^{a}	0.030	0.001
	24	0.201^{a}	0.300^{b}	0.301^{b}	0.301^{b}	0.302^{b}	0.301^{b}	0.302^{b}	0.303^{b}	0.303^{b}	0.202^{a}	0.300^{b}	0.302^{b}	0.301^{b}	0.300^{b}	0.302^{b}	0.203^{a}	0.020	0.001
	48	0.101^{a}	0.202^{b}	0.302^{c}	0.300^{c}	0.201^{a}	0.300^{c}	0.201^{b}	0.203^{b}	0.200^{b}	0.202^{b}	0.203^{b}	0.301^{c}	0.302^{c}	0.201^{b}	0.300^{c}	0.200^{b}	0.020	0.001

Means within row with different superscripts letters are different (P<0.05); CON, control; CAR, 3-carene; ALA, 4-Allylanisole; ANE, *trans*-anethole; BIS, (-)-α-bisabolol; BOR, (-)-borneole; CPY, (-)-*trans*-caryophyllene; CIN, *trans*-cinnamaldehyde; CIT, (S)-(-)-β-citronellol*;* EUC, eucalyptol*;* LIM, (R)-(+)-limonene*;* MEN, menthol*;* MYT, myrtenol*;* CYM, *P*-cymene*;* VAN, (-)-α-thujone*;* vanillin; *n*-6/*n*-3= sum of *n*-6 divided by sum of *n*-3 fatty acids, P/S= sum of PUFA divided by sum of SFA; FA= fatty acids**.**

Table 3.11 Effects of EOCs on the biohydrogenation (g/100 g) of PUFA in cultures at 12, 24 and 48 h *in vitro* incubation

		EOCs																	
FA	**Time (h)**	**CON**	**3-CAR**	**ALA**	**ANE**	**BIS**	**BOR**	**CPY**	**CIN**	**CIT**	**EUC**	**LIM**	**MEN**	**MYT**	**CYM**	**THU**	**VAN**	**sed**	**P-values**
C18:2 *n*-6	12	73.4^{a}	68.4^{a}	54.2^{be}	61.8^{c}	68.9^{a}	63.9^{c}	70.1^{a}	47.8^{e}	50.1^{e}	71.8^{a}	66.4^{a}	60.1^{c}	56.1^{bc}	60.6^{c}	60.0^{c}	73.8^{a}	3.67	0.001
	24	85.5^{a}	78.6^{b}	69.7^{c}	73.2^{d}	81.3^{e}	77.6^{b}	85.3^{a}	77.4^{b}	70.6^{c}	82.9^{a}	78.9^{b}	75.3^{df}	76.9^{f}	72.9^{d}	75.3^{df}	85.2^{a}	1.59	0.001
	48	90.0^{a}	83.8^{bd}	74.4^{c}	79.4^{d}	85.4^{b}	82.2^{d}	88.3^{a}	85.7^{b}	85.1^{b}	87.7^{a}	84.2^{b}	81.2^{d}	84.4^{b}	77.8^{d}	81.4^{d}	89.6^{a}	1.31	0.001
C18:3 *n*-3	12	80.4^{a}	75.0^{b}	60.9^{ce}	67.8^{d}	74.2^{b}	69.2^{d}	77.8^{b}	56.3^{e}	54.7^{e}	78.0^{b}	73.6^{b}	67.0^{d}	64.2^{d}	67.2^{d}	66.8^{d}	79.6^{a}	2.49	0.001
	24	89.7^{a}	82.5^{b}	73.6^{c}	77.8^{bd}	84.3^{b}	81.7^{b}	88.8^{a}	82.9^{b}	76.9^{d}	87.7^{a}	82.7^{b}	78.7^{de}	82.1^{be}	76.4^{d}	79.5^{e}	88.9^{a}	1.36	0.001
	48	92.8^{a}	87.6^{be}	79.0^{c}	82.9^{d}	88.5^{b}	86.1^{e}	91.7^{af}	90.0^{f}	87.3^{be}	91.8^{af}	87.9^{be}	85.2^{e}	88.3^{b}	81.4^{d}	85.4^{e}	92.3^{a}	1.11	0.001
C20:5 *n*-3	12	45.2^{a}	48.5^{a}	27.7^{b}	38.7^{c}	35.4^{c}	24.2^{b}	50.7^{a}	33.3^{bc}	28.9^{b}	40.0^{a}	46.1^{a}	38.8^{c}	26.1^{b}	35.9^{c}	25.0^{b}	42.2^{a}	3.75	0.001
	24	56.5^{a}	57.7^{a}	39.2^{b}	48.9^{c}	44.8^{bc}	33.8^{b}	59.5^{a}	52.6^{ac}	50.0^{c}	47.2^{c}	56.7^{a}	49.4^{c}	39.0^{b}	49.0^{c}	36.4^{b}	52.9^{ac}	3.12	0.001
	48	71.4^{a}	67.5^{ac}	43.9^{b}	54.8^{c}	47.5^{b}	35.9^{d}	68.0^{ac}	66.6^{ac}	65.2^{ac}	51.7^{c}	67.1^{ac}	54.5^{c}	44.0^{b}	56.3^{c}	40.0^{b}	63.2^{c}	3.29	0.001
C22:6 *n*-3	12	29.8^{a}	32.6^{a}	11.3^{b}	26.9^{c}	14.6^{b}	7.4^{b}	36.0^{a}	17.6^{b}	25.6^{c}	22.0^{c}	33.5^{a}	22.5^{c}	8.7^{b}	18.7^{b}	6.1^{b}	25.3^{b}	5.21	0.001
	24	34.4^{ad}	38.1^{a}	15.8^{bc}	32.5^{a}	17.2^{b}	8.5^{c}	36.0^{a}	33.9^{ad}	37.9^{a}	23.6^{bd}	40.4^{a}	29.5^{d}	14.4^{bc}	32.8^{a}	8.3^{c}	28.8^{d}	5.17	0.001
	48	54.1^{a}	56.9^{a}	22.1^{b}	39.2^{c}	20.1^{b}	9.4^{d}	51.1^{a}	54.0^{a}	62.1^{a}	26.3^{b}	58.1^{a}	33.4^{c}	18.8^{b}	44.2^{c}	8.8^{d}	44.5^{c}	4.73	0.001

Means within row with different superscripts letters are different (P<0.05); CON, control; CAR, 3-carene; ALA, 4-Allylanisole; ANE, *trans*-anethole; BIS, (-)-α-bisabolol; BOR, (-)-borneole; CPY, (-)-*trans*-caryophyllene; CIN, *trans*-cinnamaldehyde; CIT, (S)-(-)-β-citronellol*;* EUC, eucalyptol*;* LIM, (R)-(+)-limonene*;* MEN, menthol*;* MYT, myrtenol*;* CYM, *P*-cymene*;* VAN, (-)-α-thujone*;* vanillin; FA= fatty acids

3.5.0. Discussion

3.5.1. *In vitro* fermentation parameters

In the present study, the findings are consistent with a number of previous *in vitro* studies for example, Benchaar et al. (2007) observed that inclusion of carvacrol and thymol (with similar chemical structure to ALA, ANE, CIN) in *in vitro* batch incubation impaired gas production, and Martinez et al. (2006) showed that *in vitro* DM degradability of ration (concentrate to hay ratio of 70: 30 DM basis) was decreased by addition of thymol and carvacrol (1.35 microliter/ml). Thymol and carvacrol (phenolic compounds) are in the same chemical group as ALA and ANE which are among the most inhibitory in this study. In addition, the supplementation of different doses *(*150, 300, 450 and microgram/ml) of oil of Zataria *multiflora*, which is rich in thymol and carvacrol and Siberian fir needle oil (125, 250 and 500 mg/L) proportionally reduced total gas production (Talebzadeh *et al*., 2012; Gunal *et al*., 2013). Macheboeuf *et al*. (2008) reported that more than 80% decrease in gas production was observed when EO of *Origanum vulgare* (with carvacrol and thymol as main constituents) was added to *in vitro* culture. The results also corroborate previous *in vivo* studies where 500 mg/day of cinnamaldehyde (Busquet *et al*., 2003; Calsamiglia *et al*., 2007), and a mixture of eugenol (90 mg/day) plus 180 mg/day of cinnamaldehyde (Cardozo *et al*., 2006) negatively affected VFA. Results of this experiment suggest that the EOCs used might have modified microbial species composition in the cultures resulting in decreased fermentation of substrates, consequently producing less gas relative to the control, however to different degrees. However, there are no microbial data to substantiate this claim. The differences in these EOCs to influence the amount of gas production could be due to the variation in the chemical structure of the isoprene unit which is known to play a vital role in influencing the antimicrobial activity of terpenes (Griffin *et al*., 1999). In terms of their effectiveness to modify microbial population, the phenolic compound (CIN) had the greatest inhibition of total gas production at 12 h, although its potency was not sustained to 24 and 48 h, suggesting a possibility of either the gradual degradation of the active molecules of the compound or microbial adaptation to the compound (Chizzola *et al*., 2004; Brodiscou *et al*., 2007). Other phenolics such as ALA, ANE and monoterpene alcohol (MEN) sustained inhibition throughout incubation period whilst the aldehyde (VAN) was shown to have the least potential to modify

fermentation. These findings are consistent with previous reports which ranked the antimicrobial potency of EOCs in the following descending order: phenols > cinnamic aldehyde > alcohols > aldehydes (Kalemba *et al*., 2012). The tendency for the phenolic compounds to elicit greatest inhibition of gas production could be attributed to the possession of hydroxyl group whose acidic characters are speculated to inhibit both the attachment of bacteria to insoluble fractions of feed and the digestion of the soluble components of feeds (McAllister *et al*., 1994; Aharoni *et al*., 1998). The results observed with VAN is also consistent with previous observation by Castillejos *et al*. (2006) who reported that vanillin (5, 50, 500 and 5000) did not affect rumen fermentation. It could be suggested in this study that at the dose administered, normal activity of some ruminal micro-organisms responsible for feed digestion were inhibited by majority of the EOC tested. In general, total gas was significantly reduced due to the inclusion of EOCs, which suggests impaired microbial activity.

The concentration of NH_3-N in cultures was considerably reduced by ANE and LIM after 24 h. After 48 h ANE and CIN substantially decreased NH_3-N concentration in cultures whilst other treatments maintained NH_3-N concentration similar to the control. Results of this study agree with a number of previous studies such as Cardozo *et al*. (2005), where eugenol (300 mg/l), whose chemical structure is similar to ANE and CIN reduced NH_3-N concentration in *in vitro* batch culture; where limonene (500 mg/L), guaiacol (5, 50, 500, 5000 mg/L) and eugenol (5, 50 and 500 mg/L) substantially reduced linearly the concentration of NH_3-N in culture (Castillejos *et al*., 2006; Benchaar *et al*., 2008), and Castillejos *et al*.(2006) and Benchaar *et al*. (2008), where the concentration of NH_3-N in culture was not affected by inclusion of vanillic aldehyde (5, 50 and 500 mg/L). It has been established that the hydroxyl groups in the chemical structures of phenolic compounds are vital constituent responsible for their high and broadest antimicrobial activity (Burt, 2004). In the current study, the reduced concentration of ammonia-N in cultures supplemented with ANE and CIN could be due to the inhibition of hyper ammonia producing bacteria (HAP) or proteolytic bacteria which are responsible for amino acid deamination (Chapter 1). Previous reports indicate that hyper ammonia producing bacteria, proteolytic bacteria and protozoa are the major groups of microbes involved in NH_3-N production (Mclnotch *et al*., 2003; Bach *et al*., 2005). Therefore, reduced activities of these predominant organisms could be speculated as the

consequence of the lower concentration of ammonia N in some cultures. Indeed, some EOCs have been reported to reduce the activities of HAP (Mclnotch *et al*., 2003).

The pH of ruminal cultures was increased by all EOCs at 24 and 48 h except VAN. Similar result was obtained by Chaves *et al*. (2008) who reported higher ruminal pH following inclusion of anethole, juniper berry, garlic, and cinnamon oils in culture.

The average concentration of TVFA was highest in the control and VAN, and significantly low in the remaining treatments, with the lowest concentration of TVFA observed in cultures receiving MEN, ALA, ANE and 3-CAR. These results are consistent with earlier studies where the addition of thyme oil (125, 250 and 500 mg/l) and citronella oil (125, 250 and 500 mg/l) rich in citronella, citronellol and limonene decreased TVFA (Gunal *et al*., 2013; Pandu *et al*., 2014). In the current study, VAN and CPY did not alter TVFA and did not change the pH of the fluid, hence, it could be suggested that the high TVFA concentration in cultures supplemented with VAN reduced the pH of rumen fluid. However, it can be speculated that the activities of cellulolytic bacteria may have been impaired by EOCs not pH given that ruminal pH was not reduced since cellulolytic bacteria are sensitive to low (< 6.0) rumen pH (Weimer, 1993). Therefore, reductions in TVFA could be attributed to decreased digestibility of feed which is influenced by the chemical configuration of the EOCs used. In the current study, the same set of EOCs such as ALA and ANE (phenolics) and monoterpene alcohol (MEN) were observed to cause the highest reductions of both the concentrations of TVFA and cumulative gas production. Reduced digestion of feed which is reflected by decreased production of gas can be accompanied by less production of hydrogen, CH_4 and volatile fatty acids plus a lower A/P ratio (Boggs *et al*., 1987). The significant decrease in TVFA due to supplementation of EOCs in cultures shows that at the dose used in this experiment, EOC had non-selective and general antimicrobial activity against a broad spectrum of rumen micro-organisms. As ruminants derive over 70% of their required energy from VFA (Bergman, 1990), a reduction of VFA of the magnitude observed (over 10% average reduction) with all EOCs except CPY and VAN, would suggest a significant reduction of rumen fermentation. A reduction in VFA would suggest that if similar effect is exerted *in vivo*, growth of animal would also be affected since VFA are the major sources of energy. Addition of EOC may decrease (at high doses) or have no effect (at low doses) on VFA

production (Patra and Saxena, 2010). Effects of EOC on VFA are determined by the dose, type of EO used (Bustquet *et al*., 2006), and the pH of the rumen (Cardozo *et al*., 2005).

In this study the concentration of acetate was generally increased and the levels of propionate were concomitantly reduced plus high A/P ratios with all EOCs relative to the control except CIN and MEN (which reduced acetate at 24 h), and VAN and CPY (no effect). This suggests that the inclusion of CIN and MEN in cultures could potentially modify rumen fermentation towards decreased methanogenesis (Demeyer and Van Nevel, 1995). In the rumen, anaerobic fermentation of proteins and soluble carbohydrates tends to shift fermentation towards higher propionate production and results in reduced acetate and methanogenesis (Demeyer and Van Nevel, 1995), since the predominant producers of acetate and methane are cellulolytic microbes. In agreement with the current study, Spanghero *et al.* (2008) observed that a blend of EO shifted the end products of fermentation toward a reduction in the A/P ratio and acetate proportion only at lower pH.

The results of the current study on rumen fermentation profiles showed that EOCs could be selected to manipulate rumen fermentation if the dose is optimized. In general, the degree of using EOCs to manipulate rumen fermentation activities (such as ammonia, VFA and gas production) seems to be in the following decreasing sequence: phenolic compounds such as ALA, ANE, and CIN > the monoterpene alcohols example, MEN > aldehydes such as VAN, had the least potential.

3.5.2. Effect of EOC on fatty acid metabolism

The concentrations of C14:0 were reduced in cultures where BIS and CYM were added. The EOCs ALA, ANE, BIS and MEN reduced the levels of C16:0 in cultures relative to the control. A reduction in the content of these saturated fatty acids (C14:0 and C16:0) is beneficial as they are known to have the capacity of raising plasma cholesterol through suppression and saturation of low-density lipoprotein (LDL) receptors in the blood (Keys *et al*., 1995). This suggests that a reduction in the concentration of C14:0 and C16:0 in the current study with the inclusion of those EOCs could possibly decrease the plasma levels of low-density lipoprotein (LDL) if this observation is also repeated *in vivo*.

Stearic acid (the end-product of the biohydrogenation of C18:1 *n*-9, C18:3 *n*-3 and C18:2 *n*-6) was reduced in cultures receiving CIT at 12 h whilst the remaining EOCs maintained C18:0 at levels similar to the control. The mean concentration of C18:0 tended to be increased with the addition of CYM, LIM and CIT to cultures at 24 h, and reduced in the cultures with added BOR, BIS, CIT, EUC, MEN, MYT and THU after 48 h compared to the control. The remaining treatments maintained similar levels of C18:0 to the control at both 24 and 48 h. The reduced concentration of C18:0 in cultures receiving CIT, BOR, BIS, EUC, MEN, MYT and THU is not consistent with the observed high accumulation of VA in this study. This observation suggests that these EOCs could have impaired the activity of *Clostridium proteoclasticum* (Kemp *et al*., 1975; Maia *et al*., 2007), renamed (Moon *et al*., 2008) as *Butyrivibrio proteoclasticus,* the bacteria responsible for converting VA to C18:0. It is uncertain why the high accumulation of C18:3 *n*-3 and C18:2 *n*-6 in vessels receiving ALA, ANE, CIN, LIM and CYM in this study was not accompanied with a reduction of C18:0, since C18:0 is the end product of biohydrogenation. However, the high levels of C18:0 in cultures with those EOCs despite high content of PUFA in their vessels are in agreement with previous studies with EOs (Vasta *et al*., 2013). The accumulation of PUFA (C18:3 *n*-3 and C18:2 *n*-6) without major effects on the final end product of biohydrogenation could suggest three possibilities: firstly, that other unidentified biohydrogenation intermediates were produced in these cultures (Gunal *et al*., 2013); secondly, that the observed accumulation of PUFA could be due to reduced lipolytic activities. Buccioni *et al*. (2012) suggested that if small amounts of PUFA in the diet reach the duodenum, it might be arising from a reduction in lipolysis. This suggests that these EOCs might inhibit the activities of *Butyrivibrio fibisolvens* and *Anaerovibrio lipolytica* or their lipases, which are responsible for hydrolysing the ester bonds of dietary glycolipids and triglycerides (Buccioni *et al*. 2012), potentially reducing lipolysis and isomerization of fatty acids. Thirdly, the accumulation of C18:0 in the current study despite maintenance of high levels of PUFA could be due to the concomitant higher concentrations of C18:1 *n*-9 in cultures with added ALA, ANE, CIN, LIM and CYM, as C18:0 may also result from C18:1 *n*-9. Jenkins *et al*. (2006) reported that about 70% of C18:1 *n*-9 in rumen culture was converted to C18:0 and only 30% was transformed to ketostearic acid and hydroxystearic acid. Stearic acid (C18:0) is a saturated fatty acid but without any harmful effects on human health (Grundy, 1994; Pariza, 2004; Webb and O'Neill, 2008), therefore,

stearic acid should not be included with other saturated fatty acids such as C14:0 and C16:0, in attempt to control the levels of plasma cholesterol through dietary manipulations (Cobb, 1992).

The formation of C18:1 *trans*-11 (vaccenic acid; VA) from the rumenic acid (*cis*-9 *trans* 11 CLA) is aided by the activity of the rumenic acid enzyme, the reductase enzyme (Jenkins *et al.*, 2008; Kim *et al*., 2009; Buccioni *et al*., 2012). In this study, the concentration of C18:1 *trans* 11 was increased with the addition of all EOCs at 24 and 48 except for VAN, with the greatest increase recorded in cultures with MEN and BOR compared to the control. Increased concentration of C18:1 *trans* 11 in this study is in agreement with previous reports where EOCs were observed to maintain high levels of C18:1 *trans* 11 (Lourenco *et al*., 2009). In this study, EOCs such as MEN and BOR were among the additive type with the lowest concentration of C18:0, implying that the concomitant high concentrations of C18:1 *trans* 11 could have resulted from reduced conversion of C18:1 *trans* 11 to C18:0. This might indicate that these EOCs potentially have the capacity to cause decrease activity of *Clostridium proteoclasticum* (Kemp *et al*., 1975; Maia *et al*., 2007), renamed (Moon *et al*., 2008) as *Butyrivibrio proteoclasticus,* the bacteria that is responsible for converting VA to C18:0. It could also mean that inclusion of these EOCs might have stimulated the activity of the reductase enzyme which facilitates the production of *trans*-11 18:1 (vaccenic acid; VA) from *cis*-9 *trans* 11 18:2 CLA. These results imply that the inclusion of these EOCs could also potentially increase the concentration of *cis*-9 *trans* 11 18:2 CLA since vaccenic acid is the key substrate for endogenous synthesis of CLA in animal tissues (Griinari *et al*., 2000). It is established that about 90% of *cis-9 trans-11* CLA in cow's milk and tissues resulted from the desaturation of *trans*-11 18:1 through the Δ-9 desaturase enzyme (Piperova *et al*., 2002; Kay *et al*., 2004).

In the present study, no effect of supplementing EOCs was observed on the levels of *cis*-9 *trans* 11 18:2 CLA at all times of incubation except for 48 h where MEN, BOR and BIS significantly increased *cis*-9 *trans* 11 18:2 CLA. The increased effects of MEN, BOR and BIS on the levels of cis-9 *trans* 11 18:2 CLA, relative to the control, is consistent with previous studies (Whitney *et al*., 2011). As mentioned previously, *cis*-9 *trans* 11 18:2 CLA is formed in the first step during the transformation of *cis*-9, *cis*-12 18:2 by the linoleic acid isomerase (LA-I) (Jenkins *et al*., 2008; Kim *et al*., 2009; Buccioni *et al*., 2012). Results of the current study suggest that all but MEN, BOR and BIS at 48 h could have impaired the

activities of the linoleic acid isomerase which facilitates the formation of *cis*-9 *trans* 11 18:2 CLA from *cis*-9, *cis*-12 18:2.

The content of C18:2 *n*-6 rapidly decreased with time, being highest at 12 h and lowest at 48 h, in both the CON and in the presence of all EOCs. The biohydrogenation of C18:2 *n*-6 was comparable to, but slightly lower than that of C18:3 *n*-3, and in line with results of previous *in vitro* (Beam *et al*., 2000) and *in vivo* (Wachira *et al*., 2000) studies. The effects of the added EOCs on the biohydrogenation of C18:2 *n*-6 was similar to their effects on the biohydrogenation of C18:3 *n*-3 (discussed later), with ALA, ANE, CIT and MEN being the most effective EOCs to reduce the extent of biohydrogenation of C18:2 *n*-6.

In the current study, the content of C18:3 *n*-3 rapidly decreased with time, being highest at 12 h and lowest at 48 h, in both the CON and in all EOCs, evidently reflecting the biohydrogenation of C18:3 *n*-3 in cultures over time. This rapid biohydrogenation of C18:3 *n*-3 from ground linseed which exceeded 80 g/100 g at 24 h agrees with previous *in vivo* studies (Wachira *et al*., 2000; Scollan *et al*., 2001, Wang *et al*., 2002; Chikunya *et al*., 2004) and *in vitro* studies (Sinclair *et al*., 2005). Relative to the control, within the EOCs, the concentration of C18:3 *n*-3 was highest in ALA, ANE, MEN and CIT and consistently lowest in VAN and CPY during the first 24 h. At 48 h, the highest levels of C18:3 *n*-3 were still found in cultures with ALA and ANE. In this experiment, the EOCs which possess phenolic moieties in their chemical structures such as ALA and ANE known to express the broadest antimicrobial effects, caused high accumulation of C18:2 *n*-6 and C18:3 *n*-3. This is consistent with previous report by Vasta *et al*. (2013) who added Artemisia EO (containing phenolic compounds such as thymol and carvacrol), and Lourenco *et al*. (2008) who used cinnamaldehyde (500 mg/L). The antibacterial activity and specific mode of action of an individual EOC is influenced by the chemical structure of the compound (Dormans and Deans, 2000). Although, CIN (another phenolic compound) had the highest content of C18:3 *n*-3 and C18:2 *n*-6 at 12 h, its potency was not sustained to 24 and 48 h, suggesting the possibility of gradual degradation of the active molecules of the compound or microbial adaptation to the compound (Chizzola *et al*., 2004; Brodiscou *et al*., 2007). The possibility of ALA and ANE to exert the greatest antimicrobial characteristic by maintaining higher levels of C18:3 *n*-3 than other EOCs could be due to the possession of hydroxyl group whose acidic characters are speculated to have broad inhibition of microbial activities.

This could suggest that ALA and ANE probably had higher capacity to reduce the biohydrogenation of C18:3 *n*-3 and C18:2 *n*-6 compared to the rest of the treatments leading to high accumulation of PUFA in their cultures. EOs containing high proportion of phenolic compounds such as thymol, carvacrol and eugenol (2-methoxy-4-(2-propenyl)phenol as components of their chemical structure exhibit the strongest antimicrobial properties against food borne pathogens (Cosentino *et al*., 1999; Juliano *et al*., 2000; Lambert *et al*., 2001). The mechanisms of action of phenolic compounds are thought to generally include disruption of proton motive force (PMF), coagulation of cell contents, disturbance of cytoplasmic membrane and disruption of active transport and electron flow (Sikkema *et al*., 1995; Davidson, 1997).

In the literature, formaldehyde treatment of fat sources has been described as effective in increasing the flow of C18:3 *n*-3 to the abomasum of goats (Scott *et al*., 1971), and in reducing the *in vitro* biohydrogenation of C18:2 *n*-6 (Gulati *et al*., 1997). However, beside the fact that the use of formaldehyde would potentially be criticized by regulatory authorities, the inconsistency of using it to reduce the biohydrogenation of either C18:3 *n*-3 or C18:2 *n*-6 was reported by Sinclair *et al*. (2005). These authors reported that formaldehyde treatment of whole linseed alone slightly reduced the biohydrogenation of either C18:3 *n*-3 or C18:2 *n*-6. Hence, they proposed that pre-treatment of whole linseed with NaOH and formic acid could be more effective because about 443 and 307 g/kg of C18:3*n* - 3 were protected from biohydrogenation when whole linseed was pre-treated with NaOH and formic acid respectively, prior to treatment with formaldehyde. In the current study the content of C18:3 *n*-3 or C18:2 *n*-6 at 24 h decreased significantly in response to EOCs in the progression: ALA and ANE (phenylpropanoids), MEN (monoterpene alcohol) and CIT (monoterpene) > 3-CAR and BOR (bicyclic monoterpenes), CIN (phenylpropanoid), LIM, MYT, CYM and THU (monoterpenes) > BIS (sesquiterpene) and EUC (monoterpene ethers) > CPY (bicyclic sesquiterpene) and VAN (aldehydes). These findings are consistent with previous reports ranking the antimicrobial potency of EOCs in the following descending order: phenols > cinnamic aldehyde > alcohols > aldehydes (Kalemba *et al*., 2012).

The extent of disappearance of C20:5 *n*-3 and C22:6 *n*-3 in vessels increased as the time of incubation progressed, being lowest at 12 h and highest at 48 h. After 48 h, the quantity of the content of C20:5 *n*-3 and C22:6 *n*-3 in vessel which disappeared was highest in the control, with only 28.6 g/100 g and 45.9

g/100 g of C20:5*n* -3 and C22:6*n* -3 respectively, remaining in vessels. These findings support previous reports from *in vivo* studies where the biohydrogenation of C20:5 *n*-3 and C22:6 *n*-3 in fish oil range from 72 to 93 g/100 g TFA (Wachira *et al*., 2000; Scollan *et al*., 2001; Chikunya *et al*., 2004), and contrast *in vitro* reports where the biohydrogenation of C20:5 *n*-3 and C22:6 *n*-3 was less than 50 g/100 g (Ashes *et al*., 1992; Sinclair *et al*., 2005). Within the EOCs, the extent of inhibition of the biohydrogenation of C20:5 *n*-3 and C22:6 *n*-3 differs and can be ranked in the following decreasing order: ALA, MYT, CIT, CIN, BOR and THU > ANE, BIS, MEN and CYM > 3-CAR, CPY, LIM, VAN. In general, the biohydrogenation of the fish oil fatty acids (C20:5 *n*-3 and C22:6 *n*-3) was less compared to the ruminal disappearance of C18 fatty acids (C18:3 *n*-3 and C18:2 *n*-6). This inability of microbes to hydrogenate the fish oil fatty acids is not due to the difference in the lipase activities but because microbes lack the enzymes necessary to hydrogenate the long chain *n*-3 PUFA (Ashes *et al*., 1992). It should be remembered that the biohydrogenation pathways of C20:5 *n*-3 and C22:6 *n*-3 are not yet fully established (section 1.7.8).

No effect of treatment on ∑SFA was found after 24 h. Therefore a further calculations of ∑SFA without 18:0 (i.e. ∑SFA-18:0) was made but results were similar to the reported effects on ∑SFA. The lack of difference on the content of ∑SFA and (∑SFA-C18:0) could be due to the reported high levels of C18:0 which possibly results from the metabolism of oleic acid. However, there was reduction on the ∑SFA in cultures with added BOR, CIT, MEN, MYT and THU compared to the control after 48 h. These reductions in the content of ∑SFA in these EOCs coincided with the high levels of *trans*-11 18:1 and decreased concentration of 18:0 and oleic acid in those cultures.

The content of ∑PUFA decreased with increase in the time of incubation (i.e. highest at 12 h and lowest at 48 h). After 48 h, the levels of ∑PUFA observed in cultures with ALA, ANE and MEN, which were the most effective EOCs, was more than doubled the concentration observed in cultures with the control. The decrease in the levels of ∑PUFA with time is understood from the fact that the concentration of individual PUFA also decreased with time. The highest content of ∑PUFA in cultures treated with ALA, ANE and MEN is also expected as the highest levels of individual PUFA were also observed in those

EOCs. The aim of increasing the content of ∑PUFA in diet is to reduce the risks of coronary heart diseases in humans (as described in Chapter 1).

In the report presented here no effect of inclusion of VAN and CIN was recorded on TFA at 12, 24 and 48 h. However, all other EOCs reduced TFA relative to the control. Reduction of TFA could suggest that the reported decrease in TVFA and ammonia concentration had reduced the microbial *de novo* fatty acids synthesis (Sauvant and Bas, 2001), leading to reductions in the levels of TFA. The strength of this conclusion lies in the fact that VAN which did not affect the TFA concentration also did not change the TVFA and ammonia concentrations in cultures in this study.

The content of ∑*n*-6/∑*n*-3 was consistently reduced in cultures with BIS, BOR, EUC, MYT, THU and VAN at both 24 and 48 h relative to the control. In this study, the major thrombotic fatty acid (C20:4 *n*-6; Kinsella *et al.*, 1990) was not affected by treatments.

The ratio of PUFA to SFA (P: S) in cultures decreased with time of incubation, and it was increased with the addition of all EOCs except EUC and VAN relative to the control at 12 and 24 h. At 48 h of incubation, the ratio of P/S in cultures with ALA, ANE, BOR, MEN, MYT and THU (EOCs with the highest levels of P/S) tripled the content of P/S in the control. In this study, only the inclusion of ALA, ANE, BOR, MEN, MYT and THU in cultures at 12 h had P/S of 0.40 which is close to the value of 0.45 recommended in the guidelines of the Department of Health (1994).

3.6. Conclusion

In the current study, the content of C18:3 *n*-3 or C18:2 *n*-6 at 24 h increased significantly in the progression: ALA and ANE (phenylpropanoids), MEN (monoterpene alcohol) and CIT (monoterpene) > 3-CAR and BOR (bicyclic monoterpenes), CIN (phenylpropanoid), LIM, MYT, CYM and THU (monoterpenes) > BIS (sesquiterpene) and EUC (monoterpene ethers) > CPY (bicyclic sesquiterpene) and VAN (aldehydes). These results showed that the phenylpropanoids (ALA and ANE) and monoterpene alcohols (MEN) had the greatest potential compared to the rest of the EOCs to reduce the disappearance

of *n*-3 PUFA and suggest that their use could enhance the concentrations of *n*-3 PUFA in ruminant food products if this is confirmed *in vivo*. However, this needs to be balanced against their effects on VFA. It is also worthwhile to investigate the mechanism of action and the stability of these EOCs in the rumen.

In the event of introducing these substances to the public or to livestock industries, the whole essential oils might stand a better chance of acceptance compared with the individual EOCs. The whole EOs have been used by man since antiquity in the area of aromatherapy and food industries, hence, are likely to encounter less regulatory hassles compared to individual EOCs. In addition, the synergistic effect between the minor and major components in the whole oils is expected to improve the effectiveness of the whole oils against biohydrogenating bacteria over the individual constituent compounds. These considerations would lead us to the second experiment using the parent whole oils of the ten most effective EOCs. Therefore, the next experiment (discussed in Chapter 4) is to evaluate the potential of the whole oils on rumen fermentation and biohydrogenation of PUFA.

Chapter 4

Effects of ten whole essential oils on rumen fermentation and biohydrogenation of *n*-3 polyunsaturated fatty acids by rumen microorganisms *in vitro*

Abstract

The effects of ten whole essential oils (EO) on the fermentation activities of rumen microbes and the biohydrogenation (BH) of *n*-3 polyunsaturated fatty acids (PUFA) were evaluated *in vitro* using batch culture system. Rumen fluid was collected from four Hartline × Texel cross cull ewes offered grass hay (*Lolium perenne*) and water *ad libitum* and supplemented with additional 400 g/sheep/day of lamb finisher cubes. A basal feedstock comprising of 70:30 grass hay and concentrate was formulated. Serum bottles were incubated at 39^0C; each bottle contained 1 g of feed substrate, 80 ml buffer, 20 ml inoculum, then supplemented with 300 mg/l of EOCs and repeated twice. There were 11 treatments, with six replicates per treatment as follows: control (CON), whole oils of anise (ANO), cassia (CSO), citronella (CTO), clove (CLO), cornmint (CMO), eucalyptus (ETO), juniper berry (JPO), lavender (LVO), mandarin (MDO) and rosemary (RMO). Fermentation was stopped at 12, 24 and 48 h, and samples were collected to analyse ammonia N, total VFA and molar proportions of individual VFA; and concentration of PUFA including intermediates of BH. Relative to the control, CSO was the only EO that reduced ($P<0.001$) ammonia concentration in culture by a magnitude of 33%, whilst others maintained similar ammonia levels. With the exception of ETO and LVO, the addition of all EO decreased ($P<0.001$) total VFA compared to the control, with CSO being the most inhibitory, inducing a 27% reduction. The concentrations of C18:2 *n*-6 and *n*-3 PUFA (C18:3 *n*-3, C20: 5*n*-3 and C22: 6*n*-3) were maintained at higher levels ($P<0.001$) with addition of all EO, and were highest ($P<0.001$) in cultures where ANO and CSO were added. Except for CTO and CLO, the inclusion of EO did not affect the concentration of 18:2 *cis*-9 *trans* 11. The levels of C18:1 *trans* 11 were significantly increased ($P<0.001$) by supplementing with either CSO, CTO, CMO or RMO. There was no effect of EOs inclusion on C18:0 except for CSO (12 h) and RMO (24 h) which reduced ($P<0.001$) the level of C18:0. The potential of whole EOs used in this study to inhibit BH at 24 h can be ranked as follows: ANO and CSO > MDO and CMO > CTO and RMO > JPO, LVO, CLVO and ETO. These results suggest that the use of CSO and ANO have the greatest potential to enhance the concentrations of *n*-3 PUFA in ruminant food products if this is also confirmed *in vivo*. However, optimum doses are needed to balance their effects on VFA.

4.1. Introduction

The health benefits of *n*-3 PUFA such as C18:3 *n*-3, 20:5 *n*-3 and 22:6 *n*-3 have been described (see section 1.2.2). Briefly, this category of fatty acids has been reported to have positive effects such as reducing the risk of coronary heart disease in humans (Department of Health, 1994; de Lorgeril *et al*., 1999). These health benefits of *n*-3 PUFA have raised the need to increase the levels of these fatty acids in ruminant food products such as meat and milk. Because the fatty acid composition of ruminant food products depends on the metabolism of dietary lipids in the rumen (Kim *et al*., 2009); the manipulation of rumen biohydrogenation of PUFA has received significant attention within the scientific community (Scollan *et al*., 2001, Wachira *et al*., 2000; Wang *et al*., 2002; Chikunya *et al*., 2004; Scollan *et al*., 2006; Shingfield and Griinari, 2007; Gunal *et al*., 2013).

The possibility of using whole EOs and their constituent compounds (EOCs) in ruminant fermentation have also been examined previously (Calsamiglia *et al*., 2007; Benchaar *et al*., 2008; Hart *et al*., 2008; Chapter 1). The huge number (3000) of commercially available EOs makes it practically impossible to evaluate the effects of EOs all by *in vivo* trials. Therefore, the use of *in vitro* experimental models to investigate effects of EOs and consequently predict their *in vivo* effects has become a common phenomenon for researchers. In our previous *in vitro* study (Chapter 3; Eburu and Chikunya, 2014), the effects of 15 EOCs on the metabolism of PUFA was evaluated. The study found that some individual EOCs such as anethole, menthol, 4-allylanisole and *p*-cymene have the potential to reduce the extent of ruminal biohydrogenation of PUFA. In another *in vitro* study, Sgwane *et al*. (2013) reported that the EOCs pinene, linalyl acetate, L-menthone and pulegone reduced the extent of ruminal biohydrogenation of PUFA. However, the use of whole essential oils as opposed to individual EOCs has a number of attractions. They have been used by man since antiquity in the area of aromatherapy and food industries, hence, are likely to encounter less regulatory hassles compared to the EOCs. In addition, the potential synergistic action between the minor and major constituents in the whole oils might improve the effectiveness of the whole oils against biohydrogenating bacteria over the individual constituent compounds. The aim of this study was therefore to establish whether the parent oils from which some of the individual EOCs showing potential are the predominant compounds, are equally as effective at

inhibiting the biohydrogenation of PUFA and increasing the accumulation of biohydrogenation intermediates.

4.2. Materials and methods

4.2.1. Animal management and collection of rumen fluid

In this experiment, four cull-ewes (Hartline × Texel cross, mean weight 41.5 ±1.7kg) were used as rumen fluid donors. Details of animal housing, experimental diets, feeding regime, duration of adaptation and collection of rumen fluid are as described in section 2.1.

4.2.2. Basal feedstock, treatments and *in vitro* incubation

The basal feedstock was mixtures of good quality rye-grass hay (*Lolium perenne*), lamb finisher concentrate, whole ground linseed and fish oil. Details of the basal feedstock used in this *in vitro* incubation are as described in the general material and methods (see section 2.3). The ingredients, chemical and fatty acid composition of the diet are shown in Table 2.2.

The effects of 10 whole essential oils were evaluated using the *in vitro* gas production batch culture method described by Theodorou *et al*. (1994), which is outlined in section 2.4 of the general materials and methods.

All EOs used were purchased from Sigma-Aldrich Co. Ltd., UK and were stored at the required temperatures as specified on delivery notes prior to use. The description of the EOs as purchased from Sigma-Aldrich is shown in Table 4.1. The typical constituent component composition of the whole essential oils used in this study is shown in Table 4.2.

There were 11 treatments and six replicates as follows: Control (CON), anise oil (ANO), cassia oil (CSO), citronella oil (CTO), clove oil (CLO), cornmint oil (CMO), eucalyptus oil (ETO), juniper berry oil (JPO), lavender oil (LVO), mandarin oil (MDO) and rosemary oil (RMO).

Incubation was done in 125 ml clear glass type 1 serum bottles (R & L Slaughter Ltd, Essex, UK) for 48 h in each run and repeated twice. In each run 268 (11 x 6 +4) serum bottles were incubated, each bottle

contained 1 g of feed substrate, 300 mg/l of EO, 80 ml anaerobic buffer (see Table 2.1) and 20 ml inoculum and the bottle sealed with rubber cork before incubation.

Table 4.1 Description of the essential oils used in this experiment as purchased from Sigma-Aldrich

Name of EO	Abbreviation	Catalogue number	Type	Density
Anise oil	ANO	10521	Anise seed oil, natural identical	0.980 g/ml
Cassia oil	CSO	W225800	Cassia oil	1.058 g/ml
Citronella oil	CTO	W230812	Java natural, 85/35%	0.888 g/ml
Clove oil	CLO	W232300	Clove bud oil, natural (US), FCC, FG	1.05 g/ml
Cornmint oil	CMO	W521604	Natural Chinese type	-
Eucalyptus oil	ETO	W246611	80-85%, FG	0.909 g/ml
Juniper berry oil	JPO	W260401	FCC type	0.863 g/ml
Lavender oil	LVO	61718	Oil from *Lavandula angustifolia*	0.879 g/ml
Mandarin oil	MDO	W265713	Italian FC type	0.846 g/ml
Rosemary oil	RMO	W299200	FCC type	0.908 g/ml

Supplier: Sigma-Aldrich Co. Ltd., UK; FCC = food chemicals codex; FG = food grade

Table 4.2 Typical constituent component composition of the whole essential oils used in this study

Common name	Botanical name	Composition (%) of major constituent compounds	References
Anise oil	*P. anisum*	*Trans*-anethole (82.7%), carryophyllene (3.8%), limonene (2.3%)	Soher *et al*. (2014)
Cassia oil	*C. cassia*	*Trans*-cinnamaldehyde (70-90%), *trans*-2-methoxycinnamaldehyde (3-5%), cinnamyl acetate (1-6%), eugenol (0.5%)	EP 5; Kalemba *et al*. (2012)
Citronella oil	*Cymbopogon winterianus*	Citronella (31.5%), geraniol (19.2%), citronellol (5.4%), limonene (2.2%)	Pandu *et al*. (2014)
Clove oil	*Eugenia caryophyllus*	Eugenol (75-85%), eugenal acetate (8-15%)	Bauer *et al*. (2001)
Cornmint oil	-	Menthol (30-55%), menthone (14-32%), methyl acetate (2.8-10%), menthofuran (1-9%)	EP 5; Kalemba *et al*. (2012)
Eucalyptus oil	*Eucalyptus citriodora* Hook	1, 8-cineole (68%), α –pinene (3.7%), *p*-cymene (1.7%)	Elaissi *et al*. (2011)
Juniper berry oil	*Juniperus oxycedrus spp*	α –pinene (39.8%), manoyl oxide (10.2%)	Sofia *et al*. (2002)
Lavender oil	*Lavandula angustifolia*	Linalool (42.74%), linalyl acetate (23.25%), camphor (8.03%), borneol (7.1%)	Danh *et al*. (2012)
Mandarin oil	*Citrus reticulate* Blanco	Limonene (75%), linalool (5.1%), γ- terpinene (2.1%)	Yu *et al*. (2007); EP 5
Rosemary oil	*Rosmarinus officinalis*	1, 8-cineole (3-89%), α-pinene (2-25%), camphor (2-14%), Bornyl acetate (0-17%)	Pintore *et al*. (2002)

4.2.3. Sample collection and preservation

Experimental details relating to gas pressure measurement, stopping times, collection and storage of samples are as described in the general material and methods (see section 2.4).

4.2.4. Chemical analysis

The concentration of NH_3-N in digesta was analysed using the phenol-hypochlorite method (Weatherburn, 1967; Broderick and Kang, 1980) adapted for use on the plate reader as described in general materials and methods (see section 2.5.4).

The concentration of volatile fatty acid (VFA) was determined by Gas chromatography (GC) as described by Ottenstein and Bartley (1971). Details of this method are outlined in the general materials and methods (see section 2.5.6).

The concentration of fatty acids in feed and freeze dried samples were extracted by direct saponification method described by Enser et al. (1998). See section 2.5.7 of the general materials and methods for detailed description of the techniques.

4.2.5. Experimental design and statistical analysis

This study was a completely randomized design (CRD) experiment. The objective was to examine the effects of ten EOs on the extent of rumen biohydrogenation of *n*-3 PUFA *in vitro*, including their effects on fermentation parameters (gas production, NH_3-N concentration and on concentrations of TVFA and molar proportions of individual VFA. The null hypothesis was that inclusion of 300 mg/L of EOs would have no effect on fermentation and biohydrogenation data. The alternative hypothesis was that the EOs would affect (decrease or increase) fermentation activities. Data were analysed (separately for each time point) by one-way analysis of variance (ANOVA) using GenStat 16th edition (VSN international Ltd, Registered to: Writtle College) with experimental runs as a blocking factor. Differences between treatments were declared by least significance difference (LSD) and significance was declared at $P< 0.05$.

4.4.0. RESULTS

4.4.1. *In vitro* fermentation parameters

The effects of EO on cumulative gas production (ml/g OM) and concentration of NH_3-N (mM) are shown in Table 4.3. At 12 h of incubation, the levels of gas production in the control and five of the added EOs (ANO, CTO, ETO, JPO and LVO) were not significantly different (P=0.004), but the rest of the treatments reduced gas production compared to the control. Relative to the control, about half of the added EOs (CLO, CMO, MDO and RMO) caused inhibition of gas production (mean 17%) at 24 h, whilst the rest of the treatments (ANO, CTO, ETO, JPO and LVO) did not affect gas production at that time. However, at 48 h, gas production was reduced (P<0.001) with the inclusion of majority of the additives, with CLO being the most inhibitory (149.8 ml/g OM compared to 192.8 ml/g OM for the control), whilst the addition of CSO, CTO, JPO or LVO maintained similar gas levels to the control.

During the first 12 h of incubation, NH_3-N concentration was reduced (P<0.001) with the addition of all but three EOs (LVO, MDO and RMO), with ANO, CSO and CLO causing the greatest reduction (mean 33%). With the exception of CSO which maintained significant (P<0.001) inhibition (33%) of ammonia production after 24 h, all other EOs did not affect the concentration of NH_3-N in culture, but LVO increased NH_3-N (48%). There was no effect of treatments on the ruminal concentration of ammonia N after 48 h.

The concentration of total VFA and proportion of individual VFA are presented in Table 4.4. At 24 h, the concentration of total VFA was reduced (P<0.001) by the addition of all EOs except the inclusion of ETO and LVO, with CSO and ANO being the most inhibiting EOs, inducing 27% and 15% inhibition, respectively. At 48 h effects of the addition of EOs on total VFA was similar to the reported effects at 24 h, with ANO causing the highest reduction. Of the ten EOs screened in this study, CSO was the only additive that reduced the molar proportion of acetate relative to the control, the rest of the extracts increased acetate with CTO showing the highest increase (mean 3.5%). The molar proportion of propionate was not affected by 50% of the EOs (ANO, ETO, JPO, MDO and RMO) at 24 h, but was reduced by other additive type, with only CSO increasing (P<0.001) the molar proportion of propionate

(mean increase 2.5%). The proportion of butyrate was reduced in cultures supplemented with JPO and RMO (mean 8%) and increased with the addition of CSO and CMO (mean 21%), but other EOs did not affect the molar proportion of butyric acid in cultures at all times of incubation. The ratio of acetate to propionate (A: P) was not affected by JPO and RMO at 24 h but it was reduced by CSO, with the remaining EOs increasing A: P, with LVO causing the highest (mean 11%) increase. At 48 h period of incubation, the A/P ratio was highest in cultures with MDO and ANO (means 2.9) and lowest in CSO (2.6).

Table 4.3 Effects of EOs on cumulative gas production (ml/g OM) and ammonia concentration NH_3-N (mM) in cultures during 48 h *in vitro* incubation

		EOs												
Parameters	**Time (h)**	**CON**	**ANO**	**CSO**	**CTO**	**CLO**	**CMO**	**ETO**	**JPO**	**LVO**	**MDO**	**RMO**	**S.E.D**	**P-Values**
Gas production														
	12	96.2^{a}	95.1^{a}	77.0^{b}	95.5^{a}	80.1^{b}	81.6^{b}	97.0^{a}	102.3^{a}	97.6^{a}	80.6^{b}	84.4^{b}	7.37	=0.004
	24	150.3^{a}	143.6^{a}	141.1^{a}	146.9^{a}	123.9^{b}	124.1^{b}	145.5^{a}	149.5^{a}	143.6^{a}	119.7^{b}	130.7^{b}	8.93	=0.003
	48	192.8^{a}	172.2^{b}	191.5^{a}	194.6^{a}	149.8^{c}	151.7^{c}	178.8^{b}	182.7^{a}	176.3^{ab}	146.6^{c}	162.1^{bc}	9.93	<0.001
NH_3-N														
	12	4.8^{a}	3.3^{b}	3.2^{b}	3.5^{b}	3.2^{b}	3.8^{b}	3.8^{b}	3.5^{b}	5.6^{a}	5.6^{a}	4.9^{a}	0.44	<0.001
	24	4.5^{a}	3.4^{ab}	3.0^{b}	3.6^{ab}	4.2^{a}	4.2^{a}	4.6^{a}	4.4^{a}	6.6^{c}	4.8^{a}	3.9^{ab}	0.56	<0.001
	48	8.2	7.2	6.7	7.3	7.5	8.0	7.6	7.5	7.1	7.1	7.5	0.45	NS

Means within row with different superscripts letters are different (P<0.05); Control (CON), anise oil (ANO), cassia oil (CSO), citronella oil (CTO), clove oil (CLO), control (CON), cornmint oil (CMO), eucalyptus oil (ETO), juniper berry oil (JPO), lavender oil (LVO), mandarin oil (MDO) and rosemary oil (RMO); NS = not significant; EOs= essential oils

Table 4.4 Effects of EOs on concentration of total (mM) and proportions of individual volatile fatty acids (mM/mol TVFA) in cultures during 48 h *in vitro* incubation

		EOs												
Parameters	**Time (h)**	**CON**	**ANO**	**CSO**	**CTO**	**CLO**	**CMO**	**ETO**	**JPO**	**LVO**	**MDO**	**RMO**	**S.E.D**	**P-Values**
TVFA														
	24	70.0^{a}	60.6^{b}	51.0^{c}	61.1^{b}	65.6^{de}	64.1^{d}	68.3^{ae}	64.9^{d}	68.2^{ae}	63.5^{d}	63.2^{d}	1.37	<0.001
	48	83.5^{a}	71.0^{b}	75.7^{ce}	77.7^{c}	76.1^{c}	77.8^{c}	81.1^{d}	76.1^{c}	77.9^{e}	73.7^{e}	79.2^{d}	1.09	<0.001
Acetate														
	24	62.2^{a}	63.3^{bf}	60.1^{c}	64.1^{d}	62.9^{b}	61.3^{e}	62.7^{a}	63.0^{b}	63.7^{f}	63.2^{b}	63.1^{b}	0.33	<0.001
	48	61.6^{a}	64.0^{be}	63.3^{c}	64.0^{be}	62.4^{d}	61.6^{a}	61.8^{a}	63.3^{c}	63.8^{b}	64.3^{e}	63.3^{c}	0.17	<0.001
propionate														
	24	24.7^{a}	24.3^{ad}	25.7^{b}	23.6^{c}	24.0^{d}	23.9^{d}	24.6^{a}	24.9^{a}	23.2^{c}	24.3^{ad}	25.1^{a}	0.25	<0.001
	48	24.2^{a}	21.9^{b}	24.4^{a}	22.8^{c}	23.4^{d}	22.6^{c}	24.2^{a}	23.0^{d}	22.4^{e}	22.5^{c}	23.2^{d}	0.23	<0.001
Butyrate														
	24	10.1^{a}	9.7^{a}	12.5^{b}	9.5^{a}	10.1^{a}	11.9^{b}	9.8^{a}	9.3^{c}	10.1^{a}	9.7^{a}	9.3^{c}	0.28	<0.001
	48	10.2^{ae}	10.3^{a}	9.0^{b}	9.7^{cf}	10.3^{a}	11.8^{d}	10.2^{a}	10.0^{e}	10.1^{e}	9.6^{c}	9.8^{f}	0.08	<0.001
Acetae:propionate														
	24	2.5^{a}	2.6^{b}	2.3^{c}	2.7^{d}	2.6^{b}	2.6^{b}	2.6^{b}	2.5^{a}	2.8^{e}	2.6^{b}	2.5^{a}	0.04	<0.001
	48	2.5^{a}	2.9^{b}	2.6^{c}	2.8^{d}	2.7^{e}	2.7^{e}	2.6^{c}	2.8^{d}	2.8^{d}	2.9^{b}	2.7^{e}	0.03	<0.001

Means within row with different superscripts letters are different (P<0.05); Total volatile fatty acid (TVFA), control (CON), anise oil (ANO), cassia oil (CSO), citronella oil (CTO), clove oil (CLO), control (CON), cornmint oil (CMO), eucalyptus oil (ETO), juniper berry oil (JPO), lavender oil (LVO), mandarin oil (MDO) and rosemary oil (RMO); EOs= essential oils

4.4.2. Effect of EOs on fatty acid metabolism

The effects of EOs on concentration of selected C14 and C16 fatty acids in cultures are summarized in Table 4.5. There was no effect of treatments on the concentration of C14 at 12 and 24 h period of incubation. However, half of the added EOs (ANO, CSO, CLO, JPO and MDO) reduced ($P<0.001$) the concentration of C14 after 48 h.

Although the levels of C16:0 were not significantly different between the control and the added oils at 12 h period, it was reduced ($P<0.001$) with the addition of CSO, CMO, ETO and RMO (mean 14.0 g/100 g TFA) compared to the control (15.0 g/100 g TFA) at 24 h. However, at 48 h, the content of C16:0 was decreased with the inclusion of all EOs in cultures (mean 6.0 g/100 TFA) except the cultures supplemented with CSO which did not affect the levels of C16:0 relative to the control (7.0 g/100 g TFA).

Relative to the control (2.7 g/100 TFA), the concentration of C16:1 at 12 h was increased by all treatments except CLO, JPO and MDO, with CSO showing the highest increase (3.3 g/100 TFA). At 24 h the content of C16:1 in cultures was increased with the inclusion of all EOs except ANO, JPO and LVO which maintained the levels of C16:1 similar to the control. Except the addition of CLO, CTO and JPO which did not affect the concentration of C16:1 compared to the control (2.7 g/100 g TFA), the levels of C16:1were increased with all additives, with ANO the highest (3.0 g/100 TFA).

Indicated in Table 4.6 are concentrations of selected C18 fatty acids in cultures during incubation. Except for CSO (12 h) and RMO (24 h) which reduced ($P<0.001$) the content of 18:0 compared to the control, there was no effect of EOs inclusion on the production of stearic acid. At 48 h, the vessels with added MDO and CTO had the highest content of C18:0 (Mean values 12.6 g/100 g TFA) compared to the control (6.9 g/100 g TFA).

The concentration of C18:1 *n*-9 at 12 h was significantly highest in CSO (8.4 g/100 g TFA) followed by ANO and CTO (means 7.9 g/100 g TFA) compared to the control (7.4 g/100 g TFA). At 24 h period of incubation, the fermentation vessel content of C18:1 *n*-9 was highest ($P<0.001$) in ANO, CSO, CTO and MDO (means 8.4 g/100 g TFA) and lowest ($P<0.001$) in RMO, ETO and CLO (means 7.3 g/100 g TFA)

relative to the control (6.0 g/100 g TFA). After 48 h, the level of C18:1 *n*-9 in cultures was not affected with the inclusion of five of the extracts (CSO, CLO, ETO, JPO and LVO) but it was significantly increased with the rest of the treatments.

The levels of C18:1 *trans* 11 were increased (P<0.001) with all EOs at 12 h relative to the control (1.1 g/100 g TFA), with the highest content found in cultures supplemented with CSO (1.6 g/100 g TFA). At 24 h period of incubation, the content of C18:1 *trans* 11 was highest in CSO relative to the control. Majority of the added EOs increased (P<0.001) the concentration of C18:1 *trans* 11 in cultures after 48 h, with JPO, CTO and LVO being the most effective EOs (means 3.1 g/100 g TFA), compared to the control (1.96 g/100 g).

Except for CSO, CTO and ANO at 12, 24 h and 48 h respectively, which increased (P<0.001) the content of *cis*-9 *trans* 11 CLA, there was no effect of treatment on the content of *cis*-9 *trans*-11 18:2 CLA during incubation.

The content of C18:2 *n*-6 decreased with time, being highest at 12 h and lowest at 48 h, in both the control and the EOs cultures. At 24 h, relative to the control, the inclusion of all EOs maintained higher (P<0.001) the content of C18:2 *n*-6 in cultures though at varying levels, being highest in CSO (6.0 g/100 g TFA) and ANO (5.4 g/100 g TFA), and lowest in CLO, ETO and LVO (means 3.3 g/100 g TFA), compared to the control (2.2 g/100 TFA). At 48 h, ANO had the highest (P<0.001) concentration of C18:2 *n*-6 (2.8 g/100 g TFA) and the lowest levels were observed in CSO, CTO and ETO (means 1.5 g/100 g TFA). The level of C18:2 *n*-6 in cultures with other additives was intermediate between the highest and the lowest treatments.

The vessel content of C18:3 *n*-3 also decreased with time, being highest at 12 h and lowest at 48 h, in both the CON and in all EOs cultures. Relative to the control, within the EOs, the concentration of C18:3 *n*-3 was consistently highest (P<0.001) in ANO and CSO (mean values were more than 2-fold), and consistently lowest (P<0.001) in ETO and JPO at 12 and 24 h. At 48 h, the content of C18:3 *n*-3 was maintained at the highest levels (P<0.001) with the addition of ANO (4.2 g/100 g TFA), a level which is

more than twice compared to the least effective EO (CSO; 1.8 g/100 g TFA), and the CON (1.6 g/100 g TFA).

The concentrations of selected C20 fatty acids are presented in Table 4.7. At 12 and 24 h, the level of C20:4 *n*-6 was decreased with the addition of all EOs except CLO, ETO, JPO and LVO (12 h) and CLO, ETO and JPO (24 h), relative to the control. At 48 h, it was observed that ANO and MDO had the lowest concentration of C20:4 *n*-6 (means 0.4 g/100 g TFA) compared to the control (0.7 g/100 g TFA).

In both the control and EOs, the concentration of C20:5 *n*-3 decreased with increase in the time of incubation, being highest at 12 h (means 3.6 g/100 g TFA) and lowest at 48 h (means 1.0 g/100 g TFA). The concentrations of C20:5 *n*-3 at 12 and 24 h of incubation were highest in cultures supplemented with ANO, CSO and CMO (means 3.1 and 2.5 g/100 g TFA for 12 and 24 h respectively) and lowest in ETO and JPO (means 2.2 and 2.0 g/100 g TFA for 12 and 24 h respectively) compared to the CON (1.7 and 1.4 g/100 g TFA for 12 and 24 h respectively). At 48 h, the most effective EO was ANO, which had about a four-fold increase (1.9 g/100 g TFA) in the content of C20:5 *n*-3 compared to the control (0.5 g/100 g TFA), whilst the vessel effluent content of C20:5 *n*-3 in the least effective extract (CSO) was 0.6 g/100 g TFA. Increasing the time of incubation also decreased the content of C22:6 *n*-3 in cultures, being highest in ANO and CMO (means 1.7 g/100 g TFA) and lowest in CSO (0.8 g/100 g TFA) after 48 h, relative to the control (0.9 g/100 g TFA). Other added extracts maintained intermediate effect between ANO and CSO on the concentration of C22:6 *n*-3 in cultures.

The concentrations of sums of fatty acids (g/100 g TFA) and content of total fatty acid (mg/g) are presented in Table 4.8. The levels of remaining fatty acids (RFA) were significantly reduced in cultures with added EOs at all times of incubation except ETO and RMO (24 h), and CSO, CLO and ETO (48 h).

The levels of ∑SFA in cultures at 12 h were increased by all EOs except CSO which reduced ($P<0.001$), and RMO, which had a similar level of ∑SFA to the control. At 24 h the content of ∑SFA was highest in cultures with CTO, MDO and LVO, but ∑SFA was not different between the control and majority of the extracts after 48 h period of incubation. However, the levels of SFA at 48 h were significantly reduced ($P<0.001$) with the addition of all EOs when SFA was calculated without C18:0 (i.e. ∑SFA-C18:0).

At both 12 and 24 h, the content of ∑MUFA in cultures with CLO and LVO was similar to the control (18.6 and 21.8 g/100 g TFA), but reduced with the addition of other EOs, being lowest in ANO (16.8 and 18.8 g/100 TFA, for 12 and 24 h respectively). At the last hour of incubation (48 h), majority of the treatments (CTO, CLO, CMO, ETO, LVO and RMO) did not affect the levels of ∑MUFA in cultures but it was reduced by the rest of the added EOs.

The content of ∑PUFA) decreased with increase in the time of incubation (i.e. highest at 12 h and lowest at 48 h), and was highest ($P<0.001$) in ANO (11.1 g/100 g TFA) and lowest in CSO and CTO (mean values 5.4 g/100 g TFA) at 48 h, relative to the control (4.8 g/100 TFA).

At 24 h, the amount of TFA (mg/g) was significantly reduced ($P<0.001$) by majority of the EOs relative to the control, except ANO and RMO which did not affect this concentration (Table 4.8). However, at 48 h, the inclusion of ANO, LVO and MDO (means 38.1 mg/g) reduced ($P<0.001$) the content of TFA compared to the control (43.5 mg/g), with other treatments showing no effect.

The ∑*n*-6/∑*n*-3 was lowest in cultures with ANO (24 and 48 h), CSO, CTO, CMO, ETO, MDO and RMO (24), and consistently highest in the control at all times of incubation (Table 4.9).

The ratio of PUFA to SFA ratio (P: S) in cultures decreased with time, and was consistently highest in vessels with ANO and CSO at both 12 and 24 h (means 0.7 and 0.75, for 12 and 24 h, respectively) relative to the control (0.5 and 0.3 for 12 h and 24 h respectively, Table 4.9). At 48 h of incubation, the ratio of P/S in cultures with ANO was double (0.4) the content of P/S in the control (0.2).

Table 4.10 shows the biohydrogenation (g/100 g) of C18:2 *n*-6 and *n*-3 PUFA. The extent of biohydrogenation of linoleic acid in the fermentation vessels increased as the time of incubation progressed, being lowest at 12 h and highest at 48 h. At 24 h, the biohydrogenation of C18:2 *n* -6 was lowest ($P<0.001$) in ANO and CSO (mean values 40.8 g/100 g), and highest in ETO (69.4 g/100 g), relative to the control (76.1 g/100 g), with the rest of the treatments showing effects which are intermediate between the control and the most effective treatments (ANO and CSO). At 48 h of incubation, the biohydrogenation of C18:2 *n*-6 was highest in the control, CSO and ETO (means 86.7

g/100 g), followed by CTO, CMO, JPO and RMO (means 79.8 g/100 g), and lowest in ANO (68.6 g/100 g).

The pattern of effects of EOs on biohydrogenation of C18:3 *n*-3 was similar to their reported effects on the disappearance of vessel content of C18:2 *n*-6, being lowest in ANO (69.5 g/100 g), followed by CLO and MDO (means 78.0 g/100 g), and highest in the control, CSO and ETO (means values 89.3 g/100 g) at 48 h time of incubation.

The extent of disappearance of C20:5 *n*-3 in vessels increased as the time of incubation advanced, being lowest at 12 h and highest at 48 h. After 48 h, the quantity of the content of C20:5 *n*-3 in vessel which disappeared was highest in the control and CSO (means 85.2 g/100 g), and lowest in ANO (42.0 g/100 g), with the remaining treatments having intermediate values. The trend of biohydrogenation in the vessel content of C22:6 *n*-3 was similar to the reported trend in the biohydrogenation of C20:5 *n*-3, with ANO showing the highest ($P<0.001$) potential to reduce the disappearance of C22:6 *n*-3 after 24 and 48 h, whilst control and CSO, had the lowest potential at 48 h.

Table 4.5 Effects of whole EOs on C14:0, C16:0 and C16:1 fatty acids concentration (g/100 g total fatty acids) in *in vitro* cultures at 12, 24 and 48 h incubation

Fatty acids	Time (h)	EOs											S.E.D	P-Values
		CON	ANO	CSO	CTO	CLO	CMO	ETO	JPO	LVO	MDO	RMO		
C14:0	12	5.00	5.01	5.02	5.01	5.02	5.02	5.01	5.00	5.00	5.01	5.02	0.102	NS
	24	4.99	5.00	5.01	5.01	4.98	5.00	5.02	5.00	5.01	4.96	5.01	0.102	NS
	48	5.01^{a}	4.02^{b}	4.00^{b}	5.01^{a}	4.02^{b}	5.00^{a}	5.01^{a}	4.02^{b}	5.01^{a}	4.01^{b}	5.00^{a}	0.100	<0.001
C16:0	12	15.00	15.03	15.02	15.01	15.00	15.02	15.02	15.00	15.03	15.03	15.02	0.300	NS
	24	15.02^{a}	15.02^{a}	14.00^{b}	16.01^{c}	15.01^{a}	14.03^{b}	14.03^{b}	15.04^{a}	15.02^{a}	15.01^{a}	14.02^{b}	0.400	<0.001
	48	7.00^{a}	6.03^{b}	7.02^{a}	6.00^{b}	6.02^{b}	6.05^{b}	6.04^{b}	6.01^{b}	6.02^{b}	6.03^{b}	6.01^{b}	0.300	=0.002
C16:1	12	2.70^{a}	3.00^{b}	3.30^{c}	3.00^{b}	2.80^{a}	3.10^{bc}	3.10^{bc}	2.80^{a}	3.01^{b}	2.80^{a}	3.10^{bc}	0.110	<0.001
	24	2.90^{ae}	3.00^{ab}	3.20^{bd}	3.10^{bd}	3.10^{bd}	3.30^{d}	3.30^{d}	3.00^{ab}	3.01^{ab}	2.70^{e}	3.20^{bd}	0.110	<0.001
	48	2.10^{a}	3.01^{b}	1.70^{c}	2.30^{a}	2.20^{a}	2.50^{de}	2.40^{d}	2.20^{a}	2.70^{e}	1.80^{c}	2.50^{de}	0.120	<0.001

Means within row with different superscripts letters are different (P<0.05); control (CON), anise oil (ANO), cassia oil (CSO), citronella oil (CTO), clove oil (CLO), control (CON), cornmint oil (CMO), eucalyptus oil (ETO), juniper berry oil (JPO), lavender oil (LVO), mandarin oil (MDO) and rosemary oil (RMO), NS = not significant.

Table 4.6 Effects of whole EOs on C18:0, C18:1 *n*-9, C18:1 trans 11, C18:2 c9 t11 CLA, C18:2 *n*-6 and C18:3 *n*-3 fatty acids composition (g/100 g total fatty acids) in *in vitro* cultures at 12, 24 and 48 h incubation

		EOs												
Fatty acids	**Time (h)**	**CON**	**ANO**	**CSO**	**CTO**	**CLO**	**CMO**	**ETO**	**JPO**	**LVO**	**MDO**	**RMO**	**S.E.D**	**P-Values**
C18:0	12	10.3^{a}	12.0^{b}	8.4^{c}	11.5^{b}	10.6^{a}	10.7^{a}	10.7^{a}	11.2^{b}	10.5^{a}	12.4^{b}	9.9^{ac}	0.57	<0.001
	24	7.0^{a}	8.5^{b}	7.0^{a}	9.9^{bc}	7.0^{a}	6.3^{a}	5.7^{a}	7.4^{a}	10.0^{c}	10.7^{c}	5.4^{d}	0.71	<0.001
	48	6.9^{a}	10.8^{b}	12.7^{c}	11.3^{bc}	8.9^{d}	9.2^{b}	8.5^{d}	9.2^{b}	9.0^{b}	13.8^{c}	8.6^{ad}	0.89	<0.001
C18:1 *n*-9	12	7.4^{a}	7.8^{b}	8.4^{c}	8.0^{b}	7.7^{ab}	7.7^{ab}	7.6^{ab}	7.5^{ab}	7.7^{ab}	7.6^{ab}	7.6^{ab}	0.16	<0.001
	24	6.0^{a}	8.2^{b}	8.2^{b}	8.6^{b}	7.4^{c}	7.5^{c}	7.2^{c}	8.3^{b}	7.6^{c}	8.4^{b}	7.3^{c}	0.22	<0.001
	48	1.9^{a}	3.2^{b}	2.6^{ab}	3.0b	2.8^{ab}	3.3^{b}	2.4^{ab}	2.6^{ab}	2.6^{ab}	3.9^{b}	3.5^{b}	0.56	=0.062
C18:1 trans 11	12	1.1^{a}	1.2^{b}	1.6^{c}	1.3^{d}	1.3^{d}	1.3^{d}	1.3^{d}	1.2^{b}	1.4^{e}	1.2^{b}	1.3^{d}	0.05	<0.001
	24	1.26^{a}	1.31^{ad}	1.70^{b}	1.36^{d}	1.32^{ad}	1.37^{d}	1.33^{ad}	1.3^{ad}	1.28^{a}	1.26^{a}	1.4^{e}	0.05	<0.001
	48	1.96^{a}	2.58^{bc}	2.67^{bc}	3.04^{b}	2.37^{ac}	2.36^{ac}	2.48^{ab}	3.08^{b}	3.03^{b}	2.82^{bc}	2.46^{ab}	0.29	=0.005
C18:2 c9 t11 CLA	12	0.16^{a}	0.14^{a}	0.21^{b}	0.14^{a}	0.16^{a}	0.13^{a}	0.15^{a}	0.17^{a}	0.17^{a}	0.16^{a}	0.15^{a}	0.017	=0.005
	24	0.1^{a}	0.11^{a}	0.12^{a}	0.16^{b}	0.13^{c}	0.11^{a}	0.12^{a}	0.10^{a}	0.11^{a}	0.12^{a}	0.12^{a}	0.011	<0.001
	48	0.10^{a}	0.14^{b}	0.10^{a}	0.08^{a}	0.11^{a}	0.08^{a}	0.09^{a}	0.09^{a}	0.08^{a}	0.11^{a}	0.06^{c}	0.012	<0.001
C18:2 *n*-6	12	3.8^{a}	5.5^{b}	6.3^{c}	5.6^{b}	4.8^{d}	5.0^{d}	4.3^{e}	4.3^{e}	4.8^{d}	5.1^{b}	4.6^{de}	0.24	<0.001
	24	2.2^{a}	5.4^{b}	6.0^{c}	4.1^{d}	3.4^{eg}	4.5^{f}	3.2^{e}	3.7^{g}	3.4^{eg}	4.6^{f}	3.9 ^{h}	0.16	<0.001
	48	1.1^{a}	2.8^{b}	1.4^{c}	1.5^{c}	1.8^{d}	2.2^{ef}	1.5^{c}	1.8^{d}	2.3^{e}	2.2^{ef}	2.0^{df}	0.11	<0.001
C18:3 *n*-3	12	5.4^{a}	8.4^{b}	9.6^{c}	8.0^{b}	7.1^{d}	7.6^{d}	6.2^{e}	6.4^{e}	7.0^{d}	7.5^{d}	6.8^{e}	0.38	<0.001
	24	2.8^{a}	8.1^{b}	9.4^{c}	5.9^{d}	4.9^{e}	6.9^{f}	4.5^{e}	5.3^{g}	4.8^{e}	7.4^{f}	5.8^{d}	0.27	<0.001
	48	1.6^{a}	4.2^{b}	1.8^{ac}	2.0^{c}	2.5^{d}	3.1^{e}	2.0^{c}	2.5^{d}	2.7^{d}	3.3^{e}	2.6^{d}	0.14	<0.001

Means within row with different superscripts letters are different (P<0.05); control (CON), anise oil (ANO), cassia oil (CSO), citronella oil (CTO), clove oil (CLO), control (CON), cornmint oil (CMO), eucalyptus oil (ETO), juniper berry oil (JPO), lavender oil (LVO), mandarin oil (MDO) and rosemary oil (RMO).

Table 4.7 Effects of whole EOs on C20:4 *n*-6, C20:5 *n*-3 and C22:6 *n*-3 long chain fatty acids composition (g/100 g total fatty acids) in *in vitro* cultures at 12, 24 and 48h incubation

		EOs												
Fatty acids	**Time (h)**	**CON**	**ANO**	**CSO**	**CTO**	**CLO**	**CMO**	**ETO**	**JPO**	**LVO**	**MDO**	**RMO**	**S.E.D**	**P-Values**
C20:4 *n*-6	12	0.6^{a}	0.5^{b}	0.5^{b}	0.5^{b}	0.6^{a}	0.4^{c}	0.6^{a}	0.6^{a}	0.6^{a}	0.5^{b}	0.5^{b}	0.04	<0.001
	24	0.8^{a}	0.6^{b}	0.6^{b}	0.7^{c}	0.8^{a}	0.6^{b}	0.7^{c}	0.7^{c}	0.6^{b}	0.6^{b}	0.7^{c}	0.05	<0.001
	48	0.7^{a}	0.4^{b}	0.5^{c}	0.6^{d}	0.7^{a}	0.6^{d}	0.7^{a}	0.7^{a}	0.6^{d}	0.4^{b}	0.6^{d}	0.03	<0.001
C20:5 *n*-3	12	1.7^{a}	3.0^{bc}	3.2^{b}	2.8^{c}	2.5^{d}	3.0^{bc}	2.1^{e}	2.2^{e}	2.3^{e}	2.6^{f}	2.9^{c}	0.13	<0.001
	24	1.4^{a}	2.4^{b}	3.0^{c}	2.2^{bd}	2.0^{bd}	2.1^{bd}	2.0^{bd}	1.9^{ad}	1.9^{e}	2.1^{bd}	2.0^{bd}	0.23	<0.001
	48	0.5^{a}	1.9^{b}	0.6^{ac}	0.7^{c}	1.0^{d}	1.3^{e}	1.0^{d}	0.9^{d}	1.2^{ef}	1.1^{f}	1.1^{f}	0.08	<0.001
C22:6 *n*-3	12	1.8^{a}	2.3^{bc}	2.4^{b}	2.1^{cd}	2.1^{cd}	2.3^{be}	2.3^{be}	2.0^{d}	2.1c^{d}	2.2^{e}	2.3^{be}	0.11	<0.001
	24	1.7^{a}	2.0^{b}	2.4^{c}	1.9^{b}	1.9^{b}	2.0^{b}	1.9^{b}	1.9^{b}	1.9^{b}	1.9^{b}	1.9^{b}	0.08	<0.001
	48	0.9^{a}	1.7^{b}	0.8^{a}	0.9^{a}	1.4^{c}	1.7^{b}	1.6^{bd}	1.5^{d}	1.5^{d}	1.2^{e}	1.6^{bd}	0.10	<0.001

Means within row with different superscripts letters are different (P<0.05); control (CON), anise oil (ANO), cassia oil (CSO), citronella oil (CTO), clove oil (CLO), control (CON), cornmint oil (CMO), eucalyptus oil (ETO), juniper berry oil (JPO), lavender oil (LVO), mandarin oil (MDO) and rosemary oil (RMO).

Table 4.8 Effects of whole EOs on the concentration of sums of fatty acids (g/100 g TFA) and total fatty acids (mg/g) in cultures at 12, 24 and 48 h *in vitro*

		EOs												
Total Fatty Acids	**Time (h)**	**CON**	**ANO**	**CSO**	**CTO**	**CLO**	**CMO**	**ETO**	**JPO**	**LVO**	**MDO**	**RMO**	**S.E.D**	**P-Values**
Remaining FA	12	37.1^{a}	31.1^{b}	31.3^{b}	31.2^{b}	32.7^{bc}	32.7^{bc}	34.1^{c}	34.9^{c}	33.4^{bc}	32.3^{b}	34.7^{c}	1.13	<0.001
	24	41.2^{a}	32.4^{bd}	30.4^{b}	31.1^{b}	36.1^{c}	34.6^{bc}	39.2^{a}	35.1^{d}	34.7^{bc}	31.0^{b}	38.4^{a}	1.45	<0.001
	48	47.5^{a}	40.3^{b}	46.5^{a}	43.0^{c}	45.6^{a}	42.7^{c}	46.0^{ad}	45.1^{d}	41.4^{b}	40.0^{b}	43.1^{c}	1.13	<0.001
∑SFA	12	29.7^{a}	32.0^{bd}	27.5^{c}	31.4^{b}	30.5^{b}	30.5^{b}	30.6^{b}	30.8^{b}	29.9^{a}	32.6^{d}	29.7^{a}	0.79	<0.001
	24	26.8ac	28.0a	26.0ac	30.4b	26.9ac	25.7cd	24.8cd	27.4ac	29.9b	30.8b	23.8d	1.10	<0.001
	48	27.4a	30.2b	31.6b	30.9b	27.6a	28.3a	27.4a	27.9a	28.7a	33.6c	28.3a	1.00	<0.001
∑SFA-C18:0	12	19.5	19.9	19.1	19.9	19.9	19.8	19.9	19.6	19.5	20.2	19.8	0.34	NS
	24	19.8^{a}	19.4^{ac}	19^{a}	20.5^{b}	19.9^{a}	19.4^{ac}	19.1^{ac}	20^{b}	19.9^{a}	20.1^{b}	18.4^{c}	0.53	=0.017
	48	20.5^{a}	19.4^{bc}	19^{bc}	19.7^{b}	18.7^{c}	19.2^{bc}	18.9^{c}	18.7^{c}	19.8^{b}	19.8^{b}	19.7^{b}	0.43	=0.002
∑MUFA	12	18.6^{a}	16.8^{b}	17.6^{c}	17.7^{c}	18.4^{a}	18.3^{a}	18.9^{a}	17.9^{c}	18.5^{a}	16.8^{b}	18.3^{a}	0.32	<0.001
	24	21.8^{a}	18.8^{b}	20.2^{c}	21.4^{a}	22.3^{a}	20.9^{c}	21.7^{a}	22.3^{a}	21.5^{a}	19.5^{b}	20.6^{c}	0.60	<0.001
	48	18.2a	15.7b	14.0b	17.1a	16.9ab	17.7a	17.1a	16.5b	18.5a	15.2b	18.3a	0.91	<0.001
∑PUFA	12	13.4^{a}	18.9^{b}	22.0^{c}	18.3^{bd}	17.1^{d}	17.3^{d}	15.2^{e}	15.3^{e}	16.8^{d}	17.2^{d}	16.0^{e}	0.89	<0.001
	24	9.0^{a}	19.5^{b}	21.8^{c}	15.7^{d}	13.4^{e}	17.4^{f}	12.9^{e}	13.9^{e}	12.7^{e}	17.4^{f}	15.7^{d}	0.58	<0.001
	48	4.9^{a}	11.2^{b}	5.2^{ac}	5.8^{c}	7.5^{df}	8.9^{e}	7.0^{d}	7.4^{df}	8.3^{e}	8.4^{e}	7.9^{f}	0.34	<0.001
TFA (mg/g)	12	41.2^{a}	44.0^{a}	35.2^{b}	37.3^{b}	39.7^{c}	40.3^{c}	40.9^{c}	40.3^{c}	39.5^{c}	41.3^{a}	42.4^{a}	1.54	<0.001
	24	37.6^{a}	36^{a}	34.1^{c}	34^{c}	37.8^{a}	38^{a}	38.3^{a}	37.8^{a}	38.3^{a}	33.9^{e}	37.9^{a}	1.19	<0.001
	48	43.5^{a}	40^{b}	43.9^{a}	40.2^{a}	42.1^{a}	42^{a}	42.5^{a}	42^{a}	36.6^{b}	37.7^{b}	43.4^{a}	1.75	<0.001

Means within row with different superscripts letters are different (P<0.05); control (CON), anise oil (ANO), cassia oil (CSO), citronella oil (CTO), clove oil (CLO), control (CON), cornmint oil (CMO), eucalyptus oil (ETO), juniper berry oil (JPO), lavender oil (LVO), mandarin oil (MDO) and rosemary oil (RMO), NS= not significant, SFA= saturated fatty acids, MUFA= monounsaturated fatty acids, PUFA= polyunsaturated fatty acids, TFA= total fatty acids.

Table 4.9 Effects of whole EOs on fatty acid ratios ∑*n*-6/∑*n*-3 and P:S in cultures at 12, 24 and 48 h *in vitro* incubation

Fatty acid ratios	Time (h)	EOs											S.E.D	P-Values
		CON	ANO	CSO	CTO	CLO	CMO	ETO	JPO	LVO	MDO	RMO		
∑*n*-6/∑*n*-3	12	0.50	0.50	0.51	0.51	0.50	0.51	0.50	0.51	0.51	0.50	0.50	0.010	NS
	24	0.50^{a}	0.41^{b}	0.41^{b}	0.40^{b}	0.51^{a}	0.41^{b}	0.40^{b}	0.50^{a}	0.51^{a}	0.40^{b}	0.40^{b}	0.010	<0.001
	48	0.62^{a}	0.41^{b}	0.60^{a}	0.63^{a}	0.51^{c}	0.50^{c}	0.50^{c}	0.51^{c}	0.60^{a}	0.51^{c}	0.50^{c}	0.030	<0.001
P:S	12	0.50^{a}	0.60^{b}	0.80^{c}	0.60^{b}	0.61^{b}	0.60^{b}	0.50^{a}	0.50^{a}	0.61^{b}	0.51^{a}	0.50^{a}	0.05	<0.001
	24	0.30^{a}	0.70^{b}	0.80^{c}	0.50^{d}	0.51^{d}	0.70^{b}	0.50^{d}	0.50^{d}	0.41^{e}	0.60^{f}	0.70^{b}	0.04	<0.001
	48	0.22^{a}	0.41^{b}	0.21^{a}	0.20^{a}	0.30^{c}	0.31^{c}	0.30^{c}	0.32^{c}	0.30^{c}	0.21^{a}	0.31^{c}	0.02	<0.001

Means within row with different superscripts letters are different (P<0.05); control (CON), anise oil (ANO), cassia oil (CSO), citronella oil (CTO), clove oil (CLO), control (CON), cornmint oil (CMO), eucalyptus oil (ETO), juniper berry oil (JPO), lavender oil (LVO), mandarin oil (MDO) and rosemary oil (RMO); *n*-6/*n*-3= sum of *n*-6 divided by sum of *n*-3 fatty acids, P/S= sum of PUFA divided by sum of SFA

Table 4.10 The effects of EOs on the biohydrogenation (g/100 g) of C18:2 *n*-6, C18:3 *n*-6, C20:5 *n*-3 and C22:6 *n*-3 PUFA in *in vitro* cultures at 12, 24 and 48 h incubation

		EOs												
Fatty acids	**Time (h)**	**CON**	**ANO**	**CSO**	**CTO**	**CLO**	**CMO**	**ETO**	**JPO**	**LVO**	**MDO**	**RMO**	**SED**	**P-Values**
C18:2 *n*-6	12	58.1^{a}	38.3^{b}	39.8^{b}	35.8^{b}	28.5^{c}	46.9^{de}	59.1^{a}	57.9^{a}	44.0^{de}	42.2^{d}	48.8^{e}	2.54	<0.001
	24	76.1^{a}	39.6^{b}	42.0^{b}	52.9^{e}	48.4^{d}	52.0^{c}	69.4^{e}	63.8^{f}	60.4^{f}	47.6^{d}	56.2^{c}	2.15	<0.001
	48	87.8^{a}	68.6^{b}	86.9^{a}	82.2^{e}	72.7^{d}	76.8^{e}	85.3^{a}	82.2^{e}	73.3^{d}	74.4^{d}	78.1^{e}	1.55	<0.001
C18:3 *n*-6	12	62.9^{a}	39.8^{b}	45.2^{c}	44.1^{e}	38.9^{b}	53.2^{d}	66.2^{a}	64.6^{a}	52.1^{d}	49.1^{e}	54.2^{d}	2.21	<0.001
	24	80.8^{a}	41.8^{b}	45.7^{bf}	59.0^{e}	57.7^{c}	57.2^{c}	75.9^{d}	70.5^{e}	67.4^{e}	49.8^{f}	61.2^{c}	2.13	<0.001
	48	89.2^{a}	69.5^{b}	89.8^{a}	86^{c}	78.3^{d}	80.9^{e}	89.0^{a}	86.4^{c}	81.6^{e}	77.3^{d}	82.6^{e}	1.13	<0.001
C20:5 *n*-3	12	49.1^{a}	9.4^{b}	17.4^{cd}	16.2^{c}	25.3^{d}	18.5^{cd}	48.9^{a}	46.7^{a}	33.8^{e}	26.2^{d}	19.3^{cd}	4.09	<0.001
	24	60^{a}	28.3^{bc}	24.4^{b}	32.9^{cbd}	38.9^{cd}	41.0^{d}	52.4^{a}	53.0^{a}	43.6^{d}	39.5^{d}	43.0^{d}	5.83	<0.001
	48	85.2^{a}	42.0^{b}	85.2^{a}	80.2^{e}	71.0^{d}	65.0^{e}	74.9^{d}	77.6^{e}	65.5^{e}	68.9^{e}	69.4^{e}	2.29	<0.001
C22:6 *n*-3	12	24.3^{a}	5.3^{b}	17.1^{c}	5.7^{b}	10.3^{bd}	12.0^{d}	20.7^{a}	33.8^{a}	8.4^{b}	11.4^{b}	6.2^{b}	3.12	<0.001
	24	28.1^{ac}	15.1^{b}	18.9^{b}	13.7^{b}	21.0^{a}	21.5^{a}	35.7^{e}	36.6^{e}	21.7^{a}	21.8^{a}	22.5^{a}	4.60	<0.001
	48	63.4^{ac}	28.6^{bd}	71.1^{a}	59.5^{e}	22.6^{b}	33.7^{d}	44.0^{e}	49.8^{ef}	36.7^{d}	52.5^{ef}	37.3^{d}	4.20	<0.001

Means within row with different superscripts letters are different (P<0.05), control (CON), anise oil (ANO), cassia oil (CSO), citronella oil (CTO), clove oil (CLO), control (CON), cornmint oil (CMO), eucalyptus oil (ETO), juniper berry oil (JPO), lavender oil (LVO), mandarin oil (MDO) and rosemary oil (RMO).

4.5. Discussion

The objective of the current study was to examine the effects of whole EOs on the extent of rumen biohydrogenation of *n*-3 PUFA. The EOs used in the current study were selected based on the 24 h results of the first experiment (described in Chapter 3; Eburu and Chikunya, 2014) which screened the effects of 15 individual essential oil's constituent compounds. On one hand, the EOCs were ranked from 1 (highest) to 15 (lowest) based on the concentration (g/100 g TFA) of C18:3 *n*-3, C18:2 *n*-6, C20:5 *n*-3, C22:6 *n*-3, C18:2 *cis*-9 *trans*-11 CLA and C18:1*trans* 11 in their cultures. On another hand, EOCs were ranked from 1 (least inhibition of VFA) to 15 (most inhibitory effect on VFA concentration). After careful consideration of their effects on fatty acid and volatile fatty acid concentrations, the overall best EOCs were then selected and the parent whole oils of the best 10 EOCs were then used in the current study. The 24 h results were used for selecting the most effective EOCs because of the assumption that, at 12 h, there might be inadequate time for interaction between the microbes and the EOCs, and at 48 h, there might be remarkable change in the microbial species composition (Personal communication with Prof John Wallace). Hence, 24 h results provide the most representative outlook of the effects of EOCs/EOs on rumen fermentation.

4.5.1. Effects of EOs on in *vitro* fermentation parameters

Within the EOs, the cumulative gas production was not affected at 24 h by more than half of the EOs (ANO, CSO, CTO, ETO, JPO and LVO), whilst the rest of the EOs reduced gas production relative to the control. However, at 48 h, majority of the extracts reduced gas production compared with the control, with CLO, CMO and MDO being the most inhibiting EOs. These findings on whole EOs are in agreement with our previous preliminary study (Chapter 3), where individual EOCs of these same EOs reduced the amount of total gas produced, with inhibition being highest in phenolic compounds. The differences in these EOs to influence the amount of gas production suggest differences in their potential to decrease microbial activity. This difference could be due to the variation in the chemical composition of individual EO as the antimicrobial activity of terpenes depends on the chemical structure of the isoprene unit (Griffin *et al*. 1999). Essential oils containing high proportion of phenolic compounds such as thymol, carvacrol and eugenol (2-methoxy-4-(2-propenyl)phenol as components of their chemical

structure exhibited the strongest antimicrobial properties against food borne pathogens (Cosentino *et al*., 1999; Juliano *et al*., 2000; Lambert *et al*., 2001). In the current study, CLO (containing a phenolic compound, eugenol as the active compound, Table 4.2), ANO (containing a phenolic compound, anethole) were among the most effective EO to reduce gas production. This further confirms the well-established contention that phenolic compounds have the greatest antimicrobial strength to modify microbial composition resulting in decreased fermentation of substrates, as reduction in fermentation is due to altered microbial species composition (Van Soest, 1994). There is a link between the inhibition of gas production by the EOCs (Chapter 3) and the whole oils in the current study. Overall, CMO and MDO had the most inhibiting effect on total cumulative gas production which is consistent with the previous study where menthol (active component of CMO, Table 4.2), cinnamaldehyde (active component of cassia, Table 4.2) and anethole (active component of ANO, Table 4.2) were among the most effective EOCs to reduce total gas production. However, comparison of the effects of EOCs (in Chapter 3, e.g. anethole) and EOs (current study, e.g. anise oil) on gas production indicate that anise oil is more inhibitory than anethole (as lower gas was produced in culture with anise oil than in anethole). The higher level of gas in cultures supplemented with anethole 300 mg/L (Chapter 3) than anise oil 300 mg/L (current study) suggest that other minor components of anise oil had significant synergistic effects on ruminal fermentation activities. These observations are consistent with previous studies where it is suggested that minor components may synergistically interact with the major components of EOs (Davidson and Naidu, 2000; Burt, 2004).

The concentration of NH_3-N in cultures was significantly reduced by majority (7 out of 10) of the EOs at 12 h. However, at 24 and 48 h, the concentration of NH_3-N in cultures was not affected by inclusion of EOs, except CSO which had significant inhibition (33%) of ammonia production at 24 h. The observed results at 12 h agree with previous studies where oregano (30 and 300 mg/L) and cinnamon oil (0.3 to 300 mg/L) decreased NH_3-N (Cardozo *et al*., 2005), where 0.22 mg/L of cinnamon oil reduced NH_3-N (Cardozo *et al*., 2004), and where a higher dose (3,000 mg/L) of cinnamaldehyde and cinnamon oil inhibited NH_3-N (Busquet *et al*., 2005; Busquet *et al*., 2006). This change in the potential of EOs to reduce NH_3-N production with time of incubation may be due to gradual adaptation of individual

microbial species to EO or shifts in microbial populations (Gladine *et al.*, 2007). The gradual decrease in the potency of EOs to inhibit ammonia N concentration with increase in time of incubation suggest that the benefits associated with the use of essential oil as feed additive may decline over time. Reduced concentration of NH_3-N (at 12 h) with the inclusion of EOs could be due to inhibition of microbial enzymes responsible for amino acid deamination. As suggested in previous studies, inhibition of amino acid deamination is the consequence of reduced proteolytic activity of the rumen (Mclnotch *et al*., 2003). Bach *et al*. (2005) reported that different groups of microbes: HAP (with lower abundance but use amino acids as energy source), proteolytic bacteria (with higher abundance and lower rate of NH_3-N producing potential), and protozoa, are the major groups of microorganisms involved in NH_3-N production. Therefore, the lower concentration of ammonia in some cultures could be due to inhibition of activities of these predominant microorganisms by the addition of EOs (Mclnotch *et al*., 2003). Cassia oil containing the phenolic compound, cinnamaldehyde (Table 4.2) had the highest inhibition of NH_3-N concentration in cultures, further suggesting that phenolic compounds have the greatest and broadest antimicrobial characteristics.

The inclusion of all EOs, except ETO and LVO at 24 h, significantly reduced TVFA at all times of incubation compared to the control. As ruminants derive the majority of their energy from VFA (Bergman, 1990), a reduction in VFA of the magnitude observed (over 10% average reduction) with all EOs except ETO and LVO would suggest a gross inhibition of rumen function. Inhibition of TVFA in the current study agrees with a number of previous studies such as Eburu and Chikunya (2014), where inclusion of 300 mg/L of EOCs reduced TVFA; Gunal *et al*. (2013), where different doses of citronella oil (125, 250 and 500 mg/L) reduced TVFA; and Agarwal *et al*. (2009), where 1.0 and 2.0 ml/L of peppermint oil depressed feed digestibility and TVFA production, and, Busquet *et al*. (2005), where inclusion of higher dose (3000 mg/L) of whole EO and individual components such as cinnamon oil and cinnamaldehyde (3000 mg/L) reduced TVFA. By contrast, the use of anise at 0.22 mg/L in continuous culture experiment did not change the profile of TVFA (Cardozo et al. (2004), and lavender oil (5, 50 and 500 mg/L) did not modify rumen fermentation parameters after 24 h (Castillejos *et al*., 2008). The difference in the production of TVFA between the current study and the study of Cardozo *et al*. (2004)

could be understood from the point of view of the difference in the dose of EOs. Addition of EO to cultures may decrease (at high doses) or have no effect (at low doses) on TVFA concentration, depending on the concentration of the EOs to alter feed nutrient digestibility (Patra and Saxena, 2010). In addition, previous studies suggested that effects of EO on VFA are not always consistent, but determined by the dose and type of EO (Bustquet *et al*., 2006), and the pH of the rumen (Cardozo *et al*., 2005). In the current study, the reduction on the concentration of TVFA was consistent with the reported reduction on cumulative gas production after 48 h period of incubation. Reduced digestion in feed which is reflected on decreased production of gas can be accompanied by less production of hydrogen, CH_4 and volatile fatty acids plus a lower A/P ratio (Boggs *et al*., 1987).

In the current study, CSO (with cinnamaldehyde as active component, Table 4.2) was the only additive that reduced the molar proportion of acetate relative to the control, the rest of the extracts increased acetate. This supports the finding in the first experiment (Chapter 3) where cinnamaldehyde decreased the molar proportion of acetate. In addition; the result is consistent with previous studies (Busquet et al., 2005; Gunal *et al*., 2013) where the use of either individual EOCs or whole EOs increased the molar proportion of acetate in cultures. However, using rumen fluid from beef cattle fed 10% concentrate and 90% forage in *in vitro* fermentation experiment with pH 5.5, Cardozo *et al*. (2005) reported that anise oil reduced acetate proportion. The difference in the composition of the basal diets fed to the donor animals between the current study and the study by Cardozo *et al*. (2005) could be responsible for the observed differences in the molar proportion of acetate. The proportion of propionate was not affected by 50% of the EOs (ANO, ETO, JPO, MDO and RMO) at 24 h, but reduced by the rest of the additives used, with the exception of CSO increased this molar proportion. This increase in the molar proportion of propionate with the inclusion of CSO is consistent with the findings from previous studies (Busquet *et al*., 2005; Castillejos *et al*., 2008). This suggests that the inclusion of CSO in cultures modified microbial population towards decreased methanogenesis (Demeyer and Van Nevel, 1995). In the rumen, anaerobic fermentation of proteins and soluble carbohydrates (sugars and starch) with higher propionate production results in reduced acetate and methanogenesis (Demeyer and Van Nevel, 1995), since the predominant producers of acetate and methane are cellulolytic bacteria. The reduced proportion of propionate in the

current study with the inclusion of the rest of the EOs also supports the results in Chapter 3, where EOCs reduced the molar proportion of propionate, and also consistent with earlier studies (Castillejos *et al*., 2006). The ratio of acetate to propionate (A: P) was not affected by JPO and RMO at 24 h but reduced by CSO, with the remaining EOs increasing A/P. The increased ratio of A/P with the inclusion of most of the EOs could be due to the higher molar proportion of acetate observed in those cultures, and the decrease in A/P in cultures supplemented with CSO is also due to the increased proportion of propionate in those vessels.

4.5.2. Effect of EOs on fatty acid metabolism

In the current study, the addition of ANO, CSO, CLO, JPO and MDO (at 48 h) and the inclusion of all EOs except CSO (at 48 h) reduced the concentrations of C14:0 and C16:0 respectively. Available reports suggest that these fatty acids (palmitic and myristic acids) are capable of raising plasma cholesterol through suppression and saturation of low-density lipoprotein (LDL) receptors (Keys *et al*., 1995). This suggests that a reduction in the concentration of C14:0 and C16:0 in the current study with the inclusion of all EOs except CSO could possibly decrease the plasma levels of low-density lipoprotein (LDL) if this is repeated *in vivo*.

The concentration of the last product of the biohydrogenation of C18:3 *n*-3, C18:2 *n*-6 and C18:1 *n*-9 (C18:0) was not affected by the addition of EOs to cultures. The exception to this was the reduced levels of C18:0 in CSO (12 h) and RMO (24 h). It is uncertain why the high accumulation of C18:3 *n*-3 and C18:2 *n*-6 in this study did not translate to a reduction in C18:0, since C18:0 is the end product of their biohydrogenation. However, the accumulation of C18:0 in the current study despite high levels of PUFA is in agreement with previous studies with EOs (Vasta *et al*., 2013). Also, in our previous studies with individual EOCs, the production of C18:0 was not affected by majority of the EOCs at 24 h despite higher levels of C18:3 *n*-3 and C18:2 *n*-6 (Chapter 3; Eburu and Chikunya, 2014). As suggested in Chapter 3, three possibilities were discussed as potential reasons for the high accumulation of PUFA without major effects on the end product of biohydrogenation: firstly, the high concentration of C18:0 in the current study despite high levels of PUFA could be emanating from the high concentration of C18:1 *n*-9 in cultures with added EOs. Jenkins *et al*. (2006) reported that about 70% of C18:1 *n*-9 in rumen culture was

converted to C18:0, and only 30% was transformed to ketostearic acid and hydroxystearic acid; the next possibility is that other unidentified biohydrogenation intermediates could have been produced in these cultures (Gunal *et al*., 2013); and finally, accumulation of PUFA could be due to reduced lipolytic activities. Buccioni *et al*. (2012) suggested that if small amounts of PUFA in the diet reach the duodenum, it might be arising from a reduction in lipolysis. This suggests that these EOs inhibit the activity of *Butyrivibrio fibisolvens* and *Anaerovibrio lipolytica*, which are responsible for hydrolysing the ester bond in fatty acids (Buccioni *et al*., 2012), potentially reducing lipolysis and isomerization of oil. As suggested by previous studies (Cobb 1992; Grundy, 1994; Pariza, 2004), unlike C14:0 and C16:0 fatty acids, discussion on the control of cholesterol through dietary manipulations should not be focused on stearic acid as it is a saturated fatty acid but without any harmful effects on human health.

The activity of the rumenic acid enzyme, the reductase enzyme, aids the formation of *trans*-11 18:1 (vaccenic acid; VA) from *cis*-9 *trans* 11 18:2 CLA (Jenkins *et al*., 2008; Kim *et al*., 2009; Buccioni *et al*., 2012). In the current study, majority of the added EOs increased the concentration of C18:1 *trans* 11 in cultures after 48 h, JPO, CTO and LVO being the most effective EOs, in agreement with Lourenco *et al*. (2009). This suggests that inclusion of EOs stimulates the activity of the reductase enzyme, and also indicate that inclusion of these EOs could increase the concentration of *cis*-9 *trans* 11 18:2 CLA. Vaccenic acid represents a substrate for endogenous synthesis of CLA in animal tissues through the Δ-9 desaturase enzyme (Griinari *et al*., 2000); hence, increasing the level of VA would potentially have a linear effect on the amount of *cis*-9 *trans* 11 18:2 CLA in animal tissues (Griinari *et al*., 2000). Previous studies (Piperova *et al*., 2002; Kay *et al*., 2004) reported that about 90% of *cis-9 trans-11* CLA in cow's milk resulted from the desaturation of VA. In addition, high accumulation of C18:1 *trans* 11 in the current study suggests that the addition of EOs could likely impair the activity of *Clostridium proteoclasticum* (Kemp *et al*., 1975; Maia *et al*., 2007), renamed as *Butyrivibrio proteoclasticus* (Moon *et al*., 2008), the bacteria responsible for converting VA to C18:0.

The current study observed that only the inclusion of CTO (24 h) and ANO (48 h) significantly increased the content of *cis*-9 *trans* 11 18:2 CLA, relative to the control, which is consistent with previous studies (Whitney *et al*., 2011); the rest of the treatments did not affect this intermediate of biohydrogenation. As

mentioned previously, *cis*-9 *trans* 1118:2 CLA is formed in the first step during the transformation of *cis*-9, *cis*-12 18:2 by the linoleic acid isomerase (LA-I) (Jenkins *et al*., 2008; Kim *et al*., 2009; Buccioni *et al*., 2012). It can be suggested from the results of the current study that the activities of the lipase (LA-I) which facilitate the formation of *cis*-9 *trans* 11 CLA from *cis*-9, *cis*-12 18:2 were impaired by all but CTO and ANO at the times indicated.

The content of C18:2 *n*-6 in both the CON and in all EOs rapidly decreased with time, being lowest at 48 h and highest at 12 h. Although the biohydrogenations of C18:2 *n*-6 and C18:3 *n*-3 were similar, that of C18:2 *n*-6 was slightly lower than that of C18:3 *n*-3. These biohydrogenation values conform to results of previous *in vitro* (Beam *et al*., 2000) and *in vivo* (Wachira *et al*., 2000) studies. The effects of the added EOs on the biohydrogenation of C18:2 *n*-6 and C18:3 *n*-3 (discuss later) were similar, with ANO and CSO being the most effective EOs to reduce the extent of biohydrigenation of C18:2 *n*-6.

As a direct reflection of the biohydrogenation of C18:3 *n*-3 in cultures, the content of C18:3 *n*-3 rapidly decreased with time, being highest at 12 h and lowest at the end of incubation (48 h). This rapid biohydrogenation of C18:3 *n*-3 from whole ground linseed which was more than 80 g/100 g at 24 h agrees with the results obtained with the EOCs in Chapter 3. This biohydrogenation values are also in support of other results of previous *in vivo* studies (Wachira *et al*., 2000; Scollan *et al*., 2001, Wang *et al*., 2002) and *in vitro* studies (Sinclair *et al*., 2005). In the first 24 h, the concentration of C18:3 *n*-3 was significantly highest in ANO and CSO and consistently lowest in ETO and JPO, relative to the control. At 48 h, the highest levels of C18:3 *n*-3 were still observed in cultures with ANO. These findings are in agreement with our previous study where anethole and cinnamaldehyde, the active components of anise and cassia EOs respectively, were among the most effective compounds to maintain the highest concentrations of C18:3*n* -3 and C18:2 *n*-6 (Chapter 3, Eburu and Chikunya, 2014). The most active component of ANO (anethole) and CSO (cinnamaldehyde) possess phenolic moieties in their chemical structures (Calsamiglia *et al*., 2007; Bakkali *et al*., 2008). As mentioned previously, the antibacterial activity and specific mode of action of an individual EO is influenced by the chemical structure of EO components (Dormans and Deans, 2000). The possibility of ANO and CSO to exert the greatest antimicrobial characteristic by maintaining higher levels of C18:3 *n*-3 than other EOs could be due to

their phenolic properties. This could suggest that ANO and CSO probably reduced isomerization of C18:3 *n*-3 compared to the rest of the treatments. EOs containing high proportion of phenolic compounds such as thymol, carvacrol and eugenol (2-methoxy-4-(2-propenyl)phenol as components of their chemical structure exhibit the strongest antimicrobial properties against food borne pathogens (Cosentino *et al*., 1999; Juliano *et al*., 2000; Lambert *et al*., 2001). The mechanisms of action of phenolics are thought to generally include disruption of proton motive force (PMF), coagulation of cell contents, disturbance of cytoplasmic membrane and disruption of active transport and electron flow (Sikkema *et al*., 1995; Davidson, 1997). By contrast, the use of six different essential oils (citronella, rosemary, sage, Siberian fir needle oil, clove and white thyme) at 125, 250 and 500 mg/L did not affect the biohydrogenation of C18:3*n* -3 (Gunal *et al*., 2013). The possible explanation to the difference in results obtained between the study of Gunal *et al*. (2013) and the current study could be the difference in the maturity stage of the parent plant at which the oil was obtained before use, and the part of the plant from which the oils were extracted. This is because maturity stage and the part of the plant from where oil is extracted have been reported to influence the antimicrobial potency of oils (McGimpsey *et al*., 1994; Cosentino *et al*., 1999; Lis-Balchin *et al*., 1999; Marino *et al*., 1999; Delaquis *et al*., 2002). This indicates wide variation between the compositions of EOs obtained from different parts of the same plant, which could translate to their different antimicrobial characteristics. Comparison between the effects of EOCs (in Chapter 3, e.g. anethole) and EOs (the current study, e.g. anise oil) on concentrations and biohydrogenation of C18:3 *n* -3 and C18:2 *n*-6 suggest that anise oil and its predominant active compound (anethole) had similar effects on rumen concentration of C18:3 *n*-3. This observation is consistent with previous studies where it is established that anethole is the main compound in anise oil (Davidson and Naidu, 2000). However, the higher concentrations of C18:3 *n*-3 and C18:2 *n*-6 in cultures supplemented with anise oil 300 mg/L (current study) than anethole 300 mg/L (Chapter 3) suggest that other minor components of anise oil had significant synergistic effects on ruminal fermentation activities.

In our previous study (Chapter 3; Eburu and Chikunya, 2014), the use of anethole and 4-allylanisole, the two most effective EOCs, protected 22.2 and 26.4 g/100 g of C18:3 *n*-3 from biohydrogenation. In the current study however, the use of ANO and CSO, the two most effective EOs, protected 58.2 and 54.3

g/100 g respectively, from biohydrogenation at 24 h. As mentioned previously, the possible reason why the use of the whole EOs in the current study had higher potential to protect C18:3 *n* -3 or C18:2 *n* -6 from biohydrogenation could be that there is some form of synergism between the minor and major compounds in the whole EOs (Davidson and Parish, 1989; Burt, 2004).

The extent of disappearance of C20:5 *n*-3 and C22:6 *n*-3 in vessels increased as the time of incubation progressed, being highest at 48 h and lowest at 12 h. The quantity of the content of C20:5 *n*-3 and C22:6 *n*-3 in vessel which disappeared after 48 h was highest in the control. These results support previous reports from *in vivo* studies where the biohydrogenation of C20:5 *n*-3 and C22:6 *n*-3 in fish oil range from 72 to 93 g/100 g TFA (Wachira *et al*., 2000; Scollan *et al*., 2001; Chikunya *et al*., 2004). However, these findings contrast *in vitro* reports where the biohydrogenation of C20:5 *n*-3 and C22:6 *n*-3 was less than 50 g/100 g (Ashes *et al*., 1992; Sinclair *et al*., 2005). Similar to the results obtained in Chapter 3, the potential inhibition of the biohydrogenation of C20:5 *n*-3 and C22:6 *n*-3 differs within the EO group; with biohydrogenation being lowest in ANO whose most active compound (anethole) possesses phenolic moieties. As discussed in Chapter 3, the biohydrogenation of the fish oil fatty acids (C20:5 *n*-3 and C22:6 *n*-3) was less compared to the ruminal disappearance of C18 fatty acids (C18:3 *n*-3 and C18:2 *n*-6). This inability of microbes to hydrogenate the fish oil fatty acids is not due to the difference in the lipase activities but because microbes lack the enzymes necessary to hydrogenate the long chain *n*-3 PUFA (Ashes *et al*., 1992).

There were no clear effects of treatments on the content of ∑SFA after 48 h; therefore, ∑SFA was calculated without C18:0 (I.e. ∑SFA-C18:0) since C18:0 does not have any harmful effects in human health (discussed previously). After 48 h the inclusion of all EOs in cultures reduced the content of ∑SFA-C18:0 compared with the control at 48 h, which is consistent with the higher accumulation of ∑PUFA in cultures with added EOs.

The content of ∑PUFA declined with increase in the time of incubation, and was highest in ANO at 48 h, relative to the control. The decrease in the levels of ∑PUFA with time agrees with the corresponding

decrease in the concentration of individual PUFA. The highest content of the ∑PUFA in vessels treated with ANO also conformed to the highest levels of individual PUFA.

In the report presented here, the content of TFA was significantly reduced by majority of the EOs relative to the control, except ANO and RMO (both at 24 h) which did not affect the concentration of TFA. Reduction of TFA could suggest that the reported decrease in TVFA and ammonia concentration (mostly at 12 h) had reduced the microbial *de novo* fatty acids synthesis (Sauvant and Bas, 2001), leading to reductions in the levels of TFA.

In this study, the major thrombotic fatty acid (C20:4 *n*-6; Kinsella *et al*., 1990), was beneficially reduced by most of the added EOs and was lowest in ANO and MDO after 48 h. If ∑*n*-6/∑*n*-3 is an indicator of the antithrombotic effect, then cultures with ANO would have the highest antithrombotic strength.

The ratio of PUFA to SFA (P: S) in cultures decreased with time, and was consistently highest in vessels with ANO and CSO. After 48 h of incubation, only cultures with ANO had P/S close to the value of 0.45 recommended in the guidelines of the Department of Health (1994).

4.6. Conclusion

In the current study, the potential of whole EOs used in this study to inhibit BH of *n*-3 PUFA at 24 h can be ranked as follows: ANO and CSO > MDO and CMO > CTO and RMO > JPO, LVO, CLVO and ETO. These results showed that ANO and CSO (rich in phenolic compounds) have the greatest potential to modulate the biohydrogenation of *n*-3 PUFA in the rumen. However, it is uncertain whether these changes are replicated in *in vivo* experiment. If this is confirmed, then the levels of PUFA in ruminant food products (meat and milk) could be enhanced by supplementing ruminant diet with cassia and anise oils. However, lower doses should be tested to establish optimum levels of inclusion at which feed degradability and VFA production would not be impaired. It is also worthwhile to investigate the mechanism of action and the stability of these oils in the rumen. Therefore, the next study (discussed in Chapter 5) would investigate effects of varying doses (0, 100, 200 and 300 mg/L) of the two most effective oils (anise and cassia) and the two most effective EOCs (anethole and 4-allylanisole).

Chapter 5

The effects of graded doses of 4-allylanisole, anethole, anise oil and cassia oil on fermentation and biohydrogenation of *n*-3 polyunsaturated fatty acids by rumen microorganisms *in vitro*

Abstract

Effects of varying levels of inclusion of 4-Allyanisole (ALA), anethole (ANE), anise oil (ANO) and cassia oil (CSO) on the fermentation activities of rumen microbes and the biohydrogenation (BH) of *n*-3 PUFA were evaluated *in vitro* using batch culture system. Rumen fluid was collected from six Hartline × Texel cross cull ewes. A basal feedstock comprising of 70:30 grass hay (*Lolium perenne)* and concentrate (lamb finisher cubes) was used. Serum bottles were incubated at 39^0C; each bottle contained 1 g of feed substrate, 80 ml buffer, 20 ml inoculum, then supplemented with increasing concentrations (0, 100, 200 and 300 mg/l) of each EOs/EOCs. There were 16 treatments (with 6 replicates per treatment) as follows: ALA, ANE, ANO and CSO. Fermentation was stopped after 12 and 24 h, and samples were collected to analyse NH_3-N, total and molar proportions of individual VFA; and concentration of PUFA including intermediates of BH. Relative to the control, CSO (all doses), ANE (at 100), and ALA at 300 reduced NH_3-N levels relative to the control, whilst concentration was similar in all doses of ANO. Irrespective of oil, the highest dose (300 mg/L) of all substances with the exception of ANE induced the most inhibition (average 12%) on TVFA. At 200 mg/L, ANE, ALA and ANO only slightly (by about 2%) reduced TVFA relative to the control, but CSO reduced TVFA by 10%. The level of C18:0 was reduced by ANO (at 100 mg/L), ALA (at 300 mg/L) and CSO (at 200 mg/L). All doses of all substances maintained higher ($P<0.001$) the concentrations of 18:2 *cis*-9 *trans* 11 CLA and all PUFA in a dose-dependent manner, suggesting that higher doses afforded better protection. At 200 and 300 mg/L all substances substantially maintained higher concentration of PUFA relative to the control. Therefore, considering the effects of the tested EOs and EOCs and at different doses in this study, it appears that the administration of ALA, ANE and ANO at 200 mg/L seems to give best balance between PUFA protection and minimal disturbance to VFA concentration. However, CSO requires a dose more than 100 mg/L but less than 200 mg/L to attain the same level of protection and minimising disruption of fermentation. It is worthwhile to investigate whether these effects are sustained *in vivo* including testing the possibility of microbial adaptation to these substances at these doses.

5.1. Introduction

In two separate preliminary investigations, it was established that including both individual essential oil compounds (EOCs) particularly 4-allylanisole and anethole (Chapter 3; Eburu and Chikunya, 2014) and whole essential (EOs) such as oils of anise and cassia (Chapter 4; Eburu and Chikunya, 2015a) at 300 mg/L significantly inhibits the disappearance of C18:2 *n*-6 and *n*-3 polyunsaturated fatty acids (PUFA; C18:3 *n*-3, C20:5 *n*-3 and C22:6 *n*-3) from rumen contents. However, at this level of inclusion (300 mg/L) there was a concomitant significant suppression of VFA levels (over 10% average reductions with the addition of both EOs and EOCs). Volatile fatty acids are the fundamental sources of energy for ruminants and their ruminal concentrations reflect the extent of degradation of nutrients in the used feed (Bergman, 1990; Szumacher-Strabel and Cieslak, 2012). The reduced concentrations of VFA in our previous studies (Chapters 3; 4) would suggest that if similar effect is exerted *in vivo*, animal performance would be significantly negatively affected. Previous studies suggested that the benefits of using EOs and EOCs to decrease ruminal NH_3-N production can be counterbalanced by a reduction in feed digestibility and decreased concentration of VFA if their inclusion levels are not optimized (Busquet *et al*., 2006; Martinez *et al*., 2006). Therefore, using these additives at optimum dosages could reduce the negative effects on VFA concentration. Because the aim of using EOs and EOCs as feed additive is to improve the efficiency of rumen fermentation processes and not inhibition, information on their optimum doses is vital to maximize ruminant performance. McIntosh *et al*. (2003) observed that EOs affect microbial population in a dose-dependent manner. The aim of the current study was to establish the optimal doses of the four substances (4-allyanisole, anethole, anise and cassia) which were identified in our preliminary studies (Chapters 3; 4) to be most effective at inhibiting PUFA disappearance. This would enable the achievement of satisfactory inhibition of PUFA biohydrogenation but without significant reductions in VFA concentration. In the current study, these additives were evaluated at graded levels of inclusion.

5.2.0. Materials and methods

5.2.1. Animal management and collection of rumen fluid/sampling

In this experiment, six Hartline × Texel cross cull ewes (mean weight 70.2 ±7.5kg) were used as rumen fluid donors. Experimental details relating to housing, experimental diet, feeding, duration of adaptation and collection of rumen fluid are described in section 2.1.

5.2.2. Basal feedstock, treatments and *in vitro* incubation

The basal feedstock was mixtures of good quality rye-grass hay (*Lolium perenne*), lamb finisher concentrate, whole ground linseed and fish oil. The supplier details and components of the basal feedstock used in this *in vitro* incubation are as described in the general material and methods (see section 2.3). The ingredient content, chemical and fatty acid composition of the basal feedstock are shown in Table 2.2.

The effects of the 2 EOCs and 2 whole essential oils were evaluated using the *in vitro* gas production batch culture method described by Theodorou *et al*. (1994). All EOCs and EOs used were purchased from Sigma-Aldrich Co. Ltd., UK and were stored at the required temperatures specified on delivery notes prior to use. See Tables 3.2 and 3.3 for a description of the EOCs and Tables 4.1 and 4.2 for a description of the EOs used in this study. There were 16 treatments and 6 replicates per treatment as follows: 4-Allyanisole (ALA: 0, 100, 200 and 300 mg/L), anethole (ANE: 0, 100, 200 and 300 mg/L), anise oil (ANO: 0, 100, 200 and 300 mg/L) and cassia oil (CSO: 0, 100, 200 and 300 mg/L). In total 292 serum bottles were incubated, each bottle contained 1 g of feed substrate, 300 mg/l of EOC, 80 ml anaerobic buffer (see Table 2.1) and 20 ml inoculum and the bottle sealed with rubber cork before incubation.

5.2.3. Sample collection and preservation

Experimental procedures relating to cumulative gas pressure measurements, incubation stopping times, collection and storage of samples in this study were as described in the general material and methods (see section 2.4).

5.2.4 Chemical analysis

The concentration of NH_3-N in digesta was analysed using the phenol-hypochlorite method (Weatherburn, 1967; Broderick and Kang, 1980) adopted for use on the plate reader as described in general materials and methods (see section 2.5.4).

The concentrations of total volatile fatty acid (VFA) and molar proportion of VFA was determined by gas chromatography (GC) as described by Ottenstein and Bartley (1971). Details of this method are outlined in the general materials and methods (see section 2.5.6).

The concentration of fatty acids in feed and freeze dried samples were extracted using the direct saponification method described by Enser *et al*. (1998). See section 2.5.7 of the general materials and methods for detailed description of the techniques.

5.2.5. Experimental design and statistical analysis

This study was a randomized complete block design (RCBD) experiment. The objective was to examine the effects of increasing doses of EOCs/EOs on the extent of rumen biohydrogenation of *n*-3 PUFA *in vitro*. The null hypothesis was that inclusion of varying doses of EOs/EOCs would have no effect on fermentation and biohydrogenation data. The alternative hypothesis was that the different doses of EOCs/EOs would affect (decrease or increase) fermentation activities.

The study was a 4 (EOs/EOCs: ALA, ANE, ANO and CSO) × 4 (dose: 0, 100, 200 and 300 mg/L) factorial design experiment. Therefore data were analysed by TWO-WAY analysis of variance (ANOVA) using GenStat 16th edition (VSN international Ltd, Registered to: Writtle College). The main effects were EOs/EOCs, dose and oil × dose interaction. Differences between treatments were declared by least significance difference (LSD) and significance was declared at $P< 0.05$. Data were analysed separately for each time point (12 and 24 h).

5.3.0. RESULTS

5.3.1 *In vitro* fermentation parameters

Effects of treatments on cumulative gas production (ml/g OM) and NH_3-N (mM) are presented in Table 5.1a, whilst the significance of the main effects and interactions are shown in Table 5.1b.

At 12 h, the levels of total gas was decreased ($P<0.001$) with the supplementation of all doses (100, 200 and 300 mg/L) of all EOCs/EOs in a dose response manner, relative to the control. Regardless of oil, the 300 mg/L (highest dose) of all substances caused the most reduction (average 22%) on total gas. At 200 mg/L, all treatments caused marginal inhibition of total gas (average 6%), except for CSO which inhibited by 15%. There was a significant interaction between dose levels and EOs/EOCs ($P<0.001$). It was observed that supplementing CSO 300 mg/L induced the highest inhibition of total gas relative to the 300 mg/L of the rest of the treatments (ALA, ANE and ANO). Mean values were 103.5 (average across all controls), 84.1, 82.5, 94.3 and 62.9 ml/g OM (sed= 4.02, $P<0.001$, Table 5.1a), for ALA 300, ANE 300, ANO 300 and CSO 300, respectively. At 24 h, irrespective of oil, increasing the dose of all substances progressively reduced the amount of total gas observed in fermentation vessels. The lowest dose (100 mg/L) of all substances elicited the least reduction (average 4%) on total gas, but no interaction was observed at this point.

At 12 h, the addition of ANE (at 100 and 200 mg/L) and ANO (at 300 mg/L) reduced ($P<0.001$) NH_3-N concentration relative to the control. In contrast, the supplementation of cultures with ALA (at 100, 200 and 300 mg/L) and CSO (at 200 and 300 mg/L) increased ($P<0.001$) the mean concentration of NH_3-N. There was a significant interaction between the oils and the dose such that ANE 200 mg/L decreased NH_3-N concentration compared to the 200 mg/L of the rest of the treatments (ALA, ANO and CSO). Mean values were 3.6 (mean across all controls), 3.8, 3.1, 3.8 and 3.7 mM (sed= 0.22, $P<0.001$, Table 5.1a). After 24 h, the concentration of NH_3-N in fermentation vessels was reduced ($P<0.001$) with the supplementation of ALA (at 300 mg/L) and CSO (at 100, 200 and 300 mg/L) but increased in vessels where ANE (100 mg/L) and ANO (at 200 mg/L) were added. The addition of CSO at 100 had the lowest level of ammonia compared with ALA 100, ANE 100 and ANO 100, relative to the control. Average values were 4.8, 4.4, 5.6, 4.6 and 3.4 mM (sed= 0.44, $P<0.018$, Table 5.1a), for the control, ALA 100,

ANE 100, ANS 100 and CAS 100, respectively. Similar effect was true for ALA 200, ANE 200, ANO 200 and CSO 200. However, ALA 300 and CSO 300 caused 25% average NH_3-N reduction relative to the control, whilst ANE 300 and ANO 300 maintained the concentration of NH_3-N at similar levels to the control. Mean values were 4.8, 3.4, 4.8 and 3.8 mM (sed= 0.44, P<0.018, Table 5.1a), for the control, ALA 300, ANE 300, ANO 300 and CSO 300, respectively.

Effects of treatments on the concentration of TVFA (mM) and the molar proportions of individual VFA (mM/mol TVFA) are presented in Table 5.2a and the significance of the main effects and interactions are in Table 5.2b. In this study, both EOCs and EOs used all reduced the mean (mean across all doses) concentration of TVFA, with the overall highest reductions observed in vessels with CSO (14%) and ALA (11%). All doses of all EOs and EOCs maintained a dose dependent response effect on TVFA with the exception of ANE, such that vessels that received the highest dose (300 mg/L) irrespective of additive type, induced the highest inhibition (average 12%) on TVFA (Table 5.2a). Relative to the control, supplementing ALA 300 caused the highest decrease on TVFA compared to 300 of ANE, ANO and COS (Table 5.1a). Average inhibitions were 18%, 2%, 10.7% and 15.1% for ALA 300, ANE 300, ANO 300 and CSO 300, respectively. At 200 mg/L, ANE, ALA and ANO only marginally reduced TVFA (by about 2%) relative to the control, but CSO reduced TVFA by 10%. Mean values were 92.5 (mean of all controls), 87.3, 103.5, 91.8 and 82.9 mM (sed= 2.62, P<0.001, Table 5.2a), for the control, ALA, ANE, ANO and CSO, respectively. Compared to the control, the 100 mg/L of ANO and ANE caused approximately 7% and 12% increases in TVFA, respectively (Table 5.2a).

The proportion of acetate was reduced (P<0.001) in CSO (at 100, 200 and 300 mg/L); ALA and ANO (at 300 mg/L); and in ANE (100 mg/L). By contrast, ANE (200 and 300 mg/L) and ANO (at 100 and 200) increased the proportion of acetate, relative to the control. All doses (at 100, 200 and 300 mg/L) of CSO recorded the lowest molar proportion of acetate relative to the corresponding doses of other substances (Table 5.2a, b). Overall mean across all doses showed that the proportion of propionate was increased (17%) in vessels where CSO was added and reduced (12%) where ANE was added, but ALA and ANO maintained the concentration of propionate similar to the control. At 100 mg/L, the proportion of propionate was increased with the addition of CSO compared to the 100 mg/L of the remaining

treatments. At 200 mg/L, CSO and ALA increased the concentration of propionate but ANO and ANE reduced it. Supplementing 300 mg/L of CSO increased the concentration of propionate relative to 300 mg/L of other treatments (ANO, ANE and ALA). Average values were 17.2 (mean across all controls), 17.8, 17.4, 16.4 and 20.5 (100 mg/L); 16.8, 14.2, 16.1 and 20.3 (200 mg/L); and 300 mg/L were 17.2, 17.6, 14.6, 16.4 and 19.5 mM/mol TVFA (sed= 0.54, $P<0.001$, Table 5.2a and b), for the control, ALA, ANE, ANO and CSO, respectively. Although the proportion of butyrate was not affected with higher doses (200 and 300 mg/L) of CSO and lower doses of ALA (100 and 200 mg/L), it was reduced ($P<0.001$) where higher doses of ANE (200 and 300 mg/L) and lower doses of ANO (100 and 200 mg/L) were added. The acetate to propionate ratio (A: P) was reduced with all doses of CSO (reduction being highest in the lower doses, average 21%) and the lowest dose (100 mg/L) of ALA (5%) and ANE (3%). However, all doses of ANO (100, 200 and 300 mg/L) and higher doses of ANE (200 and 300 mg/L) increased A: P (Tables 5.2a; b). Supplementation of EOCs/EOs elicited minor increases and decreases on the molar proportions of the branched-chain VFA (BCVFA) such as iso-valeric, iso-butyric, valeric and caproic acids (Tables 5.2a and b). Generally, the lowest proportions of iso-valeric, iso-butyric and caproic acids were observed in CSO (200 and 300 mg/L), relative to the control.

Table 5.1a Effects of EOs and EOCs on gas production (ml/g OM) and ammonia (mM) during 24 h *in vitro* incubation

		EOs and EOCs																	
		4-Allylanisole (mg/L)				Anethole (mg/L)				Anise (mg/L)				Cassia (mg/L)				S.E.D	
Variables	Time(h)	0	100	200	300	0	100	200	300	0	100	200	300	0	100	200	300	O or D	O × D
Gas	12	103.5	104.3	96.8	84.1	103.2	100.0	94.3	82.5	103.9	102.6	100.9	94.3	103.4	95.0	88.0	62.9	2.01	4.02
	24	147.6	145.2	132.3	117.7	147.6	140.1	129.5	113.6	147.5	142.4	136.5	128.5	147.8	138.0	131.8	108.3	2.46	4.92
NH_3-N	12	3.6	4.1	3.8	4.0	3.6	3.2	3.1	4.6	3.5	3.7	3.8	3.2	3.6	3.6	3.7	4.2	0.11	0.22
	24	4.8	4.4	4.4	3.4	4.8	5.6	4.2	4.8	4.7	4.7	5.2	4.9	4.8	3.4	4.1	3.8	0.22	0.44

S.E.D= Standard error of difference, O or D= Oil or dose; O × D = Oil by dose interaction. NB. S.E.D for oil and dose were similar that is why oil or dose is used in the table; EOCs= essential oil compounds; EOs= essential oils

Table 5.1b Effects of EOs and EOCs on gas production (ml/g OM) and ammonia (mM) during 24 h *in vitro* incubation (Significance of main effects and interactions)

Parameter	Time (h)	Oil	Dose	Oil × Dose
Gas production	12	<0.001	<0.001	<0.001
	24	NS	<0.001	NS
NH_3-N (mM)	12	<0.001	<0.001	<0.001
	24	<0.001	=0.046	=0.018

NS= Not significant (P<0.05); O × D = Oil by dose interaction; EOs= essential oils; EOCs= essential oil compounds

Table 5.2a Effects of EOs and EOCs on total (mM) and molar proportions of VFA (mM/mol TVFA) at 24 h *in vitro* incubation

	EOs and EOCs																S.E.D	
	4-Allylanisole (mg/L)				Anethole (mg/L)				Anise (mg/L)				Cassia (mg/L)					
Fatty acid	**0**	**100**	**200**	**300**	**0**	**100**	**200**	**300**	**0**	**100**	**200**	**300**	**0**	**100**	**200**	**300**	O or D	O × D
TVFA	92.5	91.6	87.3	75.9	92.6	86.6	103.5	91.1	92.7	98.9	91.8	82.6	92.2	85.3	82.9	78.5	1.31	2.62
Acetate	66.4	66.1	66.7	64.0	66.4	65.5	70.9	69.3	66.5	68.0	68.1	65.5	66.3	62.9	63.7	63.9	0.33	0.66
Propionate	17.2	17.8	16.8	17.6	17.0	16.4	14.2	14.6	17.3	16.4	16.1	16.4	17.3	20.5	20.3	19.5	0.27	0.54
Butyrate	8.2	8.1	7.9	8.7	8.2	7.7	6.9	7.4	8.2	7.0	7.0	8.2	8.1	8.4	7.8	7.9	0.16	0.32
Iso-butyric	0.8	0.7	0.7	0.8	0.8	0.8	0.6	0.6	0.8	0.8	0.8	0.8	0.8	0.7	0.6	0.6	0.01	0.03
Iso-valeric	0.9	0.6	0.7	0.7	0.9	1.2	1.2	0.9	0.8	1.1	1.0	1.0	0.8	0.6	0.5	0.5	0.02	0.05
Valeric	1.0	1.0	1.1	1.1	1.1	0.9	1.0	1.0	1.0	1.0	0.9	1.0	0.9	1.0	0.9	1.0	0.01	0.02
Caproic	0.4	0.2	0.5	0.6	0.4	0.6	0.4	0.6	0.4	0.7	0.7	1.6	0.4	0.0	0.0	0.3	0.07	0.13
A/P ratio	3.7	3.7	4.0	3.7	3.7	3.8	5.0	4.8	3.7	4.2	4.2	4.0	3.6	3.1	3.1	3.3	0.09	0.17

O or D= Oil or dose; O × D = Oil by dose interaction. NB. S.E.D for oil and dose were similar that is why oil or dose is used in the table

Table 5.2b Effects of EOs and EOCs on total VFA (mM) and molar proportions of VFA (mM/mol TVFA) at 24 h *in vitro* incubation **(**Sig.of O×D**)**

	Oil	**Dose**	**O×D**
TVFA (mM)	<0.001	<0.001	<0.001
Acetate	<0.001	<0.001	<0.001
Propionate	<0.001	<0.001	<0.001
Butyrate	<0.001	<0.001	<0.001
Iso-butyric	<0.001	<0.001	<0.001
Iso-valeric	<0.001	<0.001	<0.001
Valeric	<0.001	<0.001	<0.001
Caproic	<0.001	<0.001	<0.010
A/P ratio	<0.001	<0.001	<0.001

NS= Not significant (P<0.05); EOs= essential oils; EOCs= essential oil compounds

5.3.2. Effect of EOC on fatty acid metabolism

Effects of treatments on the concentrations of selected C14 and C16 are presented in Table 5.3a and the significance of the main effects and interactions are shown in Table 5.3b.

At 12 h, the concentration of C14:0 decreased in ALA (at 200 mg/L) and in CSO (at 100, 200 and 300 mg/L). However, mean levels of C14:0 was not affected with the supplementation of ALA (100 and 300 mg/L) and ANE and ANO (at 100, 200 and 300 mg/L) (Table 5.3a, b). There was a significant interaction between oils and dose levels. Relative to the control, all doses (100, 200 and 300 mg/L) of CSO and ALA 200 reduced the levels of C14:0 compared to the respective doses of other treatments (Table 5.3a; b). After 24 h, the addition of EOCs/EOs did not change the concentration of C14:0 in the vessels.

The concentration of C16:0 at both 12 and 24 h decreased ($P<0.001$) as the dose of all substances (ALA, ANE, ANO and CSO) increased. Regardless of the substance, the lowest levels of C16:0 were observed in vessels in which the 300 mg/L of both EOCs and EOs were added. Although the interaction between oil and dose was not significant at 12 h, rates were different after 24 h. After 24 h, the addition of ANE 100 increased the concentration of C16:0 compared with the supplementation of 100 mg/L of other treatments (ALA, ANO and CSO), relative to the control. Average values for 100 mg/L were 15.8 (mean of all controls), 15.6, 17.7, 15.5 and 15.9 g/100 g TFA (sed= 0.51, $P<0.001$, Table 5.3a), for the control, ALA, ANE, ANO and CSO, respectively.

The addition of all doses of all substances increased ($P<0.001$) the concentration of C16:1, with increases being proportional to the dose of oils (Table 5.3a; b). After 24 h, CSO 300 mg/L reduced (by about 4%) the concentration of C16:1 relative to the 300 mg/L of the rest of the treatment which increased it (average 13%), compared with the control.

Effects of EOCs and EOs on concentrations of selected C18 fatty acids are presented in Table 5.4a and significance of the main effects and interactions are shown in Table 5.4b.

At 12 h, the fermentation vessel concentrations of C18:0 were decreased in ANO (at 100 mg/L, by 6%); increased in ALA and ANE (at 100, 200 and 300 mg/L), ANO (at 300 mg/L) and CSO (at 200 and 300 mg/L); whilst CSO (at 100 mg/L) maintained similar to the control. Fermentation vessels that received

ANO 100 mg/L reduced the content of C18:0 compared with the 100 mg/L of the rest of the treatments (ALA, ANE and CSO), whilst vessels with added ANE 100 had the highest concentration of C18:0, relative to the control. Mean values for 100 mg/L were 12.1 (average of controls), 12.6, 13.1, 11.4 and 12.3 g/100 g TFA (sed= 0.27, P<0.001, Table 5.4a, b), for the control, ALA 100, ANE 100, ANO 100 and CSO 100 mg/L, respectively. After 24 h, the concentration of C18:0 was reduced (P<0.001) in vessels that were supplemented with ALA (at 300 mg/L) and CSO (at 200 mg/L). In contrast, the level of C18:0 was increased in cultures where ANE (300 mg/L) and ANO (at 200 and 300 mg/L) were added, but concentration was not affected in vessels that received ALA (200 and 300 mg/L), ANE (100 and 300 mg/L), ANO (at 100 mg/L) and CSO (at 100, 200 and 300 mg/L).

Anise oil had the overall lowest mean of C18:1 *n*-9 relative to the control after 12 h. Average values were 7.9 (mean across all controls), 7.8, 7.7, 7.4 and 7.9 g/100 g TFA (sed= 0.06, P<0.001, Table 5.4a, b), for the control, ALA, ANE, ANO and CSO, respectively. After 24 h, irrespective of oil, the highest dose (300 mg/L) of all substances with the exception of ANO had the highest levels of C18:1 *n*-9. The addition of ANO 300 had the lowest content of C18:1 *n*-9 compared with 300 mg/L of ALA, ANE and CSO, relative to the control. Mean values expressed as % increase to the control (5.3) were 35.8%, 30.2%, 15.1% and 41.5% (Table 5.4a), for ALA, ANE, ANO and CSO, respectively.

At 12 h, the mean (across all doses) concentration of C18:1 *trans* 11 was highest in vessels supplemented with CSO, lowest in fermentation vessels with ALA and ANO, whilst ANE maintained it similar to the control. Mean values across all doses were 1.8, 1.7, 1.8, 1.7 and 2.0 g/100 g TFA (sed= 0.02, P<0.001, Table 5.4a, b), for the control, ALA, ANE, ANS and CAS, respectively. The observed concentration of C18:1 *trans* 11 at 12 h was proportional to the dose level of all additive type, such that the 300 mg/L (the highest dose) of all substances recorded the highest (P<0.001) level of C18:1 *trans* 11 (Table 5.4a). Supplementation of CSO 300 mg/L caused 17% increase on 18:1 *trans* 11 compared to 300 mg/L of the rest of the treatments (ALA, ANE and ANO). Mean values for 300 mg/L were 1.8 (mean of all controls), 1.8, 1.8, 1.8 and 2.1 g/100 g TFA (sed= 0.04, P<0.001, Table 5.4a, b), for the control, ALA, ANE, ANO and CSO, respectively. However, after 24 h, effects of dose, oil and interactions were not significant.

At 12 h, effects of EOCs/EOs, dose and the interactions between oils and dose levels on the concentration of C18:2 *cis*-9 *trans* 11 CLA were not significant (Table 5.4a, b). However, after 24 h, the overall concentration of C18:2 *cis*-9 *trans* 11 CLA was maintained at higher levels ($P<0.001$) with the supplementation of all substances. The level of C18:2 *cis*-9 *trans* 11 CLA increased as the dose of oil progressed, such that the highest dose (300 mg/L) of all substances irrespective of oil, recorded the highest level of C18:2 *cis*-9 *trans* 11 CLA, relative to the control. The minimum doses of all substances needed to maintain higher concentration of C18:2 *cis*-9 *trans* 11 CLA relative to the control were ANO (at 100, 200 and 300 mg/L), ALA (at 200 mg/L), ANE and CSO (at 300 mg/L). Fermentation vessels which received all the doses of ANO (100, 200 and 300 mg/L) contained the highest levels of C18:2 *cis*-9 *trans* 11 CLA compared to the respective doses of other treatments, relative to the control. Mean values were 0.15 (average of controls); 0.15, 0.14, 0.16 and 0.14 (100 mg/L); 0.16, 0.15, 0.18 and 0.15 (200 mg/L) and 300 mg/L were 0.18, 0.16, 0.19 and 0.18 g TFA (sed= 0.010, $P<0.001$, Table 5.4a, b), for the control, ALA, ANE, ANO and CSO, respectively.

All doses (of both EOCs and EOs) maintained the concentrations of C18:2 *n*-6 and C18:3 *n*-3 in a dose related response. Irrespective of additive type, the highest concentrations of C18:2 *n*-6 and C18:3 *n*-3 were observed in vessels supplemented with the highest dose (300 mg/L) of all the substances. At 100 mg/L, there was a significant ($P<0.001$) but marginal increases on the concentrations of both C18:2 *n*-6 and C18:3 *n*-3, relative to the control. The minimum dose of all the oils required to maintain a substantial concentrations of both C18:2 *n*-6 and C18:3 *n*-3 relative to the control was 200 mg/L. The average (12 and 24 h) effects of treatments indicated that the mean concentrations of C18:2 *n*-6 and 18:3 *n*-3 were highest in vessels supplemented with ANO 200 relative to the 200 mg/L of other substances and the control. Mean values (12 and 24 h) for 200 mg/L were 3. 0 (average of controls), 3.8, 3.7, 3.9 and 3.7 (C18:2 *n*-6) and for C18:3 *n*-3 were 3.7 (mean of controls), 5.0, 5.0, 5.3 and 4.8 g/100 g TFA (Table 5.4a, b), for the control, ALA, ANE, ANO and CSO, respectively. The corresponding % increases relative to the control were 26.7%, 23.3%, 30% and 23.3% (18:2 *n*-6) and for C18:3 *n*-3 were 35.1%, 35.1%, 43.2% and 29.7%, for ALA, ANE, ANO and CSO, respectively.

The effects of EOCs and EOs on the concentrations of selected C20 and C22 are presented in Table 5.5a and significance of the main effects and interactions are indicated in Table 5.5b.

At 12 h, the addition of EOCs/EOs did not affect the concentration of of C20:4 *n*-6, relative to the control (Table 5.5a, b). However, after 24 h, the concentration of C20:4 *n*-6 decreased ($P<0.001$) in ALA and ANE (at 100, 200 and 300 mg/L). The lowest concentration of C20:4 *n*-6 was observed in vessels where ANE 300 was added relative to 300 mg/L of other treatments (ALA, ANO and CSO) and the control. Mean values for 300 mg/L were 0.5 (mean of control), 0.4, 0.3, 0.5 and 0.5 (sed= 0.04, $P<0.023$, Table 5.5a, b), for the control, ALA 300 mg/L, ANE mg/L, ANO mg/L and CSO mg/L, respectively.

Relative to the control, overall means indicated that all substances (ALA, ANE, ANO and CSO) increased ($P<0.001$) the concentrations of both C20:5 *n*-3 and C22:6 *n*-3 in vessels at 12 and 24 h. The levels of C20:5 *n*-3 and C22:6 *n*-3 observed in fermentation vessels were proportional to the doses of all substances used in this study. Regardless of substances, the highest concentrations of C20:5 *n*-3 and C22:6 *n*-3 were recorded in vessels where 300 mg/L of all substances were added (Table 5.5a). Although the concentrations of C20:5 *n*-3 and C22:6 *n*-3 were higher ($P<0.001$) at 100 mg/L of all substances, the values were marginal relative to the control. There was a significant interaction between oils and dose at both 12 and 24 h time points. The average (12 and 24 h) effects of oils on the concentration of C20:5 *n*-3 indicated that the levels of C20:5 *n*-3 was highest ($P<0.001$) in vessels with all doses of ANO (100, 200 and 300 mg/L) compared with the corresponding doses of all other additive type and the control. Mean values were 1.2 (control); 1.3, 1.3, 1.5 and 1.3 g/100 g TFA (100 mg/L); 1.4, 1.4, 1.8 and 1.5 g/100 g TFA (200 mg/L) and for 300 mg/L were 1.8, 1.6, 1.9 and 1.7 g/100 g TFA (Table 5.5a, b), for ALA, ANE, ANS and CAS, respectively. Although the interactions were not statistically significant (Table 5.5a, b), trends indicated that the average concentration of C22:6 *n*-3 was higher in cultures with ANO 200 than the 200 mg/L of ALA, ANE and CSO.

The addition of increasing doses of EOs and EOCs sequentially increased the concentrations of ∑PUFA and P/S ratio and progressively decreased the concentrations of ∑*n*-6/∑*n*-3 and ∑SFA-18:0, relative to the control (Tables 5.6a, b).

The effect of EOs/EOCs on the biohydrogenation (g/100 g) of PUFA (C18:2 *n*-6, C18:3 *n*-3, C20:5 *n*-3 and C22:6 *n*-3) and the significance of main effects and interactions are shown in Tables 5.7a and 5.7b, respectively.

Relative to the control (both at 12 and 24 h), the biohydrogenation of C18:2 *n*-6 in the fermentation vessels decreased ($P<0.001$) with the supplementation of all doses (100, 200 and 300 mg/L) of all substances, in a manner that is proportional to the dose level of the oils that were added (Tables 5.7a). The average biohydrogenation of C18:2 *n*-6 across corresponding doses of all oils at 12 h were 50.3, 46.7 and 39.8 g/100 g for 100, 200 and 300 mg/L, respectively, relative to the control (55.4 g/100 g). At 24 h, the values were 78.8, 74.5 and 67.7 g/100 g, for 100, 200 and 300 mg/L, respectively, relative to the control (82.7 g/100 g). These indicate that biohydrogenation was lowest in the presence of the highest dose (300 mg/L) but was significantly though marginally inhibited at the 100 mg/L of all oils. The interaction between oil and dose was significant at 12 h but not at 24 h. Relative to the control, the biohydrogenation of 18:2 *n*-6 was lowest in CSO 300 mg/L (Table 5.7a, b).

The biohydrogenation of C18:3 *n*-3 in fermentation vessels decreased ($P<0.001$) by the supplementation of all doses (100, 200 and 300 mg/L) of all oils in a dose dependent manner, compared to the control (Tables 5.7a). However, the inhibition of the biohydrogenation of C18:3 *n*-3 was marginally significant at 100 mg/L of all substances. The pattern of effects of oils and dose levels on the biohydrogenation of C18:3 *n*-3 was the same at both 12 and 24 h time point (i.e. both oil, dose and the interaction of dose and oils were significant at 12 and 24 h). At 12 h the mean biohydrogenation of C18:3 *n*-3 in cultures with all doses of ANO (100, 200 and 300 mg/L) was lowest compared to the respective biohydrogenation levels in corresponding doses of other substances relative to the control. Mean values were 67.9 (mean of all controls); 63.5, 65.0, 58.6 and 63.5 (100 mg/L); 59.0, 63.1.8, 52.7 and 56.6 (200 mg/L) and for 300 mg/L were 52.5, 56.3, 46.8 and 44.8 g/100 g (sed= 2.05, $P<0.001$, Table 5.7a, b), for ALA, ANE, ANO and CSO, respectively. The values for 24 h were 87.2 (mean across all controls); 83.8, 84.6, 84.3 and 84.1 (100 mg/L); 77.0, 80.4, 77.6 and 82.0 (200 mg/L) and for 300 mg/L were 69.4, 73.4, 70.9 and 75.6 g/100 g (sed= 1.07, $P<0.001$,Table 5.7a, b), for ALA, ANE, ANO and CSO, respectively.

At both 12 and 24 h, the biohydrogenation of C20:5 *n*-3 and C22:6 *n*-3 in fermentation vessels decreased ($P<0.001$) as the level (100, 200 and 300 mg/L) of all substances increased (Tables 5.7a). However, on the average, the minimum level of all substances necessary to cause substantial inhibition of the biohydrogenation of both C20:5 *n*-3 and C22:6 *n*-3 was 200 mg/L. The rate of biohydrogenation of C20:5 *n*-3 and C22:6 *n*-3 was not different between the corresponding levels of all substances (i.e. no significant interaction between oils and dose, Table 5.7b).

Table 5.3a Effects of whole EOs and EOCs on the concentration of selected C14/C16 fatty acids (g/100 g TFA) in cultures at 12 and 24 h *in vitro* incubation

		EOs and EOCs																	
		4-Allylanisole (mg/L)				**Anethole (mg/L)**				**Anise (mg/L)**				**Cassia (mg/L)**				**S.E.D**	
Fatty acid	Time (h)	**0**	**100**	**200**	**300**	**0**	**100**	**200**	**300**	**0**	**100**	**200**	**300**	**0**	**100**	**200**	**300**	O or D	O × D
C14:0	12	5.00	5.01	4.00	5.00	5.01	5.00	5.01	5.01	5.00	5.01	5.00	5.00	5.01	4.01	4.01	4.01	0.000	0.100
	24	5.00	5.01	5.01	5.02	5.01	5.01	5.02	5.00	5.00	5.01	5.02	5.00	5.00	5.00	5.00	5.01	0.100	0.200
C16:0	12	15.50	15.30	15.00	14.90	15.70	15.60	15.40	15.10	15.70	15.20	15.20	15.10	15.60	15.00	14.90	14.90	0.090	0.180
	24	15.80	15.60	15.40	14.90	15.70	17.70	15.60	15.00	15.90	15.50	15.30	15.30	15.80	15.90	15.40	15.00	0.260	0.510
C16:1	12	3.10	3.20	3.20	3.20	3.30	3.20	3.30	3.30	3.20	3.40	3.40	3.30	3.10	3.10	3.20	3.10	0.030	0.050
	24	2.60	2.80	2.80	3.10	2.50	2.90	2.70	2.90	2.60	2.80	2.80	2.80	2.70	2.70	2.60	2.50	0.060	0.130

O or D= Oil or dose; O × D = Oil by dose interaction. NB. S.E.D for oil and dose were similar that is why oil or dose is used in the table

Table 5.3b Effects of whole EOs and EOCs on concentration of selected C14/C16 fatty acids (g/100 g TFA) in cultures at 12 and 24 h *in vitro* incubation (Significance of main effects and interactions)

Fatty acid	**Time (h)**	**Oil**	**Dose**	**Oil × Dose**
C14:0	12	<0.001	=0.017	<0.001
	24	NS	NS	NS
C16:0	12	=0.004	<0.001	NS
	24	=0.028	NS	<0.001
C16:1	12	<0.001	=0.028	=0.004
	24	<0.001	<0.001	=0.011

NS= Not significant (P<0.05); O × D = Oil by dose interaction

Table 5.4a Effects of whole EOs and EOCs on the concentration of selected C18 fatty acids (g/100 g TFA) in cultures at 12 and 24 h *in vitro* incubation

Fatty acids	Time (h)	EOs and EOCs																S.E.D	
		4-Allylanisole (mg/L)				Anethole (mg/L)				Anise (mg/L)				Cassia (mg/L)					
		0	100	200	300	0	100	200	300	0	100	200	300	0	100	200	300	O or D	O × D
C18:0	12	12.1	12.6	13.2	13.0	12.0	13.1	13.1	12.8	12.2	11.4	12.4	13.0	12.1	12.3	12.6	12.7	0.14	0.27
	24	12.8	12.9	12.9	12.3	12.9	13.2	13.8	13.0	12.8	13.1	13.6	13.7	12.7	12.6	12.3	12.8	0.23	0.47
C18:1 *n*-9	12	7.7	7.8	7.7	7.9	7.7	7.9	7.6	7.6	7.5	7.3	7.5	7.5	7.9	7.8	7.9	8.0	0.06	0.12
	24	5.3	6.4	6.9	7.2	5.3	6.5	6.9	6.9	5.4	6.4	6.6	6.1	5.2	6.6	6.8	7.5	0.25	0.50
C18:1 *trans* 11	12	1.8	1.7	1.7	1.8	1.9	1.7	1.8	1.8	1.7	1.7	1.7	1.8	1.7	1.9	1.9	2.1	0.02	0.04
	24	1.2	1.3	1.3	1.3	1.3	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.1	1.4	1.4	1.5	0.17	0.34
18:2 *cis*-9 *tr*11 CLA	12	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.010	0.020
	24	0.15	0.15	0.16	0.18	0.16	0.14	0.15	0.16	0.15	0.16	0.18	0.19	0.14	0.14	0.15	0.18	0.010	0.010
C18:2 *n*-6	12	4.3	4.8	4.9	5.3	4.2	4.9	4.9	5.3	4.4	4.9	5.3	5.4	4.3	4.7	5.1	5.8	0.05	0.10
	24	1.7	2.1	2.6	3.2	1.6	2.0	2.5	3.1	1.7	2.0	2.4	2.8	1.6	2.1	2.2	2.6	0.04	0.07
C18:3 *n*-3	12	5.2	6.0	6.4	7.4	5.3	6.1	6.5	7.5	5.2	6.4	7.2	7.7	5.1	5.8	6.7	8.3	0.08	0.15
	24	2.1	2.7	3.6	4.7	2.0	2.7	3.4	4.6	2.2	2.5	3.4	4.2	2.1	2.5	2.8	3.7	0.05	0.10

O or D= Oil or dose; O × D = Oil by dose interaction. NB. S.E.D for oil and dose were similar that is why oil or dose is used in the table

Table 5.4b Effects of whole EOs and EOCs on concentration of selected C18 fatty acids (g/100 g TFA) in cultures at 12 and 24 h *in vitro* incubation (Significance of main effects and interactions)

Fatty acid	Time (h)	Oil	Dose	Oil × Dose
C18:0	12	<0.001	<0.001	<0.001
	24	<0.001	<0.001	NS
C18:1 *n*-9	12	<0.001	NS	NS
	24	NS	<0.001	<0.001
C18:1 trans 11	12	<0.001	<0.001	<0.001
	24	NS	NS	NS
C18:2 *cis*-9 tr11 CLA	12	NS	NS	NS
	24	<0.001	<0.001	<0.001
C18:2 *n*-6	12	NS	<0.001	<0.001
	24	<0.001	<0.001	<0.001
C18:3 *n*-3	12	<0.001	<0.001	<0.001
	24	<0.001	<0.001	<0.001

NS= Not significant (P<0.05), O × D = Oil by dose interaction

Table 5.5a Effects of whole EOs and EOCs on concentration of selected long chain fatty acids (g/100 g total fatty acids) in cultures at 12 and 24 h *in vitro* incubation

		EOs and EOCs																	
		4-Allylanisole (mg/L)				Anethole (mg/L)				Anise (mg/L)				Cassia (mg/L)				S.E.D	
Fatty acids	**Time (h)**	**0**	**100**	**200**	**300**	**0**	**100**	**200**	**300**	**0**	**100**	**200**	**300**	**0**	**100**	**200**	**300**	O or D	O × D
C20:4 *n*-6	12	0.51	0.50	0.50	0.51	0.51	0.50	0.52	0.40	0.52	0.50	0.50	0.51	0.51	0.51	0.50	0.50	0.010	0.010
	24	0.51	0.41	0.42	0.42	0.52	0.41	0.40	0.30	0.52	0.50	0.53	0.51	0.52	0.52	0.40	0.51	0.020	0.040
C20:5 *n*-3	12	1.70	1.80	1.90	2.20	1.80	1.80	1.90	2.00	1.70	2.20	2.60	2.60	1.70	1.80	2.00	2.20	0.060	0.120
	24	0.60	0.70	0.90	1.30	0.50	0.70	0.90	1.20	0.70	0.70	1.00	1.20	0.60	0.80	0.90	1.20	0.020	0.040
C22:6 *n*-3	12	2.00	2.10	2.10	2.20	2.00	2.00	2.01	2.10	2.00	2.10	2.20	2.20	2.00	2.10	2.10	2.10	0.020	0.030
	24	1.70	1.80	1.90	2.00	1.60	1.70	1.80	1.90	1.70	1.80	1.90	1.90	1.70	1.80	1.80	1.80	0.040	0.080

O or D= Oil or dose; O × D = Oil by dose interaction. NB. S.E.D for oil and dose were similar that is why oil or dose is used in the table

Table 5.5b Effects of whole EOs and EOCs on concentration of selected long chain fatty acids (g/100 g total fatty acids) in cultures at 12 and 24 h *in vitro* incubation (Significance of main effects and interactions)

Fatty acid	Time (h)	Oil	Dose	Oil × Dose
C20:4 *n*-6	12	<0.001	<0.001	NS
	24	<0.001	<0.001	=0.023
C20:5 *n*-3	12	<0.001	<0.001	<0.001
	24	NS	<0.001	<0.001
C22:6 *n*-3	12	<0.001	<0.001	NS
	24	NS	<0.001	NS

NS= Not significant (P<0.05), O × D = Oil by dose interaction

Table 5.6a Effects of whole EOs and EOCs on the concentration of fatty acids (g/100 g TFA) and content of total fatty acids (mg/g) at 12 and 24 h *in vitro* incubation

		EOs and EOCs																S.E.D	
		4-Allylanisole				Anethole				Anise				Cassia					
Fatty Acid	Time (h)	0	100	200	300	0	100	200	300	0	100	200	300	0	100	200	300	O or D	O × D
RFA	12	32.0	30.9	31.3	29.5	31.6	30.3	30.7	30.0	32.1	31.8	29.9	29.4	31.1	31.9	30.5	28.7	0.34	0.68
	24	40.7	36.8	35.3	34.4	40.8	36.3	35.0	35.3	40.7	37.3	36.1	34.2	40.6	36.0	36.8	35.7	0.87	1.73
∑SFA	12	32.3	32.4	32.6	32.5	32.4	33.3	33.2	32.6	32.6	31.8	32.5	33.0	32.4	31.6	32.0	32.0	0.20	0.40
	24	33.3	33.1	33.0	32.0	33.2	33.6	34.1	32.8	33.5	33.3	33.6	33.7	33.2	33.0	32.2	32.4	0.54	1.07
∑SFA-18:0	12	20.3	19.8	19.4	19.5	20.2	20.2	20.0	19.8	20.3	20.3	20.2	20.0	20.4	19.3	19.4	19.3	0.09	0.17
	24	20.5	20.3	20.1	19.7	20.4	20.3	20.2	19.8	20.6	20.2	20.0	19.9	20.5	20.4	19.9	19.5	0.32	0.65
∑MUFA	12	12.7	12.8	12.5	12.7	12.7	12.8	12.6	12.6	12.8	12.5	12.6	12.4	12.6	12.8	13.0	13.2	0.08	0.16
	24	9.1	10.5	11.1	11.6	9.3	10.6	10.8	11.0	9.1	10.4	10.7	11.0	9.2	10.7	10.9	11.5	0.25	0.49
∑PUFA	12	13.9	15.6	15.9	17.8	13.8	15.5	16.0	17.7	13.9	16.3	18.1	18.6	14.0	15.0	16.7	19.0	0.32	0.64
	24	6.7	7.8	9.5	11.7	6.7	7.6	9.1	11.3	6.7	7.6	9.4	11.0	6.7	7.7	8.3	9.9	0.09	0.19
TFA (mg/g)	12	41.3	39.0	37.6	36.2	41.2	36.1	39.1	38.3	41.3	42.0	41.3	39.4	41.4	38.5	37.7	36.7	0.83	1.66
	24	39.7	39.1	37.8	37.9	39.7	40.1	38.2	38.7	39.8	40.9	41.8	39.8	39.6	41.6	40.3	40.6	1.23	2.46
***n*-6/*n*-3**	12	0.55	0.54	0.51	0.49	0.56	0.54	0.52	0.49	0.55	0.50	0.48	0.47	0.54	0.53	0.52	0.50	0.004	0.008
	24	0.50	0.49	0.47	0.45	0.50	0.49	0.47	0.45	0.51	0.51	0.48	0.45	0.50	0.50	0.49	0.47	0.007	0.014
P/S	12	0.43	0.48	0.49	0.55	0.43	0.47	0.48	0.54	0.44	0.52	0.56	0.56	0.42	0.47	0.52	0.59	0.006	0.013
	24	0.35	0.39	0.47	0.59	0.34	0.37	0.45	0.57	0.36	0.38	0.47	0.55	0.35	0.38	0.41	0.51	0.01	0.02

O or D= Oil or dose; O × D = Oil by dose interaction. NB. S.E.D for oil and dose were similar that is why oil or dose is used in the table. RFA= remaining fatty acids, SFA= saturated fatty acids, MUFA= monounsaturated fatty acids, PUFA= polyunsaturated fatty acids, TFA= total fatty acids, *n*-6/*n*-3= sum of *n*-6 divided by sum of *n*-3 fatty acids, P/S= sum of PUFA divided by sum of SFA.

Table 5.6b Effects of whole EOs and EOCs on the concentration of fatty acids (g/100 g TFA) and content of total fatty acids (mg/g) at 12 and 24 h *in vitro* incubation **(**Significance of main effects and interactions**)**

Fatty acid	Time (h)	Oil	Dose	Oil × Dose
Remaining fatty acids	12	NS	<0.001	NS
	24	<0.001	<0.001	<0.001
∑SFA	12	<0.001	NS	NS
	24	<0.001	=0.047	=0.007
∑SFA-18:0	12	<0.001	<0.001	NS
	24	=0.007	NS	<0.001
∑MUFA	12	<0.001	NS	NS
	24	=0.029	<0.001	<0.001
∑PUFA	12	<0.001	<0.001	<0.001
	24	<0.001	<0.001	<0.001
Total fatty acid (mg/g)	12	<0.001	<0.001	NS
	24	<0.001	=0.012	=0.003
***n*-6/*n*-3**	12	<0.001	<0.001	NS
	24	0.005	0.001	0.001
P/S	12	0.001	0.001	0.001
	24	NS	0.001	0.001

NS= Not significant (P<0.05); SFA= saturated fatty acids, MUFA= monounsaturated fatty acids, PUFA= polyunsaturated fatty acids, TFA= total fatty acids, *n*-6/*n*-3= sum of *n*-6 divided by sum of *n*-3 fatty acids, P/S= sum of PUFA divided by sum of SFA.

Table 5.7a The effects of EOs on the biohydrogenation (g/100 g) of PUFA in cultures at 12 and 24 h *in vitro* incubation

		EOs and EOCs																SED	
		4-Allylanisole				Anethole				Anise				Cassia					
Fatty acid	Time (h)	0	100	200	300	0	100	200	300	0	100	200	300	0	100	200	300	O or D	O × D
C18:2 *n*-6	12	55.4	50.7	48.9	42.9	55.3	50.7	49.3	42.3	55.4	50.2	45.8	41.3	55.2	49.6	42.7	32.8	1.01	2.01
	24	82.7	79.0	72.6	66.1	82.9	78.9	75.2	66.5	82.7	79.5	74.8	69.1	82.6	77.6	75.2	69.2	0.55	0.11
C18:3 *n*-3	12	67.9	63.5	59.0	52.5	67.8	65.0	63.1	56.3	67.9	58.6	52.7	46.8	67.9	63.5	56.6	44.8	1.02	2.05
	24	87.2	83.8	77.0	69.4	87.0	84.6	80.4	73.4	87.4	84.3	77.6	70.9	87.2	84.1	82.0	75.6	0.54	1.07
C20:5 *n*-3	12	59.4	57.1	47.5	30.1	59.5	60.8	57.9	47.4	59.3	45.6	36.3	33.4	59.4	46.5	30.2	16.6	2.25	4.51
	24	84.3	84.1	75.8	59.9	84.4	85.6	80.3	68.1	84.3	83.3	76.6	67.8	84.2	77.6	70.3	56.8	1.06	2.11
C22:3 *n*-6	12	23.3	22.9	15.7	8.5	23.3	25.2	23.5	10.2	23.2	19.1	15.2	5.7	23.3	10.8	6.9	6.8	1.67	3.34
	24	39.0	34.0	25.5	16.5	39.1	36.9	34.6	21.3	39.0	32.0	27.9	13.2	38.9	22.7	16.5	11.4	2.21	4.41

O or D= Oil or dose; O × D = Oil by dose interaction. NB. S.E.D for oil and dose were similar that is why oil or dose is used in the table

Table 5.7b The effects of EOs on the biohydrogenation (g/100 g) of PUFA in cultures at 12 and 24 h *in vitro* incubation (Significance of main effects and)

Fatty acid	Time (h)	Oil	Dose	Oil × Dose
C18:2 *n*-6	12	<0.001	<0.001	<0.001
	24	NS	<0.001	NS
C18:3 *n*-3	12	<0.001	<0.001	<0.001
	24	<0.001	<0.001	<0.001
C20:5 *n*-3	12	<0.001	<0.001	NS
	24	<0.001	<0.001	NS
C22:3 *n*-6	12	<0.001	<0.001	NS
	24	<0.001	<0.001	NS

NS= Not significant (P<0.05), O × D = Oil by dose interaction

5.4. Discussion

The aim of the present study was to examine the effects of graded doses of 4-Allylanisole, anethole, anise oil and cassia oil on the extent of rumen biohydrogenation of *n*-3 PUFA. The EOCs used in the current study were selected based on the 24 h batch culture results of the first experiment described in Chapter 3 which screened the effects of 15 individual essential oil's constituent compounds on the extent of rumen biohydrogenation of *n*-3 PUFA (Eburu and Chikunya, 2014). Similarly, the EOs used in the current study were selected based on the 24 h batch culture results of the second experiment described in Chapter 4 which screened the effects of 10 whole essential oils on the extent of rumen biohydrogenation of *n*-3 PUFA (Eburu and Chikunya, 2015a). In each of those experiments, selection of both EOCs and EOs was based on two ways: on one hand, the EOCs/EOs were ranked from 1 (highest) to 15 (lowest) based on the concentration (g/100 g TFA) of C18:3 *n*-3, C18:2 *n*-6, C20:5 *n*-3, C22:6 *n*-3, C18:2 *cis-9 trans*-11 CLA and C18:1*trans* 11 in their cultures, on another hand, EOCs/EOs were ranked from 1 (least inhibitory) to 15 (most inhibitory) on VFA concentration. After considering their effects on fatty acid and volatile fatty acid concentrations, the two overall best EOCs and two best EOs were selected and investigated in the current study to establish optimum doses for them.

5.4.1. In *vitro* fermentation parameters

In this study, it was generally observed that the supplementation of all doses of EOCs/EOs progressively reduced substantially the amount of total gas. Regardless of oil, the 300 mg/L (highest dose) of all substances induced the most reduction (average 22%) on total gas. At 200 mg/L, all treatments caused marginal inhibition of total gas (average 6%), except for CSO which inhibited by 15% at this level of inclusion. There was a significant interaction between dose levels and EOs/EOCs but only at the highest level (300 mg/L) at 12 h. This interaction showed that the supplementation of CSO 300 mg/L induced the highest inhibition of total gas relative to the 300 mg/L of the rest of the treatments (ALA, ANE and ANO). However, after 24 h the interaction between dose levels and EOs/EOCs was not significant. The dose dependent effects of the substances on gas production are consistent with previous studies such as those of Talebzadeh *et al.* (2012), where the supplementation of different doses (150, 300, 450 and microgram/ml) of oil of *Zataria multiflora*, which is rich in thymol and carvacrol (phenolic compounds)

and those of Gunal *et al*., (2013), where Siberian fir needle oil (125, 250 and 500 mg/L) reduced total gas production according to concentration of EOs.

Overall, under our experimental conditions, the minimum concentration of EOCs/EOs that caused inhibition of rumen fermentation activities (gas production, concentrations of NH_3-N, VFA and PUFA) ranged from 100 mg/L to 300 mg/L for all the substances used (ALA, ANE, ANO and CSO). It is useful to point out that the uniqueness of substances in this study is that all the whole oils (anise and cassia) contained phenolic compounds as the most active components and all the essential oil compounds (4-allyanisole and anethole) are also phenolic compounds (Table 3.3; 4.2). Anethole is the main active compound in anise oil (*P. anisum*) (Soher *et al*., 2014; Davidson and Naidu, 2000, Table 4.2); cinnamaldehyde is the predominant constituent of cassia oil (EP 5; Kalemba *et al*., 2012; Table 4.2) and 4-allylanisole is the most active component of basil oil (Naidu 2000; Holley *et al*., 2005). Anethole, cinnamaldehyde and 4-allylanisole are phenylpropanoids (see Table 3.3). Hence, all the substances contained phenolic moieties in their chemical structures. The antimicrobial effects and mechanism of action of EO is determined by the chemical structure of its constituent compounds (Dorman and Deans, 2000). Hence, these phenolic compounds (in the current study) generally require about the same concentration to elicit a response. This characteristic (phenolic chemical structure) which is common to all the substances in the current study could be speculated as the reason for the lack of marked difference among them in terms of their effect on gas production after 24 h. The range of minimum concentrations of EOCs/EOs in this study (100-300 mg/L) is within the established minimum inhibitory concentration (MIC) necessary to cause inhibition of both mixtures of microorganisms and single bacterial species by other phenolic compounds or essential oils rich in phenolic compounds of this type. The MIC of carvacrol was 22.5-500 mg/L against *Escherichia coli* (Cosentino *et al*., 1999); 17.5-45 mg/L against *Staphylococcus aureus* (Lambert *et al*., 2001) and 37.5-500 mg/L against *Listeria monocytogenes* (Cosentino *et al*., 1999; Pol and Smid, 1999). The MIC for thymol was 22.5-45 mg/L against *E.coli* (Cosentino *et al*., 1999), 45.0 mg/L against *L. monocytogenes* (although these are not rumen bacteria) (Cosentino *et al*., 1999) and 14.0-22.5 mg/L against *Staph. aureus* (Cosentino *et al*., 1999; Lambert *et al*., 2001). The MIC for clove oil (whose main active compound is eugenol, a phenolic compound) against

E.coli ranges from 40-250 mg/L (Smith-Palmer *et al*., 1998) and against *L.monocytogenes* was 20 mg/L (Smith-Palmer *et al*., 1998). The MIC for thyme oil (rich in thymol and carvacrol) against *L.monocytogenes* was 30 mg/L (Smith-Palmer *et al*., 1998; Burt 2004) and against *E.coli* ranges from 45-125 mg/L (Smith-Palmer *et al*., 1998; Burt 2004). The broadest antimicrobial activities of the phenolic compounds is linked with the possession of hydroxyl group, whose acidic characters are speculated to inhibit both the attachment of bacteria to insoluble fractions of feed and the digestion of the soluble components of feeds (McAllister *et al*., 1994; Aharoni *et al*., 1998).

The concentration of NH_3-N in fermentation vessels was reduced in CSO (at 100, 200 and 300), ANE (at 100 and 200 mg/L) and ALA and ANO (300 mg/L). These results are consistent with the observations of Busquet *et al*. (2006) who reported that cassia/cinnamon oil and its main constituent compound (cinnamaldehyde) reduced ammonia concentration. However, Busquet *et al*. (2006) did not observe reduction in NH_3-N with the supplementation of ANE and ANO (at 30 or 300 mg/L). The difference in the observation between our results and those of Busquet *et al*. (2006) could be due to the variations in the environmental conditions, plant chemotype or in the preparation of the anise oil. The results when 300 mg/L of all substances was administered are also in agreement with the reports in Chapter 3 (where the levels of NH_3-N in vessels were significantly decreased with the addition of ALA and ANE), and in Chapter 4 (where the addition of CSO reduced the content of NH_3-N). These findings suggest that the inclusion of ALA and ANO (300 mg/L); ANE (at 100 and 200 mg/L) and CSO (at 100, 200 and 300) could potentially inhibit the activities of hyper ammonia producing bacteria or proteolytic bacteria (which are responsible for extensive deamination of amino acid, Mclnotch et al., 2003; Bach *et al*., 2005; section 1.7.6). It has been observed that about 75-85% of dietary N consumed by dairy cows is excreted in faeces and urine as waste (Tamminga, 1992). Therefore, in terms of practical implications of the results of the current study, it can be conceived that supplementing the diet of dairy cows with CSO (either at 100, 200 or 300 mg/L), ANE (at 100 mg/L) and ALA and ANO (at 300 mg/L) could possibly reduce the production of NH_3-N and increase ruminal escape of dietary protein, consequently improving the efficiency of protein use in the rumen (Van Nevel and Demeyer, 1988). There was a significant interaction between dose levels and EOs/EOCs, which suggest that the use of CSO 100 mg/L would

reduce more amount of ammonia than the supplementation of ANE 100 mg/L. Reduction of NH_3-N with the supplementation of ANE (at 100 mg/L) has practical benefits as its use would potentially improve the efficiency of protein in the rumen without adversely affecting VFA concentration (see VFA concentration at 100 mg/L).

Total VFA was decreased with the inclusion of all the EOCs and EOs used in this experiment, with the highest reductions being observed in vessels with CSO and ALA. All doses of all EOs and EOCs maintained a dose related response effects on TVFA with the exception of ANE, with cultures receiving the highest dose (300 mg/L) of all substances irrespective of oil inducing the highest inhibition (average 12%) on TVFA. The rate of VFA inhibition which clearly depends on doses of EOs is consistent with the reports of other studies who investigated dose related (0, 3, 30, 300 and 3000 mg/L) effects of cassia oil, anethole and anise oil on VFA concentration (Bustquet *et al*., 2006; Macheboeuf *et al*., 2008; Gunal *et al*., 2013). Furthermore, these results are in agreement with those of Gunal *et al*. (2013), where different doses of citronella oil (125, 250 and 500 mg/L) reduced TVFA; Pandu *et al*. (2014), where the addition of thyme oil (125, 250 and 500 mg/l); and Agarwal *et al*. (2009), where 1.0 and 2.0 ml/L of peppermint oil caused a dose related depression of feed digestibility and TVFA concentration. The average inhibition of TVFA with the inclusion of 300 mg/L of all substances in the current study also agree with our previous reports where the addition of 300 mg/L of EOCs (Chapter 3) and EOs (Chapter 4) caused over 10% reduction of TVFA. There was a significant interaction at 200 mg/L, which indicated that ANE, ALA and ANO only marginally reduced TVFA (by about 2%) relative to the control, but CSO reduced TVFA by 10%. Therefore, it could be suggested in this study that whilst the optimum dose for ALA, ANE and ANO could be 200 mg/L (because at this dose no substantial reduction on TVFA was observed), CSO requires less than 200 mg/L to maintain similar effect on concentration of TVFA. However, if the diets of ruminant must be supplemented with CSO 200 mg/L (due to other benefits associated with this dose), equivalent of 4.8 g/day/sheep, then ways of preventing reduction in feed intake, such as encapsulation must be developed. This findings support earlier observation that addition of EOCs/EOs may decrease (at high doses) or have no effect (at low doses) on VFA concentration (Patra and Saxena, 2010). In the current study, the effects of EOs/EOCs on the concentration of TVFA at different doses were consistent

with the reported effects on cumulative gas production. This suggests that the way a substance affects the digestion of feed (decrease/increase) directly impacts on the concentration of TVFA. Reduced digestion in feed which is reflected on decreased production of gas can be accompanied by less production of hydrogen, CH_4 and volatile fatty acids plus a lower A/P ratio (Boggs *et al*., 1987).

The molar proportion of acetate was reduced in CSO (at 100, 200 and 300 mg/L); ALA and ANO (at 300 mg/L); and in ANE (100 mg/L), and the reduction in cultures with CSO progressed as the dose of CSO decreased. There was a significant interaction at all doses which suggest that the supplementation of CSO (at 100, 200 and 300 mg/L) would possibly increase the molar proportion of propionate more than the addition of the remaining treatments. Consistent with the reduction on the molar concentration of acetate in cultures with CSO (at all doses), the A/P ratio was significantly reduced in vessels where all doses of CSO were added. As anaerobic fermentation of proteins and carbohydrates with higher propionate levels results in reduced acetate and methanogenesis (Demeyer and Van Nevel, 1995), results of this study suggest that the inclusion of CSO (100, 200 and 300 mg/L) would modify microbial population towards decreased methanogenesis. These observations are in support of previous reports with ALA and ANE (Chapter 3) and ANO and CSO (Chapter 4). The fact that ANE (at 100 mg/L) reduced the molar proportions of acetate and propionate casts doubt on its practical application as feed additive in dairy farming. Reduction in propionate and acetate, which are the main precursors in ruminant for glucose and fat synthesis, respectively, is detrimental. In terms of practical application, it implies that anethole may not be nutritionally beneficial to dairy cattle, except if it is used in conjunction with another compound. This observation with ANE is consistent with the results of Bustquet *et al*. (2006) who reported that anethole decreased both acetate and propionate. The BCVFA are derived from the ruminal catabolism of amino acid (Mackie and White, 1990). The reduced concentrations of BCVFA in vessels with all doses of CSO coincide with the decrease in NH_3-N concentration in this study.

5.4.2. Effect of EOs/EOCs on fatty acid metabolism

In this study, as evidence of biohydrogenation of PUFA in the rumen, the profiles of fatty acids of incubated diet were considerably different from those observed in the digesta. The levels of C14 were not affected by the addition of substances except ALA (at 200 mg/L) and CSO (at 100, 200 and 300 mg/L)

which reduced it at 12 h. Regardless of the substance, the lowest levels of C16:0 was observed in vessels that received the 300 mg/L of both EOCs and EOs at 12 h. Myristic (C14:0) and palmitic (C16:0) acids are saturated fatty acids having the capacity to increase the plasma cholesterol levels by suppressing and saturating low-density lipoprotein (LDL) receptors (Keys *et al*., 1995). This implies that reduced concentration of these fatty acids with the inclusion of ALA (at 200 mg/L) and CSO (at 100, 200 and 300 mg/L) would be beneficial in terms of the possibility of reducing the level of plasma cholesterol levels.

The levels of C18:0 (the end-product of the biohydrogenation of C18:1 *n*-9, C18:3 *n*-3 and C18:2 *n*-6) were reduced in ANO (at 100 mg/L), ALA (at 300 mg/L) and CSO (at 200 mg/L) but not in the corresponding doses of the other treatments. The significant interaction at 100 mg/L suggests that the low levels of C18:0 in ANO 100 mg/L could probably be emanating from reduce conversion of C18:1 *n*-9. This speculation is due to the corresponding low concentration of C18:1 *n*-9 in ANO 100 mg/L and the absent of reduction of C18:0 in the higher levels of ANO (200 and 300 mg/L). Overall, the addition of ANO reduced the level of C18:1 *n*-9 relative to the control. The concentration of C18:1 *trans* 11 at 12 h was increased with the addition of CSO 300 mg/L and maintained in vessels where 300 mg/L of the rest of the treatments were added. There seems to be a link in relation to the levels of C18:0, C18:1 *n*-9 and C18:1 *trans* 11 in vessels. There appears to be tendency that the higher the levels of C18:0 the lower would be the concentration of C18:1 *n*-9, and low content of C18:0 tended to be accompanied by high concentrations of C18:1 *trans* 11. The higher levels of C18:0 and the associated lower concentrations of C18:1 *n*-9 would suggest that the high stearic acid might be emanating from the conversion of the oleic acid during ruminal transformation of fatty acids. It has been observed that about 70% of C18:1 *n*-9 in rumen culture was converted to C18:0, and only 30% was transformed to ketostearic acid and hydroxystearic acid (Jenkins *et al*., 2006). The lower concentration of C18:0 which corresponds to high content of C18:1 *trans* 11 probably implies that the activity of *Clostridium proteoclasticum* (Kemp *et al*., 1975; Maia *et al*., 2007), renamed (Moon *et al*., 2008) as *Butyrivibrio proteoclasticus*, the bacteria which converts VA to C18:0, was inhibited in those cultures, hence the observed increased levels of C18:1 *trans* 11. It is important to mention here that evaluation of microbial species composition, which this study unfortunately did not perform, would have been able to conclusively establish whether there is a

relationship between the inhibition of these key biohydrogenation bacteria and the concentration of these intermediates. These findings are in agreement with results from our preliminary studies (Chapters 3 and 4). As C18:0 is a saturated fatty acid but without any harmful effects on human health (Grundy, 1994; Pariza, 2004), its high accumulation would not suggest any negative effects provided the levels of PUFA in the corresponding vessels are higher.

The concentration of C18:2 *cis*-9 *trans* 11 CLA was maintained at higher levels with the inclusion of all substances at all doses. The highest dose (300 mg/L) of all substances recorded the highest level of C18:2 *cis*-9 *trans* 11 CLA, relative to the control. The higher levels of C18:2 *cis*-9 *trans* 11 CLA were associated with lower content of C18:1 *trans* 11. This relationship between C18:2 *cis*-9 *trans* 11 CLA and C18:1 *trans* 11 is consistent with our previous results (described in Chapters 3 and 4). Results of this study suggest that inclusion of ALA (at 200 mg/L), ANE and CSO (at 300 mg/L) and ANO (at 100, 200 and 300 mg/L) would increase the level of C18:2 *trans* 11 CLA and potentially increase its health benefits (Banni *et al.*, 2001; Corl *et al.*, 2003). Although both ALA 200 mg/L and ANO 200 mg/L increased the concentration of C18:2 *cis*-9 *trans* 11 CLA, the significant interaction indicated that supplementation of ANO 200 mg/L would result in higher levels of C18:2 *cis*-9 *trans* 11 CLA than ALA 200 mg/L. Similarly, the concentration of C18:2 *cis*-9 *trans* 11 CLA in ANO 300 mg/L would potentially be higher than the levels in ANE and CSO 300 mg/L.

It was observed in this study that increasing the doses of both EOCs and EOs resulted to a proportional increase in the concentrations of C18:2 *n*-6 and C18:3 *n*-3, relative to the control. The highest concentrations of C18:2 *n*-6 and C18:3 *n*-3 which were observed in vessels with the highest dose (300 mg/L) of all substances were in the same range as the observed effects of these substances in our previous experiments (Chapter 3; Chapter 4). The minimum dose of all the oils required to maintain a higher concentrations of C18:3 *n*-3 relative to the control was 200 mg/L .The concentration of C18:3 *n*-3 at 200 mg/L increased in response to treatments relative to the control in the sequence: 43.2% (ANO 200 mg/L) > 35.1% (ANE 200 mg/L), 35.1% (ALA 200 mg/L) > 29.7% (CSO 200 mg/L). This significant interaction indicates that supplementation of ANO 200 mg/L would potentially cause higher concentration of C18:3 *n*-3 than the addition of either ALA or ANE 200 mg/L. Comparison between the

anethole and anise oil suggest that anise oil and its predominant active compound (anethole) had similar effects on rumen concentration of C18:3 *n*-3. This observation is consistent with previous studies where it is established that anethole is the main compound in anise oil (Davidson and Naidu, 2000). However, the slightly higher concentration of C18:3 *n*-3 in ANO 200 mg/L than ANE 200 mg/L suggests that other minor components of anise oil had insignificant but synergistic effects on ruminal fermentation activities.

The biohydrogenation of C18:3 *n*-3 which approached 90 g/100 g (87.2 g/100 g) at 24 h was comparable to the biohydrogenation of C18:3 *n*-3 in other studies where different protection methods were used such as *in vitro* (Beam *et al*., 2000; Sinclair *et al*., 2005) and *in vivo* (Wachira *et al*., 2000; Scollan *et al*., 2001, Wang *et al*., 2002) studies. The content of C18:2 *n*-6 and C18:3 *n*-3 rapidly decreased with time, being highest at 12 h and lowest at 24 h, in the control, EOCs and EOs. Relative to the control, the average concentration of C18:2 *n*-6 and C18:3 *n*-3 in cultures with the inclusion of 100 mg/L of all substances were significant but negligible. However, the average effects of treatments at 12 and 24 h indicated that the biohydrogenation of C18:2 *n*-6 and C18:3 *n*-3 were lowest in vessels with ANO 200 followed by ALA 200 than 200 mg/L of other substances relative to the control. At 24 h, biohydrogenation data indicated that the disappearance of C18:3 *n*-3 from ruminal content was reduced as follows: 23.0%, 19.6%, 25.2% and 18.0% for ALA, ANE, ANO and CSO, respectively. At 300 mg/L, the average protection was approximately 10% higher than that of 200 mg/L. These values are comparable to other protection techniques described in Chapter 2 such as where about 44.3 g/100 g and 30. 7 g/100 g of C18:3 *n* -3 were protected from biohydrogenation when whole linseed was pre-treated with NaOH and formic acid respectively, prior to treatment with formaldehyde. The fact that EOs and EOCs are extracts of plants which are part of animal diets and their consideration as generally recognized as safe (GRAS) food additives, offers the use of EOs to reduce ruminal PUFA disappearance advantage over other methods that use chemicals. However, data from this study disagree with the results from the study of Gunal *et al*. (2013) who reported that different doses (125, 250 and 500 mg/L) of six essential oils (citronella, rosemary, clove, white thyme, sage and Siberian fir needle oils) did not affect the concentration of C18:3 *n*-3 or C18:2 *n*-6 in batch culture study. It has been established that the antimicrobial effects and mechanism of action of EO is determined by the chemical structure of its constituent compounds

(Dorman and Deans, 2000), and could be responsible for the observed difference between our results and those of Gunal *et al*. (2013).

The extent of disappearance of C20:5 *n*-3 and C22:6 *n*-3 in vessels increased as the time of incubation advanced. After 24 h, the quantity of the content of C20:5 *n*-3 in vessel which disappeared was highest in the control (84.3%) and supports previous reports from *in vivo* studies where the biohydrogenation of C20:5 *n*-3 and C22:6 *n*-3 from fish oil range from 72 to 93 g/100 g TFA (Wachira *et al*., 2000; Scollan *et al*., 2001; Chikunya *et al*., 2004), and contrast *in vitro* reports where the biohydrogenation of C20:5 *n*-3 and C22:6 *n*-3 was less than 50 g/100 g (Ashes *et al*., 1992; Sinclair *et al*., 2005). The concentrations of C20:5 *n*-3 and C22:6 *n*-3 responded proportionally to the dose of both EOs and EOCs, with the highest levels of C20:5 *n*-3 and C22:6 *n*-3 observed in vessels where 300 mg/L of substances were added. However, at 100 mg/L, the levels of C20:5 *n*-3 and C22:6 *n*-3 were insignificant, relative to the control. The average (12 and 24 h) effects of substances on the concentration of C20:5 *n*-3 indicated that it was highest in vessels with all doses of ANO compared with the corresponding doses of all other additive type and the control. These significant interactions were consistent with the biohydrogenation data and suggest that supplementation of ANO (at 100, 200 and 300 mg/L) slightly had a higher potency to reduce the biohydrogenation of C20:5 *n*-3 and C22:6 *n*-3 irrespective of dose, than other substances. As mentioned previously (see section 1.7.8), it is worthy to mention that the biohydrogenation pathways of C20:5 *n*-3 and C22:6 *n*-3 are not yet fully established like that of C18:2 *n*-6 and C18:3 *n*-3.

The addition of graded doses of EOs and EOCs sequentially increased the concentrations of ∑PUFA and P/S ratio and progressively decreased the concentrations of ∑*n*-6/∑*n*-3 and ∑SFA-C18:0, relative to the control. At 200 mg/L all doses of all substances had the P/S ratio above the 0.45 recommended in the guidelines of the Department of Health (1994) except CSO which had 0.41.

It is important to emphasise that the possible reason why the effect of substances used in this study on fermentation parameters and PUFA concentration or biohydrogenation are not too distinct compared to the results of the first (Chapter 3) and second (Chapter 4) experiment is that all EOCs and EOs in the current study are rich in phenolic compounds. This group of compounds have been observed to elicit the

highest and the broadest antimicrobial potency. However, the interactions as discussed above suggest that at a particular dose, different phenolic compounds may elicit a slightly different response. If 200 mg/L is considered the optimum dose for majority of the substances in this study, it means about 4.8 g/sheep/day of the substances are required to feed a sheep producing an average of 24 litres of rumen fluid per day.

5.5. Conclusion

Although at 100 mg/L all substances did not affect the concentration of TVFA, effect on gas production, concentration and biohydrogenation of PUFA was significant but marginal, relative to the control. At 200 mg/L all substances substantially maintained higher concentrations of PUFA, CLA; and ANE, ALA and ANO only marginally reduced TVFA (by about 2%) relative to the control, but CSO reduced TVFA by 10%. At 300 mg/L the addition of all substances almost tripled the concentration of residual PUFA observed in cultures but this was accompanied by over 12% inhibition of the concentration of TVFA. Therefore, considering the effects of the tested EOs and EOCs and at different doses in this study, it appears that whilst administration of ALA, ANE and ANO at 200 mg/L seems to maintain higher PUFA levels and minimal disruption to VFA concentration *in vitro*, CSO requires a dose less than 200 mg/L to attain the same balance. However, the significant interactions at 200 mg/L with respect to CLA and PUFA concentration and biohydrogenation suggests that supplementing ANO (at 200 mg/L) is potentially more effective than the other three in both preventing PUFA disappearance and maintenance of optimal VFA concentration. It is worthwhile to investigate whether these effects also occur *in vivo* including testing the possibility of microbial adaptation to these substances. Therefore, the next study (Chapter 6) investigates the possibility of rumen adaptation to 100 mg/L of ANO for four weeks.

CHAPTER 6

Effects of four weeks of rumen adaptation to anise oil on biohydrogenation of *n*-3 polyunsaturated fatty acids

Abstract

This study evaluates effect of four weeks of microbial adaptation to anise oil (ANO) on the fermentation activities of ruminal micro-organisms and the biohydrogenation of *n*-3 polyunsaturated fatty acids (PUFA). In this experiment, six Hartline × Texel cross lambs were used. Three of the lambs were randomly assigned to the basal diet (basal diet group, BDG) and the remaining three lambs were offered basal diet with ANO (anise oil group, AOG) for four weeks. The rumen fluid donor sheep were offered water and hay *ad libitum* and supplemented with additional 400 g/sheep/day of concentrate plus 2.4 g/sheep/day of ANO (for the AOG). The ANO was thoroughly mixed with the concentrate prior to feeding. The total amount (400 g) of lamb finisher cubes offered per sheep/day was divided into two equal parts (200 g) and fed at 08.00 hours and 16.00 hours. After the four weeks adaptation period, lambs were slaughtered and ruminal fluid was collected from each of the lambs on BDG and on AOG and used in a 48 h *in vitro* batch culture system to study the fermentation of a 70: 30 grass hay (*Lolium perenne)* and concentrate (lamb finisher) diet . The study was a 2 (batches of rumen fluid: BDG and AOG) × 2 (doses of ANO: 0 and 200 mg/L) factorial design experiment. Total volatile fatty acid (TVFA) in RF extracted from lambs on the BDG (242.0 mM) was similar to that in the AOG (242.2 mM). Concentrations of TVFA in *in vitro* digests were similar between groups on the BDG (68.6 mM) and on the AOG (66.4 mM), but differed between levels of anise oil (0 versus 200 mg/L). The levels of NH_3-N were not different ($P<0.05$) between the RF collected from AOG and BDG. However, *in vitro* results showed that AOG 200 mg/L induced a 20% decrease on the concentration of NH_3-N in fermentation vessels, relative to BDG 0 mg/L. The *in vitro* digesta incubated in RF from the AOG (i.e. AOG 200 mg/L) maintained higher concentrations of PUFA (C18:2 *n*-6 and *n*-3 PUFA), *trans* vaccenic acid and lower concentration of stearic acid.This suggest that there was no rumen adaptation within the period of trial. These results indicate that anise oil is potentially a useful feed additive to optimise the fatty acid composition of ruminant food products, if these effects are repeated in meat and milk.

6.1. Introduction

In the preceding experiments (Chapters 3, 4 and 5), it has been established that certain EOCs (e.g. anethole and 4-allylanisole) and whole EOs (e.g. anise oil) when added at 200 mg/L can significantly suppress biohydrogenation of PUFA without detrimental effects to fermentation and VFA concentrations. However, it is not clear whether these effects are sustained in the medium and long term. Available evidence from continuous culture studies and long term *in vivo* trials suggest that the benefits associated with the use of essential oil as feed additives may decline due to two possibilities: (1) adaptation of individual microbial species to the use of EOs or, (2) shifts in microbial populations following long-term use of essential oil (Gladine *et al.*, 2007). In addition, only few studies have been conducted *in vivo* to investigate the possibility of microbial adaptation to essential oils in the rumen as a measure of their potential effectiveness as feed additives. Previous studies with the use of specific blend of essential oil (BEO) compounds consisting mainly of limonene, guaiacol and thymol observed that ruminal N metabolism was modified by supplementing with BEO (Mclntosh *et al*., 2003; Molero *et al*., 2004; Newbold *et al*., 2004). About four weeks period of microbial adaptation was suggested as the minimum time required to modify N metabolism in the rumen by some of these studies (Wallace *et al*., 2003; Molero *et al*., 2004; Castillejos *et al.*, 2007). However, to date, there is no similar report about the adaptation period of microbes to EOs and their corresponding effects on fatty acid metabolism in the rumen. The aim of the present study was to examine effects of four weeks period of microbial adaptation to anise oil on metabolism of PUFA and biohydrogenation end-product. Anise oil (200 mg/L) was identified as the most effective substance to both prevent PUFA disappearance and maintain optimal VFA concentration in the preceding Chapter (Chapter 5; Eburu and Chikunya (2015b)).

6.2.0. Materials and methods

6.2.1. Animal management and collection of rumen fluid/sampling

In this experiment, six Hartline × Texel cross lambs (mean weight 42.0 ±2.5kg) were used as rumen fluid donors. Details of animal housing, experimental diets and feeding are as described in section 2.1 of general material and methods. Briefly, the rumen fluid donor lambs were offered water and hay *ad libitum* and supplemented with additional 400 g/sheep/day of concentrate. The total amount (400 g) of lamb finisher cubes offered per sheep/day was divided into two equal parts (200 g) and fed at 08.00 hours and 16.00 hours. Three lambs were randomly assigned to the basal diet (basal diet group, BDG) and the remaining three lambs were fed basal diet with ANO (2.4 g/sheep/day ANO, AOG) for four weeks. The 2.4 g/day/sheep of anise oil was thoroughly mixed with the lamb finisher cubes prior to feeding. The dose (2.4 g/sheep/day) was estimated based on an estimated rumen volume of about 10 litres (as rumen volume equals body weight, Owens and Goetsch, 1988) and a dilution rate of about 100 mg/h. The total amount of fluid estimated to flow through the rumen per 24 h/day was 24 litres which equals about 100 mg/L (a dose which was observed to maintain the level of TVFA similar to the control in the previous experiment) (Chapter 5; Eburu and Chikunya, 2015b). See section 2.1 of the general materials and methods for the collection of rumen fluid and preparation for *in vitro* incubation.

6.2.2. Basal feedstock, treatments and *in vitro* incubation

The basal feedstock for *in vitro* incubation was mixtures of good quality rye-grass hay (*Lolium perenne*), lamb finisher concentrate, whole ground linseed and fish oil. Details of the basal feedstock used in this *in vitro* incubation are as described in the general material and methods (see section 2.3). The ingredients, chemical and fatty acid composition of the diet are shown in Table 2.2.

The effect of anise oil was evaluated using the *in vitro* gas production batch culture method described by Theodorou *et al*. (1994). Anise oil was purchased from Sigma-Aldrich Co. Ltd., UK and was stored at the required temperatures specified on delivery notes prior to use. See Tables 4.1 and 4.2 for a description of the ANO used in this study.

The effect of four weeks adaptation of rumen microbes to ANO was examined *in vitro*, using rumen fluid extracted from both basal diet group (BDG) and anise oil group (AOG) in a 2 (batches of rumen fluid:

BDG and AOG) × 2 (doses of ANO: 0 and 200 mg/L) factorial design experiment with six replicates as follows: (1) fluid from BDG plus 0 mg/l of anise (BDG 0 mg/l); (2) fluid from BDG plus a single dose of 200 mg/l of anise oil (BDG 200 mg/l); (3) fluid from AOG plus 0 mg/l of anise oil (AOG 0 mg/l); and (4) fluid from AOG plus a single dose of 200 mg/l of anise (AOG 200 mg/l).

In total 100 serum bottles were incubated, each bottle contained 1 g of feed substrate, 200 or 0 mg/l of anise oil, 80 ml anaerobic buffer (see Table 2.1) and 20 ml inoculum and the bottle sealed with rubber cork before incubation.

6.2.3. Sample collection and preservation

Cumulative gas pressure measurements, incubation stopping times, collection and storage of samples in this study were as described in the general material and methods (see section 2.4).

6.2.4 Chemical analysis

The concentration of NH_3-N in digesta was analysed using the phenol-hypochlorite method (Weatherburn, 1967; Broderick and Kang, 1980) adopted for use on the plate reader as described in general materials and methods (see section 2.5.4).

The concentration of volatile fatty acid (VFA) was determined by Gas chromatography (GC) as described by Ottenstein and Bartley (1971). Detail of this method in general materials and methods (see section 2.5.6).

The concentration of fatty acids in feed and freeze dried samples were extracted by direct saponification method described by Enser *et al*. (1998). See section 2.5.7 of the general materials and methods for detail.

6.2.5. Experimental design and statistical analysis

The study (RCBD) was a 2 (batches of rumen fluid: BDG and AOG) × 2 (doses of ANO: 0 and 200 mg/L) factorial design experiment. Therefore data were analysed by TWO-WAY analysis of variance (ANOVA) with batches of rumen fluid and dose of anise as the main effects using GenStat 16th edition. Differences between treatments were declared by least significance difference (LSD) and significance was declared at $P< 0.05$. Data were analysed separately for each time point (12, 24 and 48 h).

6.3. Results

6.3.1. Fermentation parameters

The effects of four weeks of microbial adaptation to anise oil on animal performance and concentrations of total volatile fatty acids (TVFA, mM), molar proportion of individual VFA (mM/mol TVFA) and NH_3-N (mM) *in vivo* are shown in Table 6.1. The dry matter intake was significantly higher in sheep that received anise oil compared to the control. Mean values were 18.5 and 18.9 kg/week (SEM= 0.05, P<0.05, Table 6.1), for the control and the anise oil group of sheep, respectively. The initial and final body weight gain were not different (P=0.05) between the two groups of lambs (Table 6.1).

The concentrations of NH_3-N, TVFA and molar proportions of individual VFA were not affected by treatments. However, the acetate to propionate ratio was lower in the AOG than the BDG. Mean values were 3.4 and 3.1 (SEM= 0.008, P=0.05, Table 6.1), for the BDG and the AOG, respectively.

The effects of treatments on cumulative gas production (ml/g OM), NH_3-N (mM), TVFA (mM) and molar proportion of individual VFA (mM/mol TVFA) *in vitro* are presented in Table 6.2.

In this experiment the fluid adapted with anise oil (AOG) and the unadapted fluid (BDG) maintained similar levels of total gas production. The total amounts of gas in vessels were maintained in a dose-response pattern, such that the higher dose of anise oil (200 mg/L), irrespective of source of innoculum, produced lower total gas relative to the control (BDG 0). Mean values were 96.2, 87.2, 96.0 and 90.4 ml/g OM (12 h, sed=, 3.08, P<0.028); 133.2, 121.6, 131.5 and 123.7 ml/g OM (24 h, sed= 2.93, P<0.004); and for 48 h were 164.8, 144.3, 159.4 and 145.4 ml/g OM (sed= 2.88, P<0.001, Table 6.2), for BDG 0, BDG 200, AOG 0 and AOG 200, respectively.

The concentration of NH_3-N in the fermentation vessels at all time points was not significantly different between the BDG and AOG. At 12 and 48 h, the vessel concentration of NH_3-N was not affected by dose of anise. However, at 24 h, the concentration of NH_3-N in vessels was maintained in a dose-response pattern, with the higher dose of anise oil (200 mg/L), regardless of fluid, containing lower ammonia relative to BDG 0 (Table 6.2). Mean values were 5.0, 4.7, 5.3 and 4.0 mM (sed= 0.29, P<0.017), for BDG 0, BDG 200, AOG 0 and AOG 200, respectively. This indicates that AOG 200 mg/L caused a 20% reduction on the ruminal concentration of ammonia.

At 24 and 48 h, the fermentation vessel concentration of TVFA was similar between BDG and AOG (Table 6.2). There was a dose dependent response effect on the levels of TVFA at 48 h but not at 24 h. At 48 h, irrespective of fluid type, the higher dose of anise (200 mg/L) reduced ($P<0.001$) the concentration of TVFA relative to the control (mean inhibition 8%). Mean values were 24 h (48 h in brackets) 70.2 (81.5), 67.0 (74.6), 67.3 (80.0) and 65.5 (75.0) mM for BDG 0, BDG 200, AOG 0 and AOG 200, respectively.

The molar proportion of acetate was significantly higher ($P<0.001$) in cultures with the AOG than the BDG. Mean values (average of doses within a fluid type) were 24 h (48 h in brackets) 61.3 (61.1) and 63.2 (62.6) mM/mol TVFA for the BDG and AOG, respectively. Although there was no observed difference between doses of anise on the proportion of acetate at 24 h, levels were different at 48 h, such that the 200 mg/L of both BDG and AOG maintained higher levels of acetate than the 0 mg/L.

The proportion of propionate was significantly lower ($P<0.001$) in cultures with the AOG compared to the BDG. Mean values (mean of doses within a fluid type) for 24 h were 20.0 and 18.1 and for 48 h were 20.2 and 18.7 mM/mol TVFA (Table 6.2), for the BDG and AOG, respectively. The proportion of propionate was not affected by the dose of anise (irrespective of fluid type) at 24 h, but levels were different at 48 h, with the 200 mg/L causing the higher reduction in propionate compared to the lower dose (0 mg/L).

The molar proportion of butyrate was not affected by treatment except at 24 h where the AOG reduced ($P<0.001$) it compared to the BDG (mean values 8.5 and 8.0 mM/mol TVFA for the BDG and AOG, respectively). The acetate to propionate ratios were maintained at higher levels in the AOG relative to the BDG at both 24 and 48 h.

Treatment effects caused minor changes to the concentrations of branched chain volatile fatty acids (BCVFA) such as isovaleric, valeric and isobutyric acids (Table 6.2).

Table 6.1 Effects of four weeks of rumen adaptation to anise oil on animal performance and concentrations of total volatile fatty acids (mM), molar proportion of individual VFA (mM/mol TVFA) and NH_3-N (mM) *in vivo*

	Treatments		SEM	Significance
	Basal diet group	**Anise oil group**		
Animal performance				
Intake (kg/week)	18.50	18.90	0.05	<0.001
Initial body weight (kg)	42.01	42.02	0.01	NS
Final body weight (kg)	44.02	44.03	0.01	NS
Volatile fatty acid concentration				
TVFA	242.00	242.2	18.6	NS
Acetate	68.30	65.40	2.57	NS
Propionate	20.00	21.6	2.49	NS
Butyrate	7.70	8.40	0.47	NS
Isobutyric	0.88	0.88	0.00	NS
Isovaleric	0.67	0.73	0.00	NS
Valeric	0.56	0.53	0.00	NS
A/P ratio	3.40	3.10	0.008	=0.05
Ammonia concentration				
Ammonia	3.2	2.6	0.61	NS

NS= Not significant at P<0.05. Note: Because of the small sample size (n=3) for the parameters measured in this table, differences between mean was calculated using the t-Test.

Table 6.2 Effects of four weeks of rumen adaptation to anise oil on total gas (ml/g OM), ammonia concentration (mM), total VFA (mM) and molar proportion of individual VFA (mM/mol TVFA) in cultures during 48 h *in vitro* incubation

		Treatments									
		Basal diet group		**Anise oil group**		**S.E.D**			**Significance**		
Parameter	Time	**0 mg/l**	**200**	**0 mg/l**	**200**	**Fluid**	**Dose**	**F × D**	**Fluid**	**Dose**	**F × D**
Gas	12	96.2	87.2	96.0	90.4	3.08	3.08	4.36	NS	=0.028	NS
	24	133.2	121.6	131.5	123.7	2.93	2.93	4.14	NS	=0.004	NS
	48	164.8	144.3	159.4	145.4	2.88	2.88	4.08	NS	<0.001	NS
NH_3-N	12	4.0	3.9	4.0	3.6	0.33	0.33	0.47	NS	NS	NS
	24	5.0	4.7	5.3	4.0	0.29	0.29	0.41	NS	=0.017	NS
	48	6.0	5.9	6.0	4.8	0.33	0.33	0.47	NS	NS	NS
TVFA	24	70.2	67.0	67.3	65.5	1.69	1.9	2.39	NS	NS	NS
	48	81.5	74.6	80.0	75.0	2.10	2.10	2.97	NS	<0.001	NS
Acetate	24	60.9	61.6	63.0	63.4	0.41	0.41	0.58	<0.001	NS	NS
	48	60.1	62.1	62.0	63.2	0.37	0.37	0.52	<0.001	<0.001	NS
Propionate	24	20.4	19.6	18.4	17.7	0.44	0.44	0.62	<0.001	NS	NS
	48	21.7	18.6	20.0	17.3	0.39	0.39	0.55	<0.001	<0.001	NS
Butyrate	24	8.6	8.4	7.9	8.0	0.19	0.19	0.27	=0.014	NS	NS
	48	8.0	7.7	7.9	7.9	0.16	0.16	0.23	NS	NS	NS
Isobutyric	24	0.87	0.83	0.87	0.82	0.013	0.013	0.019	NS	=0.003	NS
	48	0.96	0.96	0.98	0.94	0.023	0.023	0.032	NS	NS	NS
Isovaleric	24	1.12	1.07	0.98	0.88	0.027	0.027	0.038	<0.001	=0.011	NS
	48	1.31	1.43	1.21	1.04	0.049	0.049	0.070	<0.001	NS	=0.006
Valeric	24	0.98	0.97	1.00	0.99	0.011	0.011	0.015	NS	NS	NS
	48	1.05	1.19	1.19	1.31	0.022	0.022	0.031	<0.001	<0.001	NS
A/P ratio	24	3.0	3.1	3.4	3.6	0.09	0.09	0.13	<0.001	NS	NS
	48	2.8	3.3	3.1	3.7	0.09	0.09	0.12	<0.001	<0.001	NS

S.E.D= Standard error of difference; F × D = fluid by dose interaction, NS= Not significant at P<0.05

6.3.2. Effect of EOC on fatty acid metabolism

The effects of treatments on the concentration of selected fatty acids (g/100 g TFA) are presented in Table 6.3.

The levels of C14:0 were not affected by treatments (Table 6.3). The g/100 g concentration of C16:0 was not different between the BDG and AOG, but the 200 mg/L of anise (regardless of fluid) reduced the levels of C16:0 relative to the control at both 12 and 48 h time points. Mean values were 12 h (48 h in brackets) 15.0 (17.0), 14.0 (15.0), 15.0 (16.0) and 14.0 (15.0) g/100 g TFA for BDG 0, BDG 200, AOG 0 and AOG 200, respectively.

The level of C16:1 in the AOG at 12 h was higher ($P<0.001$) than the BDG. Mean values were 7.1 and 7.6 g/100 g TFA (sed= 0.02, $P<0.001$, Table 6.3), for the BDG and AOG, respectively. At the dose level, there was no difference ($P<0.05$), irrespective of fluid, between the levels of anise added. There was a significant interaction which indicates that addition of 0 and 200 mg/L of anise to the fluid adapted with anise oil (i.e. AOG 0 mg/L and AOG 200 mg/L) significantly increased the concentration of C16:1 compared with the respective 0 and 200 mg/L of the unadapted fluid (BDG) at 12 h. Mean values were 3.5, 3.6, 3.8 and 3.8 g/100 g TFA (sed= 0.03, $P<0.016$, Table 6.3), for BDG 0, BDG 200, AOG 0 and AOG 200, respectively.

The concentrations of C18:0 were reduced ($P<0.001$) in cultures with the AOG relative to the BDG (Table 6.3). Mean values were 14.2 and 11.2 g/100 g TFA (12 h, sed= 0.15, $P<0.001$), 15.5 and 11.9 g/100 g TFA (24 h, sed= 0.21, $P<0.001$) and for 48 h were 16.8 and 12.9 g/100 g TFA (sed= 0.45, $P<0.001$), for BDG and AOG, respectively. The 200 mg/L regardless of the type of fluid (BDG or AOG) maintained higher levels of C18:0 than the 0 mg/L of all fluids.

The concentrations of C18:1 *n*-9 in cultures were not different ($P<0.05$) between the BDG and AOG (Table 6.3). At the dose level, the 200 mg/L of all fluid type maintained higher levels of C18:1 *n*-9 than the 0 mg/L. Mean values were 7.9, 8.0, 7.7 and 8.2 (12 h); 6.3, 7.3, 6.1 and 7.8 (24 h) and for 48 h were 4.5, 5.6, 3.1 and 5.4 g/100 g TFA for BDG 0, BDG 200, AOG 0 and AOG 200, respectively.

The concentrations of C18:1 *trans* 11 at 12 and 24 h were significantly higher in cultures with the AOG than the BDG. Mean values were 3.6 and 3.8 (12 h, sed= 0.02, P<0.001, Table 6.3) and 24 h were 2.9 and 3.4 g/100 g TFA (sed=0.05, P<0.001, Table 6.3), for the BDG and AOG, respectively. There was no difference in the dose of anise oil at all time points except at 48 h where the AOG 200 mg/L had higher level of 18:1 *trans* 11 than the rest of the treatments. Average values were 1.4, 1.4, 1.4 and 1.6 g/100 g TFA BDG 0, BDG 200, AOG 0 and AOG 200, respectively.

The concentrations of C18:2 *cis*-9 *trans* 11CLA in cultures with BDG and AOG were similar (Table 6.3). The addition of different doses of anise oil (0 and 200 mg/L) had no effect on the levels of C18:2 *trans* 11CLA at all time of incubation except at 12 h, where the levels of C18:2 *trans* 11CLA in cultures were maintained in a linear pattern (dose-response). Mean values were 0.25, 0.26, 0.27 and 0.28 g/100 g TFA BDG 0, BDG 200, AOG 0 and AOG 200, respectively.

At both 12 and 24 h period, the unadapted fluid (BDG) and adapted fluid (AOG) maintained a similar level of C18:2 *n*-6 and C18:3 *n*-3 (Table 6.3). In contrast, the concentrations of C18:2 *n*-6 and C18:3 *n*-3 at 48 h were higher in vessels with AOG relative to BDG. Mean values were 5.1 and 6.8 (C18:2 *n*-6, sed=0.44, P<0.049) and for C18:3 *n*-3 were 5.7 and 7.5 g/100 g TFA (sed=0.36, P<0.022), for BDG and AOG, respectively. The 200 mg/L of all fluids maintained higher levels of C18:2 *n*-6 and C18:3 *n*-3 compared to the control. There was a significant interaction at 24 h time point, which showed that the concentrations of C18:2 *n*-6 and C18:3 *n*-3 in vessels where AOG 200 were added were higher than their corresponding values in BDG 200 (Table 6.3). Mean values were 2.7, 4.1, 2.6 and 4.9 g/100 g TFA (18:2 *n*-6, sed= 0.29, P<0.035) and for C18:3 *n*-3 were 3.4, 5.7, 3.3 and 7.0 g/100 g TFA (sed= 0.45, P<0.031), for BDG 0, BDG 200, AOG 0 and AOG 200, respectively.

At 12 h the concentration of C20:4 *n*-6 was higher (P<0.025) in cultures with the AOG compared to the BDG (Table 6.3). By contrast, the levels of C20:4 *n*-6 at 24 and 48 h were lower in vessels with AOG than BDG. Mean values were 1.0 and 1.1 (12 h), 1.2 and 1.0 (24 h) and 48 h were 1.2 (1.0) g/100 g TFA for BDG 0, BDG 200, AOG 0 and AOG 200 mg/L, respectively.

At 12 and 24 h time points, fluid from sheep adapted with anise oil maintained higher levels of C20:5 *n*-3 and C22:6 *n*-3 than the unadapted fluid (Table 6.3). Mean values were 12 h (24 h in brackets) 6.6 (4.0) and 7.0 (5.4) for C20:5 *n*-3 and for C22:6 *n*-3 were 5.0 (4.0) and 5.3 (4.6) g/100 g TFA for BDG and AOG, respectively. All treatments maintained a dose related effect on the levels of C20:5 *n*-3 and C22:6 *n*-3, such that the 200 mg/L of anise (the higher dose) contained higher amounts of these fatty acids compared to the control. Except at 48 h, there were no significant interactions between fluid and dose of anise. The concentration of C22:6 *n*-6 at 48 h was maintained higher ($P<0.049$) in cultures where AOG 200 mg/L was added than other treatments. Mean values were 1.9, 1.7, 1.8 and 2.1 g/100 g TFA (sed=0.18, $P<0.049$, Table 6.3), for BDG 0, BDG 200, AOG 0 and AOG 200 mg/L, respectively.

The content of ∑SFA was consistently lower in AOG compared to BDG at all time points (12, 24 and 48 h). Mean values were 52.9 and 50.8 (12 h), 56.8 and 52.8 (24 h) and for 48 h were 59.0 and 54.9 g/100 g TFA (Table 6.4), for BDG and AOG, respectively. Effects of treatments on levels of total SFA was not dependent on the dose of anise but levels are generally lower in the adapted fuid.

At 12 h, the fermentation vessel concentration of ∑MUFA was higher in the AOG than in vessels with BDG (Table 6.4). Mean values were 26.6 and 27.3 g/100 g TFA (sed= 0.06, $P<0.001$, Table 6.4), for BDG and AOG, respectively. There was significant interaction at 12 h period of incubation, which showed that supplementing cultures with AOG 200 mg/L contained higher concentration of ∑MUFA compared to the addition of BDG 200 mg/L. Mean values were 13.3, 13.3, 13.4 and 13.9 g/100 g TFA (sed= 0.08, $P<0.006$, Table 6.4), for BDG 0, BDG 200, AOG 0 and AOG 200 mg/L, respectively. However, at 24 and 48 h, the concentration of ∑MUFA in vessels was similar in the two fluids. The levels of ∑MUFA in cultures were maintained in a dose response pattern, with the higher dose of anise oil (200 mg/L) containing higher ∑MUFA than 0 mg/L.

The vessel contents of ∑PUFA were higher in the AOG than the BDG at 12 h. Mean values were 41.3 and 42.5 g/100 g TFA (sed= 0.17, $P<0.002$, Table 6.4), for BDG and AOG, respectively. However, at 24 and 48 h, the concentrations of ∑PUFA in vessels were similar between the two fluids. The levels of ∑PUFA in cultures increased as the dose of anise increased. Except at 24 h, there were no significant

interactions between fluid and dose on the content of PUFA. The addition of AOG 200 mg at 24 h had the highest content of ∑PUFA compared to the control. Mean values were 12.8, 15.0, 11.1 and 18.1 g/100 g TFA (sed= 1.43, P<0.025, Table 6.4), for BDG 0, BDG 200, AOG 0 and AOG 200 mg/L, respectively.

The fermentation vessel contents of total fatty acid (TFA) at 12 and 24 h in unadapted fluid (BDG) and adapted fluid (AOG) were not different. However, at 48 h the levels of TFA in AOG exceeded (P<0.001) the concentration in BDG (mean values were 64.4 and 75.9 mg/g for BDG and AOG respectively). Total fatty acid content (mg/g) in all fluids was not affected by the dose of anise.

The ratio of *n*-6/*n*-3 in cultures at all time points (12, 24 and 48 h) was consistently higher in the BDG than the AOG (Table 6.4). The vessel content of *n*-6/*n*-3 in cultures was higher in BDG 0 mg/L compared to the AOG 0 mg/L and this was true for BDG 200 mg/L relative to AOG 200 mg/L. Average concentrations were 0.46, 0.44, 0.44 and 0.44 (12 h, sed=0.003, P<0.001); 0.45, 0.46, 0.41 and 0.44 (24 h, sed=0.007, P<0.032), and for 48 h were 0.50, 0.46, 0.44 and 0.47 (sed=0.013, P<0.001), for BDG 0, BDG 200, AOG 0 and AOG 200 mg/L, respectively.

Although the average biohydrogenation of C18:2 *n*-6 and C18:3 *n*-3 in BDG and AOG were not different at 48 h, levels of disappearance after 12 and 24 h in vessels with AOG were lower than in BDG (Table 6.5). Mean values for C18:2 *n*-6 were 12 h (24 h in brackets) 43.7 (67.3) and 39.1 (60.8) and for C18:3 *n*-3 were 52.9 (74.3) and 48.1 (68.3) g/100 g, for BDG and AOG, respectively. The biohydrogenation of C18:2 *n*-6 and C18:3 *n*-3 in vessels with the higher dose of anise (200 mg/L) were maintained at lower levels compared to the levels of biohydrogenation in cultures with BDG 0 mg/L. There was a significant interactions at both 12 and 24 h such that the biohydrogenation of C18:2 *n*-6 and C18:3 *n*-3 increased significantly in response to treatment in the sequence: values for C18:2 *n*-6 were BDG 0 mg/L (50.5 and 74.1) > AOG 0 mg/L (48.0 and 73.3) > BDG 200 mg/L (36.9 and 60.4) > AOG 200 mg/L (30.1 and 48.3 g/100 g), for 12 (sed=1.26, P<0.05) and 24 h (sed=2.79, P<0.010, Table 6.5), respectively . Similar values for C18:3 *n*-3 were BDG 0 mg/L (60.9 and 80.5) > AOG 0 mg/L (58.4 and 80.4) > BDG 200 mg/L (44.8 and 68.0) > AOG 200 mg/L (37.8 and 56.2 g/100 g), for 12 (sed=1.37, P<0.032) and 24 h (sed=2.51, P<0.004, Table 6.5), respectively.

At 12 and 24 h, the mean biohydrogenation (mean across all doses within a fluid type) of 20:5 *n*-3 and 22:6 *n*-3 were lower in AOG relative to levels in BDG (Table 6.5). Mean values for 20:5 *n*-3 were 12 h (24 h in brackets) 27.4 (57.1) and 18.1 (37.2) and for 22:6 *n*-3 were 16.5 (34.4) and 5.9 (17.1) g/100 g for BDG and AOG, respectively. The biohydrogenation of 20:5 *n*-3 and 22:6 *n*-3 decreased as the dose of anise oil increased in fermentation vessels, such that the highest dose (200 mg/L) of all fluids maintained lower levels of disappearance compared to the control. Mean values for 20:5 *n*-3 were 31.8, 23.0, 24.7 and 11.5 g/100 g (12 h, sed=0.90, $P<0.001$); and 65.8, 48.4, 48.7, 25.7 g/100 g (24 h, sed= 3.22, $P<0.001$, Table 6.5), for BDG 0, BDG 200, AOG 0 and AOG 200 mg/L, respectively. The corresponding means for 22:6 *n*-3 were 19.7, 13.2, 7.9 and 3.9 g/100 g (12 h, sed= 1.46, $P<0.002$); and 41.2, 27.6, 22.8 and 11.4 g/100 g (24 h, sed= 2.68, $P<0.001$, Table 6.5), for BDG 0, BDG 200, AOG 0 and AOG 200 mg/L, respectively.

Table 6.3 Effects of four weeks of rumen adaptation to anise oil on concentration of selected fatty acids (g/100 g total fatty acids) in cultures during 48 h *in vitro* incubation

		Treatments									
		Basal diet group		Anise oil group		S.E.D			Significance		
Fatty acid	Time	0 mg/l	200	0 mg/l	200	Fluid	Dose	F × D	Fluid	Dose	F × D
C14:0	12	5.0	5.0	5.0	5.0	0.01	0.01	0.11	NS	NS	NS
	24	5.0	5.0	5.0	5.0	0.10	0.10	0.10	NS	NS	NS
	48	5.0	5.0	5.0	5.0	0.10	0.10	0.10	NS	NS	NS
C16:0	12	15.0	14.0	15.0	14.0	0.10	0.10	0.10	NS	<0.001	NS
	24	15.0	15.0	15.0	15.0	0.20	0.20	0.30	NS	NS	NS
	48	17.0	15.0	16.0	15.0	0.30	0.30	0.40	NS	=0.004	NS
C16:1	12	3.6	3.6	3.8	3.8	0.02	0.02	0.03	<0.001	NS	=0.016
	24	3.3	3.3	3.5	3.6	0.07	0.07	0.10	0.003	NS	NS
	48	3.0	3.2	3.2	3.3	0.09	0.09	0.13	NS	NS	NS
C18:0	12	6.9	7.3	5.3	5.9	0.15	0.15	0.21	<0.001	=0.002	NS
	24	7.4	8.1	5.3	6.6	0.21	0.21	0.30	<0.001	<0.001	NS
	48	8.0	8.8	5.8	7.1	0.45	0.45	0.64	<0.001	=0.029	NS
C18:1 *n*-9	12	7.9	8.0	7.7	8.2	0.07	0.07	0.09	NS	<0.001	NS
	24	6.3	7.3	6.1	7.8	0.32	0.32	0.45	NS	<0.001	NS
	48	4.5	5.6	3.1	5.4	0.74	0.74	1.04	NS	=0.029	NS
C18:1 trans 11	12	1.8	1.8	1.9	1.9	0.02	0.01	0.02	<0.001	NS	NS
	24	1.4	1.5	1.7	1.7	0.05	0.05	0.07	<0.001	NS	NS
	48	1.4	1.4	1.4	1.6	0.06	0.06	0.08	NS	=0.033	NS
18:2 tr 11CLA	12	0.25	0.28	0.26	0.27	0.008	0.008	0.011	NS	=0.034	NS
	24	0.17	0.18	0.17	0.17	0.013	0.013	0.019	NS	NS	NS
	48	0.15	0.16	0.16	0.18	0.016	0.016	0.023	NS	NS	NS
C18:2 *n*-6	12	5.1	6.5	5.1	6.6	0.06	0.06	0.08	NS	<0.001	NS
	24	2.7	4.1	2.6	4.9	0.20	0.20	0.29	NS	<0.001	=0.035
	48	2.3	2.8	2.3	4.0	0.44	0.44	0.63	=0.049	=0.006	NS
C18:3 *n*-3	12	6.8	9.7	7.0	9.9	0.10	0.10	0.15	NS	<0.001	NS
	24	3.4	5.7	3.3	7.0	0.32	0.32	0.45	NS	<0.001	=0.031
	48	2.1	3.6	3.0	4.5	0.36	0.36	0.50	=0.022	<0.001	NS
C20:4 *n*-6	12	0.5	0.5	0.6	0.5	0.01	0.01	0.01	=0.025	NS	NS
	24	0.6	0.6	0.5	0.5	0.02	0.02	0.02	=0.030	NS	NS
	48	0.6	0.6	0.5	0.5	0.01	0.01	0.01	<0.001	NS	NS
C20:5 *n*-3	12	3.1	3.5	3.3	3.7	0.03	0.03	0.03	<0.001	<0.001	NS
	24	1.6	2.4	2.3	3.1	0.15	0.15	0.21	<0.001	<0.001	NS
	48	1.2	1.6	1.2	2.2	0.27	0.27	0.38	NS	=0.016	NS
C22:6 *n*-3	12	2.4	2.6	2.6	2.7	0.03	0.03	0.04	<0.001	<0.001	NS
	24	1.8	2.2	2.2	2.4	0.07	0.07	0.10	<0.001	<0.001	NS
	48	1.6	1.7	1.8	2.1	0.13	0.13	0.18	NS	NS	=0.049

S.E.D= Standard error of difference; F × D = fluid by dose interaction, NS= Not significant at P<0.05

Table 6.4 Assessing the effects of rumen adaptation to anise oil on the concentration of sum of fatty acids (g/100 g TFA) and content of total fatty acids (mg/g) on animals treated with Basal or Anise oil diets for the four weeks prior to slaughter. Rumen Fluid was subsequently incubated *in vitro* for 48 hours, (reading taken at 12, 24 and 48 hours).

		Treatments				S.E.D			Significance		
		Basal diet group		Anise oil group							
Fatty acid	Time	0 mg/l	200	0 mg/l	200	Fluid	Dose	F × D	Fluid	Dose	F × D
	12	32.4	31.0	32.1	29.8	0.20	0.20	0.28	<0.001	<0.001	=0.039
RFA	24	36.2	33.9	38.1	32.2	0.97	0.97	1.37	NS	<0.001	NS
	48	37.6	34.5	42.4	37.9	1.24	1.24	1.75	=0.003	=0.006	NS
	12	26.7	26.2	25.6	25.2	0.18	0.18	0.25	<0.001	=0.020	NS
∑SFA	24	28.1	28.7	26.0	26.8	0.24	0.24	0.35	<0.001	=0.011	NS
	48	29.7	29.3	27.1	27.8	0.64	0.64	0.90	=0.005	NS	NS
	12	13.3	13.3	13.4	13.9	0.06	0.06	0.08	<0.001	<0.001	=0.006
∑MUFA	24	11.0	12.1	11.3	13.1	0.37	0.37	0.52	NS	<0.001	NS
	48	8.9	10.3	7.7	10.3	0.85	0.85	1.20	NS	=0.031	NS
	12	18.2	23.1	18.8	23.7	0.17	0.17	0.24	=0.002	<0.001	NS
∑PUFA	24	12.8	15.0	11.1	18.1	1.01	1.01	1.43	NS	<0.001	=0.025
	48	7.4	10.5	8.9	14.8	1.58	1.58	2.24	NS	=0.010	NS
	12	34.7	36.8	37.1	35.5	0.79	0.79	1.11	NS	NS	=0.029
TFA	24	35.1	33.9	35.9	36.5	0.93	0.93	1.32	NS	NS	NS
	48	33.8	30.6	39.2	36.7	1.48	1.48	2.10	<0.001	NS	NS
	12	0.46	0.44	0.44	0.44	0.002	0.002	0.003	<0.001	=0.002	<0.001
***n*-6/*n*-3**	24	0.45	0.46	0.41	0.44	0.005	0.005	0.007	<0.001	=0.002	=0.032
	48	0.50	0.46	0.44	0.47	0.009	0.009	0.013	=0.028	NS	<0.001
	12	0.68	0.88	0.73	0.94	0.010	0.010	0.015	<0.001	<0.001	NS
P/S	24	0.46	0.52	0.43	0.68	0.035	0.035	0.050	=0.092	<0.001	=0.017
	48	0.27	0.38	0.30	0.53	0.071	0.071	0.100	NS	=0.028	NS

S.E.D= Standard error of difference; F × D = fluid by dose interaction, RFA= remaining fatty acids, ∑SFA= sum of saturated fatty acids, ∑MUFA= sum of monounsaturated fatty acids, ∑PUFA= sum of polyunsaturated fatty acids, TFA= total fatty acids, P/S= ∑PUFA/∑SFA, *n*-6/*n*-3= sum of *n*-6 divided by sum of *n*-3 fatty acids, NS= Not significant at P<0.05

Table 6.5 Assessing the effects of rumen adaptation to anise oil on biohydrogenation of PUFA (g/100 g TFA) when animals were treated with basal or Anise oil diets for four weeks prior to slaughter. Rumen fluid was subsequently incubated *in vitro* for 48 hours, (reading were taken at 12, 24 and 48 hours).

		Treatments									
		Basal diet group		**Anise oil group**		**S.E.D**			**Significance**		
Fatty acid	Time	**0 mg/l**	**200**	**0 mg/l**	**200**	**Fluid**	**Dose**	**F × D**	**Fluid**	**Dose**	**F × D**
	12	50.5	36.9	48.0	30.1	0.89	0.89	1.26	<0.001	<0.001	=0.053
C18:2 *n*-6	24	74.1	60.4	73.3	48.3	1.97	1.97	2.79	=0.004	<0.001	=0.010
	48	82.8	71.0	77.4	61.4	4.51	4.51	6.38	NS	=0.006	NS
	12	60.9	44.8	58.4	37.8	0.97	0.97	1.37	<0.001	<0.001	=0.032
C18:3 *n*-3	24	80.5	68.0	80.4	56.2	1.78	1.78	2.51	=0.003	<0.001	=0.004
	48	87.7	77.0	83.1	74.4	2.18	2.18	3.08	NS	<0.001	NS
	12	31.8	23.0	24.7	11.5	0.90	0.90	1.28	<0.001	<0.001	NS
C20:5 *n*-3	24	65.8	48.4	48.7	25.7	3.22	3.22	4.56	<0.001	<0.001	NS
	48	73.7	61.1	73.2	53.0	6.00	6.00	8.48	NS	=0.013	NS
	12	19.7	13.2	7.9	3.9	1.46	1.46	2.06	<0.001	=0.002	NS
C22:6 *n*-3	24	41.2	27.6	22.8	11.4	2.68	2.68	3.79	<0.001	<0.001	NS
	48	45.5	31.1	36.1	35.3	4.15	4.15	5.87	NS	NS	NS

S.E.D= Standard error of difference; F × D = fluid by dose interaction, NS= Not significant at P<0.05

6.4.0. Discussion

The aim of the current study was to examine the possibility of rumen adaptation to anise oil within a feeding period of four weeks. Anise oil (200 mg/L) was identified in the previous study (Chapter 5) as the most effective essential oil in terms of both preventing PUFA disappearance and maintenance of optimal VFA production. In this study, six Hartline × Texel cross lambs were used. Three of the lambs were randomly assigned to the control treatment (without anise oil, BDG) and the remaining three lambs were offered feed pre-treated with ANO (2.4 g/sheep/day anise oil, AOG) for four weeks. All the six lambs used in this study were in good health condition throughout the period of the trial. Dry matter intake was marginally higher (by 2%) in sheep that received anise oil (AOG) compared to the control (BDG). However, final body weight gains were not affected by treatments (BDG and AOG). Nevertheless, it could be speculated that the AOG that had higher feed intake but without higher weight gain may have excreted more nutrients than the BDG. By contrast, previous *in vivo* studies where higher doses: 500 mg/day of cinnamaldehyde (Busquet *et al*., 2003; Calsamiglia *et al*., 2007), and a mixture of eugenol (90 mg/day) plus 180 mg/day of cinnamaldehyde (Cardozo *et al*., 2006) were used observed negative effect on feed intake. The observed difference in feed intake between the present study and some previous studies (Busquet *et al*., 2003; Cardozo *et al*., 2006; Calsamiglia *et al*., 2007) could be due to the different doses or type of EOs/EOCs or the diet used.

6.4.1. Fermentation parameters

The current study observed that although the BDG and AOG maintained similar levels of total gas production, BDG 200 and AOG 200 caused 7.9% (average) inhibition of total gas relative to the control. This level of reduction of total gas in cultures supplemented with 200 mg/L from both sources of rumen fluid (AOG and BDG) in the current study is similar and consistent with the level of inhibition of gas production when 200 mg/L of anise oil was used in the previous study (see Chapter 5).

A number of studies have indicated that different blend of essential oil decreased proteolysis and amino acid deamination (McIntosh *et al*., 2003; Molero *et al*., 2004; Newbold *et al*., 2004; Castillejos *et al*., 2007). Majority of these studies proposed that about four weeks period of microbial adaptation to blend of essential oil was the minimum period of time necessary to observe the effects of mixtures of essential oil

on protein metabolism in the rumen (Wallace *et al*., 2003; Molero *et al*., 2004; Castillejos *et al*., 2007). In the current *in vivo* study, the concentrations of NH_3-N from the two fluids (BDG and AOG) were not affected by treatments. This observation is in agreement with Newbold et al. (2004) who reported that 110 mg/d of blend of EO (Crina® ruminants; Akzo Surface Chemistry Ltd., Herfordshire, UK) did not affect the concentration of NH_3-N measured *in vitro*. Furthermore, Castillejos *et al.* (2007) observed that the concentration of NH_3-N was not affected by supplementing 110 mg/L of a blend of essential oil (Crina® Ruminant; AKZO NOBEL/CRINA S.A., Gland, Switzerland). The constituents of the Crina® Ruminant were guaiacol, thymol and limonene. However, both studies (Newbold *et al*., 2004; Castillejos *et al.,* 2007) reported that 24% and 14% respectively, of ammonia N was decreased when measured *in vitro* from rumen fluid collected from sheep adapted with blend of essential oil for four weeks. In the current *in vitro* study, we observed that the addition of AOG 200 mg/L but not of BDG 200 mg/L induced a 16% reduction on NH_3-N concentration after 24 h. This observation suggests reduced deamination or decreased concentration of HAP (although not tested). Some studies have demonstrated that some (HAP) such as *Peptostreptococcus anaerobius* and *Clostridium sticklandii* were more sensitive than others such as *Clostridium aminophilum* to the Crina blend of essential oil (McIntosh *et al*., 2003). Contrary to the results obtained in the current study, previous results (described in Chapters 4 and 5) indicate that anise oil (300 and 200 mg/L) did not change the levels of NH_3-N in rumen contents. These results indicate the possibility that the accumulated residual effects of feeding anise oil to lambs (for four weeks) resulted to the decreased concentration of ammonia, suggesting that ruminal microbes need adaptation time of about four weeks in order to change the levels of NH_3-N in the rumen.

After four weeks period of microbial adaptation to anise oil, TVFA concentrations in the rumen (*in vivo*) and *in vitro* were not different between the BDG and AOG, which implies that anise oil, at the dose tested in this study, neither affect feed intake nor alter the utilization of energy. These results are consistent with results of other previous studies (Wallace *et al*., 2002; Newbold *et al*., 2004). In addition, Castillejos *et al*. (2007) observed in a dual flow continuous culture studies that supplementing 650 mg/day of a blend of essential oil (Crina® Ruminant; AKZO NOBEL/CRINA S.A., Gland, Switzerland) did not alter the concentration of VFA (*in vitro* and *in vivo*). Since the absorption of VFA in the rumen is proportional to

its ruminal concentration (Lopez *et al*., 2003), this study suggests that absorption of VFA would also not be affected with the inclusion of 2.4 g/sheep/day of anise oil. Although the molar proportions of individual VFA were not affected by treatment *in vivo*, results were different *in vitro*. The unadapted fluid plus 200 mg/L of anise oil (BDG 200 mg/L) did not affect the molar proportions of acetate, propionate and the acetate to propionate ratio at 24 h which is consistent with previous reports described in Chapter 5 (where 200 mg/L of anise oil did not change the molar proportions of VFA). In contrast, AOG 200 mg/L increased acetate and reduced propionate. These results between *in vitro* and *in vivo* studies are in agreement with the observation of Castillejos *et al*. (2007) who reported that although blend of essential oil did not affect the concentrations of total and individual VFA *in vivo*, levels were affected when rumen fluid collected from sheep adapted to blend of essential oil was used in *in vitro* trial. The concentrations of branched-chain volatile fatty acids (BCVFA) such as isobutyric and isovaleric in AOG 200 mg/L were reduced which agreed with the reduced concentration of NH_3-N in those cultures. These results are consistent with decreased deamination process (Allison *et al*., 1962).

6.4.2. Effect of EOC on fatty acid metabolism

Saturated fatty acids such as C14:0 and C16:0 have the capacity to increase the plasma cholesterol levels by suppressing and saturating low-density lipoprotein (LDL) receptors (Keys *et al*., 1995). In the current study, the levels of C14:0 were not affected by treatments, but the g/100 g concentration of C16:0 was considerably reduced with the addition of 200 mg/L of anise oil (irrespective of fluid) relative to the control. This agrees with the previous reports described in Chapter 5 and suggests that anise oil could potentially reduce the levels of plasma cholesterol through decreasing the concentration of palmitic (C16:0) acids.

The average concentration of C18:0 was reduced in cultures supplemented with AOG relative to the BDG. The 200 mg/L regardless of the type of fluid (AOG or BDG) maintained higher levels of C18:0 than the 0 mg/L. At 12 and 24 h, the concentration of C18:1 *trans* 11 (vaccenic acid) was significantly higher in cultures with the AOG relative to the BDG. These results suggest that anise oil modified the biohydrogenation of fatty acids by reducing the production of stearic acid (the end-product of biohydrogenation) and maintained higher levels of C18:1 *trans* 11 than the control cultures. Reduction in

the content of C18:0 and higher levels of C18:1 *trans* 11 suggests that supplementing anise oil might reduce the activity of *Butyrivibrio proteoclasticus*, the ruminal microorganism responsible for converting C18:1 *trans* 11 to stearic acid during the reductase-step of biohydrogenation (Moon *et al*., 2008). These results agree with the reports from our preliminary *in vitro* studies (Eburu and Chikunya, 2014; 2015a; b). In those studies, both anise oil and its main active constituent compound (anethole) maintained high concentration of PUFA in cultures as a consequence of decreased biohydrogenation.

The concentrations of C18:2 *cis*-9 *trans* 11CLA in cultures at 12 h were maintained in a linear dose-response pattern. This is consistent with reports from our previous study where anise oil maintained higher levels of C18:2 *trans* 11CLA (Chapter 5; Eburu and Chikunya 2015a, b). This intermediate of biohydrogenation (C18:2 *cis*-9 *trans* 11 CLA) is formed in the first step during the transformation of *cis*-9, *cis*-12 18:2 by the linoleic acid isomerase (LA-I) (Jenkins *et al*., 2008; Kim *et al*., 2009; Buccioni *et al*., 2012). Results of the current study suggest that anise oil at the level tested facilitated the activities of the linoleic acid isomerase which regulates the formation of *cis*-9 *trans* 11 CLA from *cis*-9, *cis*-12 18:2.

The content of C18:2 *n*-6 and C18:3 *n*-3 rapidly decreased with time and the biohydrogenation of C18:2 *n*-6 and C18:3 *n*-3 was similar to previous *in vitro* (Beam *et al*., 2000; Sinclair *et al*., 2005) and *in vivo* (Wachira *et al*., 2000; Scollan *et al*., 2001, Wang *et al*., 2002) studies. After 48 h time point, the concentrations of C18:2 *n*-6 and C18:3 *n*-3 were higher in vessels with AOG than in BDG, and the 200 mg/L of all fluids maintained higher levels of C18:2 *n*-6 and C18:3 *n*-3 compared to the control. At 24 h time point, the concentrations of C18:2 *n*-6 and C18:3 *n*-3 in vessels with added AOG 200 mg/L were higher than their corresponding values in BDG 200 mg/L. This significant interaction proposes the possibility of the residual effects of feeding anise oil to lambs (for four weeks) on the maintenance of the concentration of PUFA by decreasing biohydrogenation. The biohydrogenation of these C-18 PUFAs was similar to their concentrations with AOG 200 mg/L indicating the highest potency to decrease the disappearance of PUFA. These results are consistent with those of Vasta *et al*. (2013) who observed that after 3 months of supplementing the diet of Barbarine lambs with 400 ppm of Artemisia essential oil, the concentrations of C18:1 *trans* 11, C18:2 *cis*-9 *trans* 11, C18:2 *n*-6, C18:3 *n*-3 and MUFA in the muscle were increased compared to the control. Futhermore, other studies reported that the fat content of milk

from dairy cows was increased by feeding 5 g/day/cow and 2/day/cow of alium and juniper essential oils, respectively (Yang *et al*., 2007). By contrast, Chaves *et al*. (2008) reported that supplementing garlic, juniper berry and cinnamaldehyde at 200 mg/kg of dry matter to growing lambs did not modify the fatty acid profile of back and liver fat. Other studies observed that monoterpenes blend consisting of β-pinene, linalool, α-pinene, *p*-cymene at 0.43 g/kg of dry matter intake did not change the fatty acid profile of milk (Malecky *et al*., 2009). In another study, supplementing the diet of dairy cows with 1 g/day of cinnalmaldehyde did not affect the fatty acid composition of milk (Benchaar *et al*., 2007b). The difference between the present study and previous studies (Chaves *et al*., 2008; Malecky *et al*., 2009) for composition of fatty acid could be due to the difference in the chemical structure of essential oils and the duration of exposure. These factors (the chemical structure of essential oils and the duration of exposure) could affect the applicability of *in vitro* results and the actual response from animal performance *in vivo* due to possible microbial adaptation. This is why other researchers suggested that sufficient time should be provided *in vitro* in order to study the possibility of such adaptations (Calsamiglia *et al*., 2007).

The extent of disappearance of C20:5 *n*-3 and C22:6 *n*-3 in vessels increased as the time of incubation progressed. After 24 h, the quantity of the content of 20:5 *n*-3 in vessel which disappeared was highest in the control (73.7%) and supports previous reports from *in vivo* studies where the biohydrogenation of C20:5 *n*-3 and C22:6 *n*-3 from fish oil range from 72 to 93 g/100 g TFA (Wachira *et al*., 2000; Scollan *et al*., 2001; Chikunya *et al*., 2004). The disappearance of C22:6 *n*-3 which was less than the biohydrogenation of C20:5 *n*-3 was also consistent with other previous *in vitro* reports where the biohydrogenation of C22:6 *n*-3 was less than 50 g/100 g (Ashes *et al*., 1992; Sinclair *et al*., 2005). Rumen fluid from sheep adapted with anise oil (AOG) maintained higher levels of C20:5 *n*-3 and C22:6 *n*-3 and reduced their biohydrogenation than BDG, and all treatments maintained a dose-related response effect on the levels of C20:5 *n*-3 and C22:6 *n*-3. As the concentrations of these fatty acids reflect the biohydrogenation data, it indicates that disappearance of these PUFA was substantially reduced in the AOG. However, there was no significant interaction between fluid and dose of anise on the concentration and biohydrogenation of these long chain PUFAs.

The addition of graded doses of anise (0 and 200 mg/L) sequentially increased the concentrations of ∑PUFA and P/S ratio and progressively decreased the concentrations of ∑*n*-6/∑*n*-3 and ∑SFA, relative to the control. These increased concentrations of ∑PUFA, P/S ratio and progressive decrease on the concentrations of ∑*n*-6/∑*n*-3 and ∑SFA with 200 mg/L of anise oil were higher in vessels with the AOG 200 mg/L relative to the BDG 200 mg/L. This interaction implies that feeding 2.4 g/day/sheep of anise oil for 4 weeks improved the concentrations of ∑PUFA, P/S ratio, ∑*n*-6/∑*n*-3 and ∑SFA than just the supplementation of cultures with 200 mg/L of anise oil.

This microbial adaptation study did not measure the stability of anise oil (the used essential oil) in the rumen. However, this assessment would have been achieved by measuring the amount of the oil in the diet and examination of the feaces for the same purpose. There is increasing evidence that essential oils and their constituent compounds are anaerobically biodegraded to a number of other compounds. This suggests that adaptation may result from the biotransformation of the active form of a compound to a less active form. In anaerobic environment, facultative bacteria such as *Alcaligenes defragrans* degrade monoterpenes such as limonene into 2-carene or α-terpinene depending on the absence or availability of nitrate, respectively (Heyen and Harder, 1998). Other microbes such as *E.coli* produce carveol, perillyl alcohol, perillic acid and carvone from the biotransformation of monoterpenes such as limonene (Cheong and Oriel, 2000; Mars *et al*., 2001).

In summary, as an indication of modified fatty acid profile in the present study, the fluid from lambs adapted with 2.4 g/sheep/day for four weeks contained higher levels of individual and total PUFA and reduced concentration of C18:0 and ∑SFA than the unadapted fluid. This study indicates that there was no microbial adaptation to anise oil (2.4 g/sheep/day) during this experimental period (four weeks), and suggests that at the dose tested, anise oil sustain microbial inhibition and prevented any form of microbial adaptation process.

6.5. Conclusion

In this four weeks adaptation study, fluid from sheep adapted with anise oil maintained higher concentrations of total and individual PUFA, *trans* vaccenic acid and lower concentration of stearic acid.

These results indicate that there was no microbial adaptation to anise oil in the rumen during the four weeks period of study. These results suggest that anise oil is potentially a useful feed additive to optimise the fatty acid composition of ruminant food products, if these effects are observed in meat and milk.

CHAPTER 7

7.1a. General discussion and recommendations for future research

The effects of essential oils and their constituent compounds on *in vitro* biohydrogenation of PUFA in the rumen were examined during the course of this PhD research. The major objective was to use EOs/EOCs to modify microbial metabolism of PUFA in order to optimize the fatty acid composition of ruminant meat and milk. The need for optimization of the fatty acid composition of ruminant food products is due to the well-known human health implications of consuming high SFA and low PUFA, which is common in ruminant food products. One of the weaknesses of EOCs or EOs in the rumen is their lack of selectivity in terms of microbial species affected; hence, dose optimization of EOCs/EOs was performed to obtain satisfactory protection of PUFA from ruminal biohydrogenation but without disrupting VFA concentration. In addition, microorganisms have the tendency to adapt to EOCs/EOs over time; therefore, the possibility of microbial adaptation to anise oil (the most effective oil identified during the *in vitro* studies) was investigated. During this PhD research, the main aims and the principal accomplishments and findings are outlined as follows:

1. Phenolic compounds from essential oils were more effective at reducing biohydrogenation of PUFA in the rumen than other classes of EOCs (Chapter 3).
2. Anethole and 4-allyanisole (at 300 mg/L) were the most effective EOCs to reduce the extent of *in vitro* rumen biohydrogenation of C18:3 *n*-3 (average values were 22.2% and 26.4%, for anethole and 4-allyanisole, respectively). However, at 300 mg/L, anethole and 4-allyanisole concomitantly induced substantial decrease (over 10% reductions) on the ruminal concentration of total VFA (Chapter 3).
3. Essential oils that are rich in phenolic compounds were more effective at reducing biohydrogenation of PUFA in the rumen than other essential oils whose active compounds are not predominantly phenolic compounds (Chapter 4).
4. Anise and cassia oils (at 300 mg/L) were the most effective whole EOs to reduce the extent of *in vitro* rumen biohydrogenation of C18:3 *n*-3 (mean values were 58.2% and 54.3% for anise and

cassia oils, respectively). However, protection of PUFA from biohydrogenation was accompanied by significant (over 10% reductions) suppression of VFA concentration in the ruminal contents.

5. Essential oils have slightly more potential to inhibit the biohydrogenation of PUFA in the rumen than their main active constituent components (Chapter 4 and Chapter 5).
6. At 200 mg/L, 4-allylanisole, anethole and anise oil seems to give best balance between PUFA protection and minimal disruption to VFA concentration *in vitro,* but cassia oil requires a dose more than 100 mg/L but less than 200 mg/L to attain the same balance (Chapter 5).
7. At 200 mg/L, anise oil was more effective than the rest of the other substances (-allylanisole, anethole and cassia oil) in both preventing PUFA disappearance and maintenance of optimal VFA production (Chapter 5).
8. *In vitro* digests from rumen fluid fed anise oil (2.4 g/sheep/day) contained higher concentrations of PUFA and biohydrogenation intermediate (C18:1 *trans 11*) with substantial reduction of C18:0 concentration (Chapter 6).
9. Microbial adaptation to anise oil did not occur within four weeks period used in adaptation study, as evidenced by high accumulation of PUFA and C18:1 *trans 11* with substantial reduction of C18:0 concentration (Chapter 6).
10. There is a possibility to select essential oils and their main constituent compounds to reduce the biohydrogenation of PUFA and to potentially use them to optimize the fatty acid composition of ruminant food products (Chapters 3; 4; 5; 6).

Results of this PhD thesis suggest that, generally, dietary addition of essential oils and their constituent compounds represent a potential effective technique to optimize the fatty acid composition of ruminant food products by reducing the extent of rumen biohydrogenation of PUFA. Whole essential oils that are rich in phenolic compounds are more effective than the individual constituent compounds. However, certain questions remain unanswered which need to be addressed in future study. Therefore, the following areas of further study are suggested.

7.1b MECHANISM OF ACTION OF EOS/EOCS

Despite the considerable increase on the concentration and the dramatic reduction in the biohydrogenation of PUFA due to the supplementation of EOs/EOCs, there were no corresponding data on their effects on specific biohydrogenating bacteria. It was not a deliberate decision not to evaluate effects of EOs/EOCs on microbial species composition, but priority in the design of the PhD work was given to comparative evaluation of effects of whole essential oils and their individual EOCs; establishment of optimum doses for both EOCs/EOs; and the possibility of rumen adaptation. Furthermore, the time consuming nature of microbial analyses and the expensive nature of microbial characterization studies were other factors that worked against microbial species composition analysis in this study.

Clostridium proteoclasticum (Kemp *et al*., 1975; Maia *et al*., 2007) which was re-classified as *Butyrivibrio proteoclasticus* (Moon *et al*., 2008), is well known as the only bacteria capable of converting C18:1 *trans* 11 to C18:0, and all bacteria in the *Butyrivibrio* group are known to have the capacity to convert linoleic acid to C18:2 *cis-9 trans-11* CLA. However, because bacteria need to be growing or active in order to cause biohydrogenation, other authors have argued that as PUFA are capable of inhibiting this growth, it is likely that other bacteria which are potential producers of C18:0 might have been inhibited (Wallace *et al*., 2006). Hudson *et al*. (1998) observed that *Streptococcus bovis* has the ability to cause hydration of linoleic acid to 13-hydroxy-9-octadecenoic acid. The study of Hudson *et al*. (1998) provide evidence to suggest that other facultative ruminal bacteria including *Lactobacillus, Staphyloccous, pediococcus* and *Enterococcus* have the ability to hydrate linoleic acid (Hudson *et al*., 2000). There is limited evidence whether there are many more bacteria involved in biohydrogentaion due to the time-consuming nature and the high cost of isolating such bacteria (Huws *et al*., 2006). Available evidence suggests that other bacteria such as R*uminococcaceae, Anaerovoax, Prevotella* as well as other clostridiales which have not been identified could also play a role in biohydrogenation pathways (Huws *et al.*, 2006, Huws *et al*., 2011). Future study with EOs/EOCs must consider examination of effects on microbial species composition, providing evidence on the abundance of key biohydrogenating bacteria in order to conclusively state the mechanism of actions of essential oils against individual microbial species.

7.1c *IN VIVO* STUDIES

Only short term *in vitro* studies up to 48 h and four weeks period of microbial adaptation effects were evaluated in this PhD. Several studies have reported wide discrepancies between *in vitro* and *in vivo* results. Malecky *et al*. (2009) observed that the fatty acid composition of milk was not affected by adding a monoterpene blend (0.43 g/kg diet) containing α-pinene, β-pinene, p-cymene and linalool to the diet. In another study, cinnamaldehyde (1 g/kg) added to the diet of dairy cattle also did not affect the profile of milk fatty acids (Benchaar and Chouinard, 2009). Others observed that the fatty acid profile of milk obtained from dairy cows offered 0.75 g/cow/day of a mixture of essential oils and compounds (Crina ruminants; CRINA S.A., Gland, Switzerland) was not affected (Benchaar *et al*., 2007a). Furthermore, Chaves *et al*. (2008) reported that supplementing garlic, juniper berry and cinnamaldehyde at 200 mg/kg of dry matter to growing lambs did not modify the fatty acid profile of back and liver fat. However, other studies observed that after 3 months of supplementing the diet of Barbarine lambs with 400 ppm of *Artemisia* essential oil, the concentrations of C18:1 *trans* 11, C18:2 *cis*-9 *trans* 11, C18:2 *n*-6, C18:3 *n*-3 and total MUFA in the muscle were increased compared to the control(Vasta *et al*., 2013). In our *in vitro* and adaptation studies, we observed dramatic changes in PUFA metabolism, resulting in higher concentration and reduced biohydrogenation of PUFA due to the supplementation of EOs/EOCs. A number of factors such as the environmental condition of the growing plant (soil temperature, composition and moisture), stage of plant growth, the species of the plant, the part of the plant from where the essential oil is extracted, and the particular method used for the extraction of oil, are all known to be responsible for variation in the composition of essential oil and by extension, their antimicrobial effects. In most cases, producers of the majority of the available commercial essential oils do not provide sufficient details about these factors; hence, the differences observed in results cannot be categorically attributed to any known factor. Therefore, in order to standardize the dose and type of essential oil in relation to a particular effect in the rumen, future research must provide details of the growing conditions of a plant, the species, the part of the plant from which oil was extracted, the method of extraction used, the purity and chemical composition of the oil and the concentration which produced a particular effect in the rumen. These data would reduce the discrepancies observed in results.

In a situation where the above factors are considered and a difference is still observed, this might be attributed to microbial adaptation to essential oil or shift in microbial species composition. In the present study, we established that there was no microbial adaptation after four weeks of feeding anise (at 2.4 g/sheep/day). We are proposing that there would be close relationship between *in vivo* and *in vitro* results if *in vivo* studies (evaluation of effects on meat and milk fatty acid composition) succeed microbial adaptation studies. In this way the minimum period of time which was established from an adaptation study could be applied in the *in vivo* study. We had established that there was no microbial adaptation to anise oil after four weeks period of feeding anise oil to lambs, further *in vivo* studies are necessary to examine the potential transfer of these fatty acid changes to meat or milk by using anise oil at the dose tested here and at the last four weeks to market weight (slaughter of the animal).

7.1d STABILITY OF ESSENTIAL OIL IN THE RUMEN

The microbial adaptation study did not measure the stability of anise oil (the used essential oil) in the rumen. But there is increasing evidence that essential oils and their constituent compounds are anaerobically biodegraded to a number of other compounds. This suggests that adaptation may result from the biotransformation of the active form of a compound to a less active form. In anaerobic environment, facultative bacteria such as *Alcaligenes defragrans* degrade monoterpenes such as limonene into 2-carene or α-terpinene depending on the absence or availability of nitrate, respectively (Heyen and Harder, 1998). Other microbes such as *E.coli* produce carveol, perillyl alcohol, perillic acid and carvone from the biotransformation of monoterpenes such as limonene (Cheong and Oriel, 2000; Mars *et al*., 2001). Facultative bacteria produce isoterpinolene from the biodegradation of isolimonene (Chang *et al*., 1995; Heyen and Harder, 1998). The biodegradation of EOs or their compounds is achieved through the re-arrangement of unsaturated monoterpenes by bacterial enzymes (Heyen and Harder, 1998). These same authors reported that products of EO/EOC biodegradation might be in the form of ionic compounds. This suggests that products of EO/EOCs biotransformation might just remain as intracellular substrate, hence, the inhibitory effect of the products might be lost. Heylemon and Harder (1999) reported that C-1 sp2 hybridization of menthadienes can be used to determine the metabolism or transformation of monoterpenes. Further study must use this method to elucidate the exact biodegradation pathways of EOs

and their compounds. Badee *et al*. (2011) suggested that the biodegradation of monoterpenes can be inhibited either by the substrate or the end product of bioconversion. Establishment of these pathways would help to suggest ways of inhibiting the activities of the biotransformation bacteria and ensure the stability and antimicrobial activity of EOs and their constituent compounds in the rumen.

7.1e OTHER AREAS OF FURTHER RESEARCH

It is a known fact that farmers would not adopt a new nutritional strategy except the cost-benefit analysis and the economic benefits for animal production are established. It is worth mentioning that because most commercial essential oils, particularly their active compounds, are very expensive (Sigma Aldrich); their effects on the welfare and productivity of animal should be established prior to their use in the farm. Therefore, the cost-benefits ratio which would take into account the cost of an essential oil, the concentration used and farm profitability (Calsamiglia *et al*., 2007), should be established in future study.

7.2. Brief summary

Optimising the fatty acid composition of ruminant derived food products for human health by altering the processes of lipolysis and subsequent biohydrogenation of free fatty acids was the principal aim of this study. Increased intakes of saturated fatty acids have been consistently associated with increased occurrence of chronic diseases such as diabetes, obesity, compromised immune system and cardiovascular diseases (Givens, 2005). In contrast, there is a positive relationship with increased intake of *n*-3 PUFA and reduced risk of coronary heart disease (CHD). This relationship between CHD and the intake of *n*-3 PUFA has led nutritional recommendation authorities to place emphasis on increasing the intakes of dietary *n*-3 PUFA and reducing the consumption of SFA (Department of Health, 1994; WHO, 2003). Indeed, ruminant derived food products are paradoxical. On one hand, they have often been blamed for containing low level of omega-3 fatty acids (about 2 g in every 100 g) and high content of SFA (about 65 g in every 100 g) resulting from extensive biohydrogenation of dietary PUFA and the *de novo* fatty acid synthesis (Gurr and Harwood, 1991). On the other hand, incomplete biohydrogenation of dietary PUFA produces a number of intermediate fatty acids such as *cis-9 trans* 11 18:2 CLA and its precursor, C18:1 *trans* 11. These fatty acids have been reported to possess a number of human health

benefits such as anti-carcinogenic, anti-inflammatory, anti-atherogenic and anti-diabetic effects (Banni *et al*., 2001; Corl *et al*., 2003). Therefore, research to manipulate rumen metabolism of fatty acid is necessary to optimize the fatty acid profile of ruminant food products and to improve human consumption of *n*-3 PUFA and *cis-9 trans* 11 18:2 CLA from ruminant food products.

One of the ways of optimising the fatty acid composition of ruminant food products is through animal dietary manipulation. A number of technologies have been developed over the years but they have been largely ineffective or not cost effective. These factors have hindered the commercialization of many technologies with the aim of optimizing the fatty acid composition of ruminant derived food products. A few *in vitro* studies have shown the potential of essential oils to alter fatty acid metabolism and cause accumulation of biohydrogenation intermediates. The effectiveness of essential oil to achieve this depends on the ability of its active compounds to penetrate microbial cell wall, damage membrane proteins, deplete the proton motive force, damage the cytoplasmic membrane, cause leakage of the cell contents and coagulate the cytoplasm (see Chapter 1.7.3). These mechanisms of actions are capable of enabling EOCs/EOs to reduce the activities of key biohydrogenating bacteria such as *Butyrivibrio proteoclasticus*. The fact that these substances are constituent of plants which are dietary components for herbivorous animals (Cowan, 1999), and also because a number of EOs/EOCs have been proposed as safe alternative to antibiotics and their addition to food are generally considered as safe (Calsamiglia *et al*., 2007), EOs/EOCs represent a potential dietary additive to achieve the right optimization of the fatty acid composition of ruminant food products.

The principal objectives of this PhD study were 1) to examine the *in vitro* effects of essential oils constituent compounds on the metabolism of *n*-3 PUFA by rumen microorganisms, 2) to further validate the *in vitro* effects of whole essential oils on rumen fermentation and biohydrogenation of *n*-3 polyunsaturated fatty acids by rumen microorganisms, 3) to establish optimum doses of supplementing essential oils and their compounds in ruminant diet and 4) to establish the potential adaptation time of the most effective essential oil on rumen fermentation and metabolism of *n*-3 PUFA.

In order to achieve these objectives three preliminary *in vitro* studies followed by feeding trial and then another *in vitro* study were conducted.

In all the *in vitro* trials in this research, the basal feedstock (a 70: 30 mixture of grass hay and concentrate, respectively) was similar and was formulated once. Details of the experimental diet and supplier of the ingredients are as described in section 2.3 (see general material and methods). The nutrient content and the fatty acid composition of the basal feedstock are shown in Table 2.2 (general materials and methods). Lipid sources in the basal feedstock were linseed (C18:3 *n*-3), grass hay (C18:3 *n*-3 and C18:2 *n*-6) and fish oil (C20:5 *n* -3 and C22:6 *n* -3). The predominant C-18 fatty acids in the basal feedstock were C18:3 *n*-3 (21.93 g/100 g TFA) and C18:2 *n*-6 (12.90 g/100 g TFA). These principal C-18 FAs account for more than 50% (59%) of the TFA in the diet. Similarly, C20:5 *n* -3 and C22:6 *n* -3 were the most abundant long chain *n*-3 PUFA, contributing 8.3% and 6.2%, respectively to the TFA composition of the diet. Hence, the fatty acid composition of the diet is a direct reflection of the sources of fatty acids in the diets (i.e. C18:3 *n*-3 and C18:2 *n*-6 from linseed and grass hay, respectively and C20:5*n* -3 and C22:6*n* -3 from fish oil). The basal feedstock was formulated by using feedstuff that are rich in C18:2 *n*-6, C18:3 *n*-3, C20:5 *n* -3 and C22:6 *n* -3 because the main aim of the study was to examine the biohydrogenation of those fatty acids over time.

The effects of fifteen essential oil constituent compounds (at 300 mg/L) on the ruminal biohydrogenation of C18:2 *n*-6 and *n*-3 PUFA was examined by means of 48 h *in vitro* batch culture system. A 70: 30 mixture of grass hay and concentrate respectively was formulated and then supplemented with additional fat from the mixture of 60% of fish oil and 40% of ground linseed. The experiment was repeated twice and the results presented were the means of two experiments. Treatments were control (no essential oil), 4-allylanisole, anethole and cinnamaldehyde (phenylpropanoids), menthol (monoterpene alcohol), 3-carene and borneol (bicyclic monoterpenes), citronellol, limonene, myrtenol, *p*-cymene and thujone (monoterpenes), bisabolol (sesquiterpene), eucalyptol (monoterpene ethers), caryophyllene (bicyclic sesquiterpene) and vanillin (aldehydes). There was rapid biohydrogenation of C18:3 *n*-3 from ground linseed which exceeded 80 g/100 g at 24 h. This observation agrees with previous studies where the biohydrogenation of C18:3 *n*-3 range from 72-95% (Wachira *et al*., 2000; Scollan *et al*., 2001). However,

supplementing cultures with EOCs considerably reduced biohydrogenation and maintained higher the concentrations of C18:2 *n*-6 and *n*-3 PUFA (Chapter 3). The content of C18:3 *n*-3 or C18:2 *n*-6 at 24 h increased significantly in the progression: ALA and ANE (phenylpropanoids), MEN (monoterpene alcohol) and CIT (monoterpene) > 3-CAR and BOR (bicyclic monoterpenes), CIN (phenylpropanoid), LIM, MYT, CYM and THU (monoterpenes) > BIS (sesquiterpene) and EUC (monoterpene ethers) > CPY (bicyclic sesquiterpene) and VAN (aldehydes). The results which indicated that phenolic compounds were the most potent compounds and that aldehydes were the least effective compounds was consistent with the findings of Kalemba et al. (2012) who ranked the antimicrobial potency of EOCs in the following descending order: phenols > cinnamic aldehyde > alcohols > aldehydes. Anethole and 4-allylanisole (phenolic compounds) were the most effective EOCs after 24 h. As a consequence of including anethole and 4-allylanisole in cultures, the concentrations of C18:2 *n*-6 and C18:3 *n*-3 (g/100 g TFA) were more than double the corresponding values in the control. Mean values were 2.7, 2.7 and 1.3 (C18:2 *n*-6) and for C18:3 *n*-3 were 4.0, 3.9 and 1.6 g/100 g TFA for 4-allylanisole, anethole and the control, respectively. The values for other EOCs were intermediate between the control and the most effective EOCs (anethole and 4-allylanisole). The biohydrogenation results showed that about 22.2% and 26.4% of alpha linolenic acid (C18:3 *n*-3) were protected from ruminal biohydrogenation in cultures supplemented with anethole and 4-allylanisole, respectively. The possibility of anethole and 4-allylanisole to exert the greatest antimicrobial characteristic by maintaining higher levels of C18:3 *n*-3 than other EOCs could be due to the possession of hydroxyl group whose acidic characters are speculated to have broad inhibition of microbial activities (Cosentino *et al.*, 1999; Juliano *et al*., 2000; Lambert *et al*., 2001). This could suggest that anethole and 4-allylanisole probably had higher capacity to reduce isomerization of C18:3 *n*-3 and C18:2 *n*-6 compared to the rest of the treatments or that they were more active against *Clostridium proteoclasticum* (Kemp *et al*., 1975; Maia *et al*., 2007), renamed (Moon *et al*., 2008) as *Butyrivibrio proteoclasticus,* the bacteria which converts C18:1 *trans* 11 to C18:0. The latter reason probably could be the option as further evaluation of the concentrations of C18:1 *trans* 11 revealed that levels were higher in fermentation vessels supplemented with anethole and 4-allylanisole. However, due to the high concentration of oleic acid (C18:1 *n*-9) which was probably converted to C18:0, corresponding reduction in C18:0 as an evidence of reduced biohydrogenation of C18:3 *n*-3 and C18:2 *n*-

6 was not observed in anethole and 4-allylanisole. Jenkins *et al*. (2006) observed that about 70% of C18:1 *n*-9 in rumen culture was converted to C18:0 and only 30% was transformed to ketostearic acid and hydroxystearic acid. Evaluation of microbial species composition, which this study unfortunately did not perform, would have been able to conclusively establish whether the increased concentration of PUFA and biohydrogenation intermediates such as C18:1 *trans* 11 concomitantly reduce the activity of *Butyrivibrio proteoclasticus* (the key biohydrogenator) or any other closely associated bacteria capable of causing biohydrogenation. However, substantial suppression of VFA was concomitantly associated with the increased concentration of PUFA. The fact that EOCs are extracts of plants which are part of animal diets (Cowan, 1999), and their consideration as generally recognized as safe food additives (GRAS, Calsamiglia *et al*., 2007), offers the use of EOCs to reduce ruminal PUFA disappearance advantage over other non-commercialized method of protecting PUFA from biohydrogenation such as the formaldehyde method, if their use is balanced against inhibition of VFA.

The potential of ten whole essential oils (at 300 mg/L) on the biohydrogenation of C18:2 *n*-6 and *n*-3 PUFA was further evaluated through *in vitro* batch culture system. The aim was to establish whether the whole essential oils from where some of the major compounds showing potential to reduce the biohydrogenation of PUFA in Chapter 3 are equally effective. The ten whole oils (anise, cassia, citronella, clove, cornmint, eucalyptus, juniper berry, lavender , mandarin and rosemary) used in this study were the parent oils whose main compounds were the best ten in Chapter 3. There was rapid biohydrogenation of C18:3 *n*-3 from ground linseed which exceeded 80 g/100 g at 24 h. This observation agrees with previous studies where the biohydrogenation of C18:3 *n*-3 range from 72-95% (Wachira *et al*., 2000; Scollan *et al*., 2001; Wang *et al*., 2002; Sinclair *et al*., 2005). Similarly, the biohydrogenation of C20:5 *n*-3 which exceeded 80 g/100 g was in agreement with previous reports from *in vivo* studies where the biohydrogenation of C20:5 *n*-3 and C22:6 *n*-3 in fish oil range from 72 to 93 g/100 g TFA (Wachira *et al*., 2000; Scollan *et al*., 2001; Chikunya *et al*., 2004), and contrast *in vitro* reports where the biohydrogenation of C20:5 *n*-3 and C22:6 *n*-3 was less than 50 g/100 g (Ashes *et al*., 1992; Sinclair *et al*., 2005). However, supplementing cultures with EOs substantially reduced biohydrogenation and maintained higher the concentration of C18:2 *n*-6 and *n*-3 PUFA, but with corresponding significant (over

10% average) suppression of VFA concentration (Chapter 4). The potential of whole EOs used in this study to reduce the biohydrogenation of *n*-3 PUFA at 24 h was as follows: ANO and CSO > MDO and CMO > CTO and RMO > JPO, LVO, CLVO and ETO. Similar to the results observed with the EOCs (Chapter 3), the whole essential oils whose main components possess phenolic moieties in their chemical structures were the most effective oils in this study. The fermentation vessels supplemented with anise (high in anethole) and cassia (high in cinnamaldehyde) oils elicited the most effective protection of C18:2 *n*-6 and *n*-3 PUFA from biohydrogenation. Supplementation of anise and cassia oils tripled the concentration of C18:2 *n*-6 and C18:3 *n*-3 relative to the control. The concentration values at 24 h were 5.4, 6.0 and 2.2 (C18:2 *n*-6) and for C18:3 *n*-3 were 8.1, 9.4 and 2.8 g/100 g TFA for anise oil, cassia oil and the control, respectively. The biohydrogenation data indicated that about 58.2% and 54.3% protection of alpha linolenic acid (C18:3 *n*-3) from ruminal biohydrogenation was obtained with the addition of anise oil and cassia oil, respectively. This probably implies that anise and cassia oils had higher capacity to either reduce isomerization of C18:3 *n*-3 and C18:2 *n*-6 compared to the rest of the treatments or that they were more active against *Clostridium proteoclasticum* (Kemp *et al*., 1975; Maia *et al*., 2007), renamed (Moon *et al*., 2008) as *Butyrivibrio proteoclasticus*, the bacteria which converts C18:1 *trans* 11 to C18:0. The concentration of C18:0 (the end-product of the biohydrogenation of C18:1 *n*-9, C18:3 *n*-3 and C18:2 *n*-6) was considerably decreased in cultures supplemented with cassia oil. This observation learns support to the possibility of these phenolic rich EO to reduce the activity of *Butyrivibrio proteoclasticus*, the key bacteria which converts C18:1 *trans* 11 to C18:0. Comparison of the effects of EOCs (Chapter 3) and whole essential oils (Chapter 4) indicates that the whole essential oils are more effective than the main active compounds in terms of inhibiting the biohydrogenation of PUFA. This suggests that although the major constituent components of EOs reflect the biological properties of the whole oils, minor components can contribute in modulating their activities such as hydrophobicity, cell penetration and fixation on cell membranes (Bakkali *et al*., 2008). This leads to the suggestion that the minor components of essential oils could be critical to the antimicrobial activity of the oil by working synergistically with the major components (Burt, 2004). Although substantial suppression of VFA was concomitantly associated with the increased concentration of PUFA, whole EOs are probably more effective than the individual EOCs, if their dose is optimized. These results show that EOs have higher

potential to reduce PUFA biohydrogenation than other PUFA protection technologies such as the formaldehyde method described in the reports of Sinclair *et al*. (2005), where pre-treatment of whole linseed with NaOH and formic acid prior to treatment with formaldehyde obtained 44.3% and 30.7% protection for C18:3 *n*-3, respectively. As shown above, the average use of whole anise and cassia oils are 32.0% and 18.8% better than the use of EOCs (Chapter 3) and the method of Sinclair *et al*. (2005), respectively. This is not weighting the economic and potential health benefits of the whole oils relative to the formaldehyde method of Sinclair *et al*. (2005).

Based on the substantial inhibition of total VFA results from using 300 mg/L of EOCs (first experiment, chapter 3) and of the whole oils (second experiment, chapter 4), a third *in vitro* study was conducted in which graded doses (0, 100, 200 and 300 mg/L) of two most effective EOCs and 2 most effective whole essential oils were evaluated. The aim was to establish a dose at which satisfactory inhibition of ruminal PUFA biohydrogenation can be obtained but without significant reduction of VFA concentration. Treatments were 4-Allyanisole (ALA 0, 100, 200 and 300 mg/L), anethole (ANE 0, 100, 200 and 300 mg/L), anise oil (ANO 0, 100, 200 and 300 mg/L) and cassia oil (CSO 0, 100, 200 and 300 mg/L). Increasing the dose of both EOs/EOCs substantially increased proportionally the concentration of PUFA (C18:3 *n*-3, C18:2 *n*-6, C20:5 *n*-3 and C22:6 *n*-3) and concomitant accumulation of biohydrogenation intermediates such as C18:1 *trans* 11 and 18:2 *cis*-9 *trans* 11 CLA (Chapter 5). The substances also had a dose related response on the concentration of total VFA. The concentrations of PUFA in all substances were significant but marginal at 100 mg/L; doubled at 200 mg/L and; levels were tripled at 300 mg/L. As all the essential oils and the EOCs used in this study had similar chemical structure (i.e. rich in phenolic moieties), general results did not observe any marked difference in response among them. However, there was a significant interaction at each level of supplementation with anise oil indicating higher potential than other three substances to protect PUFA from biohydrogenation. Except in anise oil (at 100 mg/L), 4-allylanisole (at 300 mg/L) and cassia oil (at 200 mg/L), the high accumulation of C18:1 *trans* 11 and C18:2 *cis*-9 *trans* 11 CLA was not associated with reduced concentration of C18:0, suggesting that the emergence of C18:0 may not be emanating from the biohydrogenation of C18:3 *n*-3 and C18:2 *n*-6 alone but from oleic acid (C18:1 *n*-9) as discussed previously . At 200 mg/L, administration of 4-allylanisole,

anethole and anise oil seems to give best balance between PUFA protection and minimal disruption to VFA concentration *in vitro*, but cassia requires a dose more than 100 mg/L but less than 200 mg/L to attain the same balance. At this dose (200 mg/L), the corresponding concentration of total VFA was unaffected in all substances except in cassia which caused 10% suppression of VFA level. However, cassia (at 200 mg/L) reduced NH_3-N concentration and the molar proportion of acetate and increased the proportion of propionate. The significant interaction on both PUFA and total VFA concentrations suggest that supplementation of anise oil had higher potential than other substances to protect PUFA from biohydrogenation and to maintain VFA concentration at optimum level.

There is evidence from results of continuous culture studies and long term *in vivo* studies that the benefits associated with the use of essential oil as feed additive may decline due to either microbial adaption to EOs or shifts in microbial populations (Gladine *et al*., 2007). Therefore, a four weeks *in vivo* feeding trial was conducted to test the possibility of microbial adaptation to the use of anise oil. Anise oil was identified as the most effective from *in vitro* trials in terms of PUFA protection and maintenance of ruminal VFA concentration (Chapter 5). In this experiment, six Hartline × Texel cross lambs (mean weight 44.0 ±2.5kg) were used as rumen fluid donors. The rumen fluid donor lambs were offered water and hay *ad libitum* and supplemented with additional 400 g/sheep/day of concentrate plus 2.4 g/sheep/day of ANO (for the anise oil group). After four weeks of adaptation, lambs were slaughtered and ruminal fluid collected from each of the unadapted lambs (BDG) and adapted lambs (AOG) was used *in vitro*. The study was a 2 (batches of rumen fluid: BDG and AOG) × 2 (doses of ANO: 0 and 200 mg/L) factorial design experiment. In this adaptation study, results indicated that rumen fluid from sheep adapted with anise oil for four weeks maintained higher concentrations of PUFA, *trans* vaccenic acid and lower concentration of stearic acid. These results are similar to the work reported in (Benchaar *et al*., 2006a). These authors observed that biohydrogenation was altered and the concentration of *cis*-9, *trans*-11 18:2 CLA in milk fatty acids was increased when a mixture of essential oil compounds (Crina ruminants' at 2 g/cow/day) was fed to dairy cow (Benchaar et al., 2006a). The Crina ruminants' mixture contains eugenol, thymol, limonene, guaiacol and vanillin (McIntosh *et al*., 2003; Castillejos *et al*., 2005). Furthermore, other studies observed that after 3 months of supplementing the diet of Barbarine lambs

with 400 ppm of *Artemisia* essential oil, the concentrations of C18:1 *trans* 11, C18:2 *cis*-9 *trans* 11, C18:2 *n*-6, C18:3 *n*-3 and total MUFA in the muscle were increased compared to the control(Vasta *et al*., 2013). Although the fatty acid composition of the sheep meat was not examined in the present study to confirm the transfer of this changes (increased PUFA and reduced C18:0) to meat, results of four weeks adaptation study imply that anise oil is a potential feed additive to reduce the biohydrogenation of PUFA and potentially optimize the fatty acid composition of ruminant food products. This suggest that if anise oil (at 2.4 g/sheep/day, the tested dose) is fed to sheep at least one month before market weight, there is a potential to alter the biohydrogenation of PUFA by increasing the concentration of PUFA and *trans* vaccenic acid with concomitant reduction in the concentration of stearic acid without affecting dry matter intake. There is also an indication that about 16% reduction in NH_3-N excretion would be achieved along with the benefits derivable from optimized fatty acid composition. The main effect of CRINA products had also established a minimum of four weeks adaptation period to reduce ammonia excretion (McIntosh *et al*., 2003; Molero *et al*., 2004; Newbold *et al*., 2004; Castillejos *et al*., 2007).

In conclusion, results of this PhD thesis suggest that, generally, dietary addition of essential oils and their compounds represent a potential effective technique to optimize the fatty acid composition of ruminant food products by reducing the extent of rumen biohydrogenation of PUFA. Whole essential oils are more effective than the individual constituent compounds. More precisely, whole essential oils that are rich in phenolic compounds were the most effective.

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